Reference to commercial kits does not mean their endorsement by the OIE. All commercial kits should be validated; tests on the OIE register have already met this condition (the register can be consulted at: www.oie.int).

OIE Manual of Diagnostic Tests and Vaccines for Terrestrial Animals

Manual of Recommended Diagnostic Techniques and Requirements for Biological Products:
Manual of Standards for Diagnostic Tests and Vaccines:
Fifth Edition, 2004


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The designations and denominations employed and the presentation of the material in this publication do not imply the expression of any opinion whatsoever on the part of the OIE concerning the legal status of any country, territory, city or area or of its authorities, or concerning the delimitation of its frontiers and boundaries.
FOREWORD

The Manual of Diagnostic Tests and Vaccines for Terrestrial Animals (Terrestrial Manual) aims to facilitate international trade in animals and animal products and to contribute to the improvement of animal health services world-wide. The principal target readership is laboratories carrying out veterinary diagnostic tests and surveillance, plus vaccine manufacturers and regulatory authorities in Member Countries. The objective is to provide internationally agreed diagnostic laboratory methods and requirements for the production and control of vaccines and other biological products.

This ambitious task has required the cooperation of highly renowned animal health specialists from many countries. The OIE, the World Organisation for Animal Health, is clearly the most appropriate organisation to undertake this task on a global level. The main activities of the organisation, which was established in 1924 and in 2008 comprised 172 Member Countries and Territories, are as follows:

1. To ensure transparency in the global animal disease and zoonosis situation.
2. To collect, analyse and disseminate scientific veterinary information on animal disease control methods.
3. To provide expertise and encourage international solidarity in the control of animal diseases.
4. Within its mandate under the WTO (World Trade Organization) Agreement on Sanitary and Phytosanitary Measures (SPS Agreement), to safeguard world trade by publishing health standards for international trade in animals and animal products.
5. To improve the legal framework and resources of national Veterinary Services.
6. To provide a better guarantee of the safety of food of animal origin and to promote animal welfare through a science-based approach.

The Terrestrial Manual, covering infectious and parasitic diseases of mammals, birds and bees, was first published in 1989. Each successive edition has extended and updated the information provided. This sixth edition includes new chapters on Guidelines for international standards for vaccine banks, Turkey rhinopneumonitis, Small hive beetle infestation (*Aethina tumida*) and camelpox, and *Mycoplasma synoviae* has been added to the chapter on Avian mycoplasmosis (previously the chapter focused on *Mycoplasma gallisepticum*). As a companion volume to the Terrestrial Animal Health Code, the Terrestrial Manual sets laboratory standards for all OIE listed diseases as well as several other diseases of global importance. In particular it specifies (in blue font) those “Prescribed Tests” that are recommended for use in health screening for international trade or movement of animals. The Terrestrial Manual has become widely adopted as a key reference book for veterinary laboratories around the world. Aquatic animal diseases are included in a separate Aquatic Manual.

The task of commissioning chapters and compiling the Terrestrial Manual was assigned to the OIE Biological Standards Commission by the International Committee of the OIE (General Assembly of national Delegates of Member Countries and Territories). Manuscripts were requested from specialists in each of the diseases or the other topics covered. After initial scrutiny by the Consultant Technical Editor, the chapters were sent to scientific reviewers and to experts at OIE Reference Laboratories. They were also circulated to all OIE Member Countries for review and comment. The Biological Standards Commission and the Consultant Technical Editor took all the resulting comments into consideration, often referring back to the authors for further help, before finalising the chapters. The final text has the approval of the International Committee of the OIE.

A procedure for the official recognition of commercialised diagnostic tests, under the authority of the International Committee, was finalised in September 2004. Data are submitted using a validation template that was developed by the Biological Standards Commission. Submissions are evaluated by appointed experts, who advise the Biological Standards Commission before the final opinion of the OIE International Committee is sought. All information on the submission of applications can be found on the OIE Web site.
The *Terrestrial Manual* continues to expand and to extend its range of topics covered. It is our sincere hope that it will grow in usefulness to veterinary diagnosticians and vaccine manufacturers in all the OIE Member Countries. A new paper edition of the *Terrestrial Manual* is published every 4 years. It is important to note that annual updates to the *Terrestrial Manual* will be published on the OIE website once approved by the International Committee, so readers are advised to check there for the latest information. The *Terrestrial Manual* is published in English, French and Spanish.

Doctor Bernard Vallat  
Director General, OIE

Professor Steven Edwards  
President, OIE Biological Standards Commission

January 2008
I am most grateful to the many people whose combined efforts have gone into the preparation of this Terrestrial Manual. In particular, I would like to express my thanks to:

Dr Bernard Vallat, Director General of the OIE from 2001 to the present, who gave his encouragement and support to the project of preparing the new edition of this Terrestrial Manual,

The Members of the OIE Standards Commission, Prof. Steven Edwards, Dr Beverly Schmitt, Dr Anatoly Golovko, Dr Mehdi El Harrak and Dr Santanu K. Bandhopadhyay who were responsible for commissioning chapters and, with the Consultant Technical Editor, for editing all the contributions so as to finalise this edition of the Terrestrial Manual,

The contributors listed on pages xxii to xxxv who contributed their invaluable time and expertise to write the chapters,

The expert advisers to the Biological Standards Commission’s meeting, Dr Adama Diallo and Dr Peter Wright, the OIE Reference Laboratory experts and other reviewers who also gave their time and expertise to scrutinising the chapters,

Those OIE Member Countries that submitted comments on the draft chapters that were circulated to them. These were essential in making the Terrestrial Manual internationally acceptable,

Ms Sara Linnane who, as Scientific Editor, organised this complex project and made major contributions to the quality of the text,

Dr James E. Pearson, Consultant Technical Editor of the Terrestrial Manual, who contributed hugely to editing and harmonising the contents, but also in collating and incorporating Member Country comments,

Members of both the OIE Scientific and Technical Department and the Publications Department, for their assistance.

Dr Barry O’Neill
President of the OIE International Committee

January 2008
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INTRODUCTION
(How to use this Terrestrial Manual)

• Arrangement of the Terrestrial Manual

Part 1, the beginning of this Terrestrial Manual, contains eleven introductory chapters that deal with a variety of general subjects of interest to veterinary laboratory diagnosticians. These chapters are intended to give a brief introduction to their subjects. They are to be regarded as background information rather than standards.

The main part of the Terrestrial Manual (Part 2) covers standards for diagnostic tests and vaccines for the diseases listed in the OIE Terrestrial Animal Health Code. The diseases are in alphabetical order, subdivided by animal host species. OIE listed diseases are transmissible diseases that have the potential for very serious and rapid spread, irrespective of national borders. They have particularly serious socio-economic or public health consequences and are of major importance in the international trade of animals and animal products.

Four of the diseases in Section 2.9 are included in some individual species sections, but these chapters cover several species and thus give a broader description. Some additional diseases that may also be of importance to trade but that do not have a chapter in the Terrestrial Code are also included in Section 2.9.

The contributors of all the chapters are listed on pages xxii–xxxv, but the final responsibility for the content of the Terrestrial Manual lies with the International Committee of the OIE.

There is an alphabetical index of the diseases at the end of Volume 2.

• Format of chapters

Each disease chapter includes a summary intended to provide information for veterinary officials and other readers who need a general overview of the tests and vaccines available for the disease. This is followed by a text giving greater detail for laboratory workers. In each disease chapter, Part A gives a general introduction to the disease, Part B deals with laboratory diagnosis of the disease, and Part C (where appropriate) with the requirements for vaccines or in vivo diagnostic biologicals. The information concerning production and control of vaccines or diagnostics is given as an example: it is not always necessary to follow these when there are scientifically justifiable reasons for using alternative approaches. Bibliographic references that provide further information are listed at the end of each chapter.

• Explanation of the tests described and of the table on pages xi–xiv

The table on pages xi–xiv lists diagnostic tests in two categories: ‘prescribed’ and ‘alternative’. Prescribed tests are those that are required by the Terrestrial Animal Health Code for the testing of animals before they are moved internationally. In the Terrestrial Manual these tests are printed in blue. At present it is not possible to have prescribed tests for every listed disease. ‘Alternative tests’ are those that are suitable for the diagnosis of disease within a local setting, and can also be used in the import/export of animals after bilateral agreement. There are often other tests described in the chapters, which may also be of some practical value in local situations or which may still be under development.
• List of OIE Reference Laboratories

A list of OIE Reference Laboratories is given in Part 3 of this Terrestrial Manual. These laboratories have been designated by the OIE as centres of excellence with expertise in their particular field. They are able to provide advice to other laboratories on methodology. In some cases standard strains of micro-organisms or reference reagents (e.g. antisera, antigens) can also be obtained from the reference laboratories.

The list of OIE Reference Laboratories will be updated by the International Committee of the OIE each year. The revised list is available on the OIE Web site.

* *
* *
The table below lists diagnostic tests in two categories: ‘prescribed’ and ‘alternative’. Prescribed tests are required by the OIE Terrestrial Animal Health Code for the international movement of animals and animal products and are considered optimal for determining the health status of animals. In the Terrestrial Manual these tests are printed in blue. At present it is not possible to have prescribed tests for every listed disease. Alternative tests are those that are suitable for the diagnosis of disease within a local setting, and can also be used in the import/export of animals after bilateral agreement. There are often other tests described in the chapters that may also be of some practical value in local situations or that may still be under development.

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* Please refer to Terrestrial Manual chapters to verify which method is prescribed.
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<td>BBAT, CF</td>
<td>Brucellin test, FPA</td>
</tr>
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<td>2.7.3/4.</td>
<td>Caprine arthritis/encephalitis &amp; Maedi-visna</td>
<td>AGID, ELISA</td>
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<td>2.7.5.</td>
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<td>2.7.6.</td>
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<td>2.7.7.</td>
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<td>Ovine epididymitis (<em>Brucella ovis</em>)</td>
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<td>2.7.10.</td>
<td>Ovine pulmonary adenocarcinoma (adenomatosis)</td>
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<td>2.7.11.</td>
<td>Peste des petits ruminants</td>
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<td>2.8.1.</td>
<td>African swine fever</td>
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<td>2.8.3.</td>
<td>Classical swine fever (hog cholera)</td>
<td>ELISA, FAVN, NPLA</td>
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<tr>
<td>Chapter No.</td>
<td>Disease name</td>
<td>Prescribed tests</td>
<td>Alternative tests</td>
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<td>2.8.4.</td>
<td>Nipah virus encephalitis</td>
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<td>2.8.5.</td>
<td>Porcine brucellosis</td>
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<td>2.8.6.</td>
<td>Porcine cysticercosis</td>
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<td>Porcine reproductive and respiratory syndrome</td>
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<td>ELISA, IFA, IPMA</td>
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<td>Swine influenza</td>
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<td>2.8.9.</td>
<td>Swine vesicular disease</td>
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<td>2.8.10.</td>
<td>Teschovirus encephalomyelitis (previously enterovirus encephalomyelitis or Teschen/Talfan disease)</td>
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<td>Bunyaviral diseases of animals (excluding Rift Valley fever)</td>
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<td>Camelpox</td>
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<td>2.9.3.</td>
<td><em>Campylobacter jejuni</em> and <em>C. coli</em></td>
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<td>2.9.4.</td>
<td>Cryptosporidiosis</td>
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<td>2.9.5.</td>
<td>Cysticercosis</td>
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<td>Agent id.</td>
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<td>2.9.6.</td>
<td>Hendra and Nipah virus diseases</td>
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<td>2.9.8.</td>
<td><em>Listeria monocytogenes</em></td>
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<td>2.9.8.</td>
<td>Mange</td>
<td>–</td>
<td>Agent id.</td>
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<td>2.9.9.</td>
<td>Salmonellosis</td>
<td>–</td>
<td>Agent id.</td>
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<tr>
<td>2.9.10.</td>
<td>Toxoplasmosis</td>
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<tr>
<td>2.9.11.</td>
<td>Verocyctotoxigenic <em>Escherichia coli</em></td>
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<tr>
<td>2.9.12.</td>
<td>Zoonoses transmissible from non-human primates</td>
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</table>

*Note:* The tests prescribed by the Terrestrial Animal Health Code for the purposes of international trade are printed in blue in this Terrestrial Manual.

**Abbreviations**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>Agent id.</td>
<td>Agent identification</td>
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<tr>
<td>Agg.</td>
<td>Agglutination test</td>
</tr>
<tr>
<td>AGID</td>
<td>Agar gel immunodiffusion</td>
</tr>
<tr>
<td>BBAT</td>
<td>Buffered Brucella antigen test</td>
</tr>
<tr>
<td>CAT</td>
<td>Card agglutination test</td>
</tr>
<tr>
<td>CF</td>
<td>Complement fixation</td>
</tr>
<tr>
<td>DTH</td>
<td>Delayed-type hypersensitivity</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>FAVN</td>
<td>Fluorescent antibody virus neutralisation</td>
</tr>
<tr>
<td>FPA</td>
<td>Fluorescence polarisation assay</td>
</tr>
<tr>
<td>HI</td>
<td>Haemagglutination inhibition</td>
</tr>
<tr>
<td>IFA</td>
<td>Indirect fluorescent antibody</td>
</tr>
<tr>
<td>IPMA</td>
<td>Immunoperoxidase monolayer assay</td>
</tr>
<tr>
<td>MAT</td>
<td>Microscopic agglutination test</td>
</tr>
<tr>
<td>NPLA</td>
<td>Neutralising peroxidase-linked assay</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PRN</td>
<td>Plaque reduction neutralisation</td>
</tr>
<tr>
<td>VN</td>
<td>Virus neutralisation</td>
</tr>
<tr>
<td>–</td>
<td>No test designated yet</td>
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### COMMON ABBREVIATIONS USED IN THIS TERRESTRIAL MANUAL

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABTS</td>
<td>2,2’-azino-di-(3-ethyl-benzthiazoline)-6-sulphonic acid</td>
</tr>
<tr>
<td>AGID</td>
<td>Agar gel immunodiffusion</td>
</tr>
<tr>
<td>ATCC</td>
<td>American type culture collection</td>
</tr>
<tr>
<td>BBAT</td>
<td>Buffered <em>Brucella</em> antigen test</td>
</tr>
<tr>
<td>BFK</td>
<td>Bovine fetal kidney (cells)</td>
</tr>
<tr>
<td>BGPS</td>
<td>Beef extract-glucose-peptone-serum (medium)</td>
</tr>
<tr>
<td>BHK</td>
<td>Baby hamster kidney (cell line)</td>
</tr>
<tr>
<td>BLP</td>
<td>Buffered lactose peptone</td>
</tr>
<tr>
<td>BPAT</td>
<td>Buffered plate antigen test</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>BSF</td>
<td>Bovine serum factors</td>
</tr>
<tr>
<td>CAM</td>
<td>Chorioallantoic membrane</td>
</tr>
<tr>
<td>CEF</td>
<td>Chicken embryo fibroblast</td>
</tr>
<tr>
<td>CF</td>
<td>Complement fixation (test)</td>
</tr>
<tr>
<td>CFU</td>
<td>Colony-forming unit</td>
</tr>
<tr>
<td>CIEP</td>
<td>Counter immunoelectrophoresis</td>
</tr>
<tr>
<td>CK</td>
<td>Calf kidney (cells)</td>
</tr>
<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>CPE</td>
<td>Cytopathic effect</td>
</tr>
<tr>
<td>CPLM</td>
<td>Cysteine-peptone-liver infusion maltose (medium)</td>
</tr>
<tr>
<td>CSY</td>
<td>Casein-sucrose-yeast (agar)</td>
</tr>
<tr>
<td>DEPC</td>
<td>Diethyl pyrocarbonate</td>
</tr>
<tr>
<td>DEAE</td>
<td>Diethylaminoethyl</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modified Eagle’s medium</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulphide</td>
</tr>
<tr>
<td>DTH</td>
<td>Delayed-type hypersensitivity</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylene diamine tetra-acetic acid</td>
</tr>
<tr>
<td>EGTA</td>
<td>Ethylene glycol tetra-acetic acid</td>
</tr>
<tr>
<td>EID</td>
<td>Egg-infective dose</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
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<tr>
<td>EMTM</td>
<td>Evans’ modified Tobie’s medium</td>
</tr>
<tr>
<td>EYL</td>
<td>Earle’s yeast lactalbumin (balanced salt solution)</td>
</tr>
<tr>
<td>FAT</td>
<td>Fluorescent antibody test</td>
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<tr>
<td>FAVN</td>
<td>Fluorescent antibody virus neutralisation</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
</tr>
<tr>
<td>FITC</td>
<td>Fluorescein isothiocyanate</td>
</tr>
<tr>
<td>FLK</td>
<td>Fetal lamb kidney (cells)</td>
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<tr>
<td>FPA</td>
<td>Fluorescence polarisation assay</td>
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<tr>
<td>G</td>
<td>Relative centrifugal force</td>
</tr>
<tr>
<td>GIT</td>
<td>Growth inhibition test</td>
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<tr>
<td>HA</td>
<td>Haemagglutination</td>
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<tr>
<td>HAD</td>
<td>Haemadsorption</td>
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<tr>
<td>HBSS</td>
<td>Hanks’ balanced salt solution</td>
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<tr>
<td>HEP</td>
<td>High-egg-passage (virus)</td>
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<tr>
<td>HEPES</td>
<td>N-2-hydroxyethylpiperazine, N-2-ethanesulphonic acid (buffer)</td>
</tr>
<tr>
<td>HI</td>
<td>Haemagglutination inhibition</td>
</tr>
<tr>
<td>HRPO</td>
<td>Horseradish peroxidase</td>
</tr>
<tr>
<td>IB</td>
<td>Immunoblot test</td>
</tr>
<tr>
<td>ICFTU</td>
<td>International complement fixation test unit</td>
</tr>
<tr>
<td>ICPI</td>
<td>Intracerebral pathogenicity index</td>
</tr>
<tr>
<td>ID₅₀</td>
<td>Median infectious dose</td>
</tr>
<tr>
<td>IFA</td>
<td>Indirect fluorescent antibody (test)</td>
</tr>
<tr>
<td>IHA</td>
<td>Indirect haemagglutination</td>
</tr>
<tr>
<td>IPMA</td>
<td>Immunoperoxidase monolayer assay</td>
</tr>
<tr>
<td>IU</td>
<td>International units</td>
</tr>
<tr>
<td>IVPI</td>
<td>Intravenous pathogenicity index</td>
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<tr>
<td>LA</td>
<td>Latex agglutination</td>
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<td>LD</td>
<td>Lethal dose</td>
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<td>LEP</td>
<td>Low egg passage (virus)</td>
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<td>LPS</td>
<td>Lipopolysaccharide</td>
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<td>MAb</td>
<td>Monoclonal antibody</td>
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<td>MAT</td>
<td>Microscopic agglutination test</td>
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<tr>
<td>MCS</td>
<td>Master cell stock</td>
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<tr>
<td>MDBK</td>
<td>Madin-Darby bovine kidney (cell line)</td>
</tr>
<tr>
<td>MDT</td>
<td>Mean death time</td>
</tr>
<tr>
<td>MEM</td>
<td>Minimal essential medium</td>
</tr>
<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
</tr>
<tr>
<td>MLV</td>
<td>Modified live virus (vaccine)</td>
</tr>
<tr>
<td>m.o.i.</td>
<td>multiplicity of infection</td>
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</table>

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1 American Type Culture Collection, P.O. Box 1549, Manassas, Virginia 20108, United States of America.
<table>
<thead>
<tr>
<th>Abbreviation</th>
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<tr>
<td>MSV</td>
<td>Master seed virus</td>
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<tr>
<td>NI</td>
<td>Neutralisation index</td>
</tr>
<tr>
<td>OGP</td>
<td>1-octyl-beta-D-glucopyranoside (buffer)</td>
</tr>
<tr>
<td>OPD</td>
<td>Orthophenylamine (chromogen)</td>
</tr>
<tr>
<td>OPG</td>
<td>Oxalase-phenol-glycerin (preservative solution)</td>
</tr>
<tr>
<td>ORF</td>
<td>Open reading frame</td>
</tr>
<tr>
<td>PAGE</td>
<td>Polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>PAP</td>
<td>Peroxidase–antiperoxidase (staining procedure)</td>
</tr>
<tr>
<td>PAS</td>
<td>Periodic acid-Schiff (reaction)</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
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<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PD</td>
<td>Protective dose</td>
</tr>
<tr>
<td>PFGE</td>
<td>Pulsed field gel electrophoresis</td>
</tr>
<tr>
<td>PFU</td>
<td>Plaque-forming unit</td>
</tr>
<tr>
<td>PHA</td>
<td>Passive haemagglutination (test)</td>
</tr>
<tr>
<td>PPD</td>
<td>Purified protein derivative</td>
</tr>
<tr>
<td>PPLO</td>
<td>Pleuropneumonia-like organisms</td>
</tr>
<tr>
<td>PRN</td>
<td>Plaque reduction neutralisation</td>
</tr>
<tr>
<td>PSG</td>
<td>Phosphate buffered saline glucose</td>
</tr>
<tr>
<td>RBC</td>
<td>Red blood cell</td>
</tr>
<tr>
<td>RFLP</td>
<td>Restriction fragment length polymorphism</td>
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<tr>
<td>RK</td>
<td>Rabbit kidney</td>
</tr>
<tr>
<td>RPM</td>
<td>Revolutions per minute</td>
</tr>
<tr>
<td>RSA</td>
<td>Rapid serum agglutination</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Reverse-transcription polymerase chain reaction</td>
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<tr>
<td>SAT</td>
<td>Serum agglutination test</td>
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<tr>
<td>SDS</td>
<td>Sodium dodecyl sulphate</td>
</tr>
<tr>
<td>SPF</td>
<td>Specific pathogen free</td>
</tr>
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<td>SPG</td>
<td>Sucrose phosphate glutamic acid</td>
</tr>
<tr>
<td>TCID&lt;sub&gt;50&lt;/sub&gt;</td>
<td>Median tissue culture infective dose</td>
</tr>
<tr>
<td>TMB</td>
<td>Tetramethyl benzidine</td>
</tr>
<tr>
<td>TSI</td>
<td>Triple sugar iron (medium)</td>
</tr>
<tr>
<td>VB</td>
<td>Veronal buffer</td>
</tr>
<tr>
<td>VBS</td>
<td>Veronal buffered saline</td>
</tr>
<tr>
<td>Vero</td>
<td>African green monkey kidney (cells)</td>
</tr>
<tr>
<td>VN</td>
<td>Virus neutralisation</td>
</tr>
</tbody>
</table>

* *
GLOSSARY OF TERMS

The definitions given below have been selected and restricted to those that are likely to be useful to users of this OIE Terrestrial Manual.

• Absorbance/optical density
Absorbance and optical density are terms used to indicate the strength of reaction. A spectrophotometer is used to measure the amount of light of a specific wavelength that a sample absorbs and the absorbance is proportional to the amount of a particular analyte present.

• Accuracy
Nearness of a test value to the expected value for a reference standard reagent of known activity or titre.

• Assay
Synonymous with test or test method, e.g. enzyme immunoassay, complement fixation test or polymerase chain reaction tests.

• Batch
All vaccine or other reagent, such as antigen or antisera, derived from the same homogeneous bulk and identified by a unique code number.

• Cell line
A stably transformed line of cells that has a high capacity for multiplication in vitro.

• Centrifugation
Throughout the text, the rate of centrifugation has been expressed as the Relative Centrifugal Force, denoted by 'g'. The formula is:

\[
\frac{(RPM \times 0.10472)^2 \times \text{Radius (cm)}}{980} = g
\]

where RPM is the rotor speed in revolutions per minute, and where Radius (cm) is the radius of the rotor arm, to the bottom of the tube, in centimetres.

It may be necessary to calculate the RPM required to achieve a given value of g, with a particular rotor. The formula is:

\[
RPM = \frac{\sqrt{g \times 980}}{0.10472 \times \text{Radius (cm)}}
\]

• Cross-reaction
See ‘False-positive reaction’.

• Cut-off/threshold
Test result value selected for distinguishing between negative and positive results; may include indeterminate or suspicious zone.
• **Dilutions**

Where dilutions are given for making up liquid reagents, they are expressed as, for example, 1 in 4 or 1/4, meaning one part added to three parts, i.e. a 25% solution of A in B.

• v/v – This is volume to volume (two liquids).
• w/v – This is weight to volume (solid added to a liquid).

• **Dilutions used in virus neutralisation tests**

There are two different conventions used in expressing the dilution used in virus neutralisation (VN) tests. In Europe, it is customary to express the dilution before the addition of the antigen, but in the United States of America and elsewhere, it is usual to express dilutions after the addition of antigen.

These alternative conventions are expressed in the *Terrestrial Manual* as ‘initial dilution’ or ‘final dilution’, respectively.

• **Efficacy**

Specific ability of the biological product to produce the result for which it is offered when used under the conditions recommended by the manufacturer.

• **Equivalency testing**

Determination of certain assay performance characteristics of new and/or different test methods by means of an interlaboratory comparison to a standard test method; implied in this definition is that participating laboratories are using their own test methods, reagents and controls and that results are expressed qualitatively.

• **False-negative reaction**

Negative reactivity in an assay of a test sample obtained from an animal exposed to or infected with the organism in question, may be due to lack of analytical sensitivity, restricted analytical specificity or analyte degradation, decreases diagnostic sensitivity.

• **False-positive reaction**

Positive reactivity in an assay that is not attributable to exposure to or infection with the organism in question, maybe due to immunological cross-reactivity, cross-contamination of the test sample or non-specific reactions, decreases diagnostic specificity.

• **Final product (lot)**

All sealed final containers that have been filled from the same homogenous batch of vaccine in one working session, freeze-dried together in one continuous operation (if applicable), sealed in one working session, and identified by a unique code number.

• **Harmonisation**

The result of an agreement between laboratories to calibrate similar test methods, adjust diagnostic thresholds and express test data in such a manner as to allow uniform interpretation of results between laboratories.

• **Incidence**

Estimate of the rate of new infections in a susceptible population over a defined period of time; not to be confused with prevalence.

• **In-house checks**

All quality assurance activities within a laboratory directly related to the monitoring, validation, and maintenance of assay performance and technical proficiency.

• **In-process control**

Test procedures carried out during manufacture of a biological product to ensure that the product will comply with the agreed quality standards.
• **Inter-laboratory comparison (ring test)**

Any evaluation of assay performance and/or laboratory competence in the testing of defined samples by two or more laboratories; one laboratory may act as the reference in defining test sample attributes.

• **Master cell (line, seed, stock)**

Collection of aliquots of cells of defined passage level, for use in the preparation or testing of a biological product, distributed into containers in a single operation, processed together and stored in such a manner as to ensure uniformity and stability and to prevent contamination.

• **Master seed (agent, strain)**

Collection of aliquots of an organism at a specific passage level, from which all other seed passages are derived, which are obtained from a single bulk, distributed into containers in a single operation and processed together and stored in such a manner as to ensure uniformity and stability and to prevent contamination.

• **Performance characteristic**

An attribute of a test method that may include analytical sensitivity and specificity, accuracy and precision, diagnostic sensitivity and specificity and/or repeatability and reproducibility.

• **Potency**

Relative strength of a biological product as determined by appropriate test methods. (Initially the potency is measured using an efficacy test in animals. Later this may be correlated with tests of antigen content, or antibody response, for routine batch potency tests.)

• **Precision**

The degree of dispersion of results for a repeatedly tested sample expressed by statistical methods such as standard deviation or confidence limits.

• **Predictive value (negative)**

The probability that an animal is free from exposure or infection given that it tests negative; predictive values are a function of the DSe (diagnostic sensitivity) and DSp (diagnostic specificity) of the diagnostic assay and the prevalence of infection.

• **Predictive value (positive)**

The probability that an animal has been exposed or infected given that it tests positive; predictive values are a function of the DSe and DSp of the diagnostic assay and the prevalence of infection.

• **Prevalence**

Estimate of the proportion of infected animals in a population at one given point in time; not to be confused with incidence.

• **Primary cells**

A pool of original cells derived from normal tissue up to and including the tenth subculture.

• **Production seed**

An organism at a specified passage level that is used without further propagation for initiating preparation of a production bulk.

• **Proficiency testing**

One measure of laboratory competence derived by means of an interlaboratory comparison; implied in this definition is that participating laboratories are using the same test methods, reagents and controls and that results are expressed qualitatively.
• **Purity**

Quality of a biological product prepared to a final form and:

a) Relatively free from any extraneous microorganisms and extraneous material (organic or inorganic) as determined by test methods appropriate to the product; and

b) Free from extraneous microorganisms or material which could adversely affect the safety, potency or efficacy of the product.

• **Reference animal**

Any animal for which the infection status can be defined in unequivocal terms; may include diseased, infected, vaccinated, immunised or naïve animals.

• **Reference Laboratory**

Laboratory of recognised scientific and diagnostic expertise for a particular animal disease and/or testing methodology; includes capability for characterising and assigning values to reference reagents and samples.

• **Repeatability**

Level of agreement between replicates of a sample both within and between runs of the same test method in a given laboratory.

• **Reproducibility**

Ability of a test method to provide consistent results when applied to aliquots of the same sample tested by the same method in different laboratories.

• **Room temperature**

The term ‘room temperature’ is intended to imply the temperature of a comfortable working environment. Precise limits for this cannot be set, but guiding figures are 18–25°C. Where a test specifies room temperature, this should be achieved, with air conditioning if necessary; otherwise the test parameters may be affected.

• **Safety**

Freedom from properties causing undue local or systemic reactions when used as recommended or suggested by the manufacturer and without known hazard to in-contact animals, humans and the environment.

• **Sample**

Material that is derived from a specimen and used for testing purposes.

• **Sensitivity (analytical)**

Synonymous with ‘Limit of Detection’, smallest detectable amount of analyte that can be measured with a defined certainty; analyte may include antibodies, antigens, nucleic acids or live organisms.

• **Sensitivity (diagnostic)**

Proportion of known infected reference animals that test positive in the assay; infected animals that test negative are considered to have false-negative results.

• **Sensitivity (relative)**

Proportion of reference animals defined as positive by one or a combination of test methods that also test positive in the assay being compared.

• **Specific pathogen free (SPF)**

Animals that have been shown by the use of appropriate tests to be free from specified pathogenic microorganisms, and also refers to eggs derived from SPF birds.
• **Specificity (analytical)**
  Degree to which the assay distinguishes between the target analyte and other components in the sample matrix; the higher the analytical specificity, the lower the level of false-positives.

• **Specificity (diagnostic)**
  Proportion of known uninfected reference animals that test negative in the assay; uninfected reference animals that test positive are considered to have false-positive results.

• **Specificity (relative)**
  Proportion of reference animals defined as negative by one or a combination of test methods that also test negative in the assay being compared.

• **Specimen**
  Material submitted for testing.

• **Standard Reagents**

  • **International Standard Reagents**
    Standard reagents by which all other reagents and assays are calibrated; prepared and distributed by an International Reference Laboratory.

  • **National Standard Reagents**
    Standard reagents calibrated by comparison with International Standard Reagents; prepared and distributed by a National Reference Laboratory.

  • **Working Standards (reagents)**
    Standard reagents calibrated by comparison with the National Standard Reagent, or, in the absence of a National Standard Reagent, calibrated against a well-characterised in-house standard reagent; included in routine diagnostic tests as a control and/or for normalisation of test results.

• **Sterility**
  Freedom from viable contaminating microorganisms, as demonstrated by approved and appropriate tests.

• **Test method**
  Specified technical procedure for detection of an analyte (synonymous with assay).

• **Tests**

  • **Prescribed**
    Test methods that are required by the OIE *Terrestrial Animal Health Code* for the international movement of animals and animal products and that are considered optimal for determining the health status of animals.

  • **Alternative**
    Test methods considered in this *Terrestrial Manual* to be suitable for the diagnosis of disease in a local situation, and that can also be used for import/export by bilateral agreement.

  • **Screening**
    Tests of high diagnostic sensitivity suitable for large-scale application.

  • **Confirmatory**
    Test methods of high diagnostic specificity that are used to confirm results, usually positive results, derived from other test methods.

• **Working seed**
  Organism at a passage level between master seed and production seed.
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The chapters in the Terrestrial Manual are prepared by invited contributors. In accordance with OIE standard procedure, all chapters are circulated to OIE Member Countries and to other experts in the disease for comment. The OIE Biological Standards Commission and the Consultant Editor then modify the text to take account of comments received. Once this review process is complete and the text is finalised, the Terrestrial Manual is presented to the OIE International Committee during its annual General Session for adoption before it is printed. The Terrestrial Manual is thus deemed to be an OIE Standard Text that has come into being by international agreement. For this reason, the names of the contributors are not shown on individual chapters but are listed below. The Biological Standards Commission greatly appreciates the work of the following contributors:

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2.7.7. Enzootic abortion of ewes (ovine chlamydiosis)  
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Moredun Research Institute, International Research Centre, Pentlands Science Park Bush Loan, Penicuik EH26 0PZ, UK.
2.7.8. Nairobi sheep disease (see chapter 2.9.1) Dr G.H. Gerdes
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2.7.9. Ovine epididymitis (Brucella ovis) Dr B. Garin-Bastuji
EU Community/OIE & FAO Reference Laboratory for Brucellosis, Unité Zoonoses Bactériennes, AFSSA, 94706 Maisons-Alfort Cedex, France.

2.7.10. Ovine pulmonary adenocarcinoma (adenomatosis) Dr M.J. Sharp
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2.7.11. Peste des petits ruminants Dr A. Diallo
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2.7.12. Salmonellosis (S. abortusovis) (see chapter 2.9.9) Dr R. Davies
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2.7.13. Scrapie Dr D. Matthews, Dr M.M. Simmons, Mr M. Stack & Prof. G.A.H. Wells
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2.7.14. Sheep pox and goat pox Dr R.P. Kitching
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2.8.1. African swine fever Dr C.A.L. Oura
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2.8.2. Atrophic rhinitis of swine Dr K.B. Register
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2.8.3. Classical swine fever (hog cholera) Dr T. Drew
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2.8.4. Nipah virus encephalitis (see chapter 2.9.6)
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2.8.5. Porcine brucellosis
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2.8.6. Porcine cysticercosis (see chapter 2.9.5)
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2.8.7. Porcine reproductive and respiratory syndrome
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2.8.8. Swine influenza
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2.8.10. Teschovirus encephalomyelitis (previously enterovirus encephalomyelitis or Teschen/Talfan disease)
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2.8.11. Transmissible gastroenteritis

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2.9.1. Bunyaviral diseases of animals (excluding Rift Valley fever)

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2.9.2. Camelpox

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2.9.4. Cryptosporidiosis

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2.9.5. Cysticercosis

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2.9.6. Hendra and Nipah virus diseases

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2.9.9. Salmonellosis

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2.9.10. Toxoplasmosis

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2.9.11. Verocytotoxigenic Escherichia coli

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2.9.12. Zoonoses transmissible from non-human primates

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SECTION 2.4.

BOVIDAE

CHAPTER 2.4.1.

BOVINE ANAPLASMOSIS

SUMMARY

Definition of the disease: Bovine anaplasmosis results from infection with Anaplasma marginale. A second species, A. centrale, has long been recognised. Whether it truly represents a separate species is unclear. Anaplasma marginale is responsible for almost all outbreaks of clinical disease. A third species, A. phagocytophilum, has recently been reported to infect cattle. However, natural infection appears to be rare and A. phagocytophilum does not cause clinical disease. The organism is classified in the genus Anaplasma belonging to the family Anaplasmataceae of the order Rickettsiales.

Description of the disease: Anaemia and jaundice are characteristic signs of anaplasmosis, but the clinical disease can only be confirmed by identifying the organism. Once infected, cattle may remain carriers for life, and identification of these animals depends on the detection of specific antibodies using serological tests, or of rickettsial DNA using amplification techniques.

Identification of the agent: Microscopic examination of blood or organ smears stained with Giemsa stain is the most common method of identifying Anaplasma in clinically affected animals. In these smears, A. marginale appear as dense, rounded, intraerythrocytic bodies approximately 0.3–1.0 µm in diameter with most situated on or near the margin of the erythrocyte. Anaplasma centrale is similar in appearance, but most of the organisms are situated away from the margin of the erythrocyte. It can be difficult to differentiate A. marginale from A. centrale in a stained smear, particularly with low levels of rickettsmia. Commercial stains that give very rapid staining of Anaplasma are available in some countries.

It is important that smears be well prepared and free from foreign matter. Smears from live cattle should preferably be prepared from blood drawn from the jugular vein or another large vessel. For post-mortem diagnosis, smears should be prepared from internal organs (including liver, kidney, heart and lungs) and from blood retained in peripheral vessels. The latter are particularly desirable if post-mortem decomposition is advanced.

Serological tests: A competitive enzyme-linked immunosorbent assay (C-ELISA) has been demonstrated to have good sensitivity in detecting carrier animals. Card agglutination, indirect ELISA, dot ELISA and indirect fluorescent antibody tests also can be used. The complement fixation (CF) test is no longer considered a reliable test for disease certification of individual animals due to variable sensitivity. Cross reactivity between Anaplasma spp. can complicate interpretation of serological tests. In general, the C-ELISA has the best specificity, with well characterized cross-reactivity only between A. marginale and A. centrale.

Nucleic-acid-based tests have been used experimentally, and are capable of detecting the presence of low-level infection in carrier cattle and tick vectors. Caution is warranted with polymerase chain reaction-based assays when used diagnostically, as a nested reaction is necessary to identify low-level carriers and nonspecific amplification can occur.

Requirements for vaccines and diagnostic biologicals: Live vaccines are used in several countries to protect cattle against A. marginale infection. A vaccine consisting of live A. centrale is most widely used and gives partial protection against challenge with virulent A. marginale.
Anaplasma centrale vaccine is provided in chilled or frozen forms. Quality control is very important as other blood-borne agents that may be present in donor cattle can contaminate vaccines and be disseminated broadly. For this reason, frozen vaccine is recommended as it allows thorough post-production quality control, which limits the risk of contamination with other pathogens.

Anaplasma centrale vaccine is not entirely safe. A practical recommendation is to restrict its use, as far as possible, to calves, as nonspecific immunity will minimise the risk of some vaccine reactions that may require treatment with tetracycline or imidocarb. Partial immunity develops in 6–8 weeks and lasts for several years after a single vaccination.

A. INTRODUCTION

Outbreaks of bovine anaplasmosis are usually due to infection with *Anaplasma marginale*. *Anaplasma centrale* is capable of producing a moderate degree of anaemia, but clinical outbreaks in the field are extremely rare. Appendages associated with the *Anaplasma* body have been observed in certain isolates of *A. marginale* (19); although this parasite has been termed *A. caudatum*, it is not considered to be a separate species. A third species, *A. phagocytophilum*, has recently been reported to infect cattle. However, natural infection appears to be rare and *A. phagocytophilum* does not cause clinical disease (8, 20).

*Anaplasma marginale* occurs in most tropical and subtropical countries, and in some more temperate regions. *Anaplasma centrale* was first described from South Africa. The organism has since been imported by other countries – including Australia and some countries in South America, South-East Asia and the Middle East – for use as a vaccine against *A. marginale*.

*Anaplasma* species were originally regarded as protozoan parasites, but later research showed they had no significant attributes to justify this description. Since the last major accepted revision of the taxonomy in 2001 (9), the Family *Anaplasmataceae* (Order *Rickettsiales*) is now composed of four genera, *Anaplasma*, *Ehrlichia*, *Neorickettsia*, and *Wolbachia*. The genus *Aegyptianella* is retained within the Family *Anaplasmataceae* as genus *incertae sedis*. The revised genus *Anaplasma* now contains *Anaplasma marginale* as the type species, *A. phagocytophilum* (formerly *Ehrlichia phagocytophila*, *E. equi* and the unclassified agent of human granulocytic ehrlichiosis), *A. platys*, and *A. bovis*. *Haemobartonella* and *Eperythrozoon* are now considered most closely related to the mycoplasmas.

*Anaplasma* species are transmitted either mechanically or biologically by arthropod vectors. Reviews based on careful study of reported transmission experiments list up to 19 different ticks as capable of transmitting *A. marginale* experimentally (15, 21). These are: *Argas persicus*, *Omithodoros lahorensis*, *Boophilus annulatus*, *B. calcatus*, *B. decoloratus*, *B. microplus*, *Dermacentor albipictus*, *D. andersoni*, *D. hunterni*, *D. occidentalis*, *D. variabilis*, *Hyalomma excavatum*, *H. rufipes*, *Ixodes ricinus*, *I. scapularis*, *Rhipicephalus bursa*, *R. evertsi*, *R. sanguineus* and *R. simus*. The authors concluded that some of these reports, including those of *R. bursa*, *H. excavatum* and *O. lahorensis*, were not entirely convincing, and that the ticks identified as *A. persicus* were probably either *A. sanchezi* or *A. radiatus*. Intrastadial or transstadial transmission is the usual mode, even in the one-host *Boophilus* species. Male ticks may be particularly important as vectors; they can become persistently infected and serve as a reservoir for infection (17). Experimental demonstration of vector competence does not necessarily imply a role in transmission in the field. However, *Boophilus* species are clearly important vectors of anaplasmosis in countries such as Australia and countries in Africa, and some species of *Dermacentor* are efficient vectors in the United States of America (USA).

Various other biting arthropods have been implicated as mechanical vectors, particularly in the USA. Experimental transmission has been demonstrated with a number of species of *Tabanus* (horseflies), and with mosquitoes of the genus *Psorophora* (15, 33). The importance of biting insects in the natural transmission of anaplasmosis appears to vary greatly from region to region. *Anaplasma marginale* also can be readily transmitted during vaccination against other diseases unless a fresh or sterilised needle is used for injecting each animal. Similar transmission by means of unsterilised surgical instruments has been described.

The main biological vectors of *A. centrale* appear to be multihost ticks peculiar to Africa, including *R. simus*. The common cattle tick (*B. microplus*) has not been shown to be a vector. This is of relevance where *A. centrale* is used as a vaccine in *B. microplus*-infested regions.

B. DIAGNOSTIC TECHNIQUES

The most marked clinical signs of anaplasmosis are anaemia and jaundice, the latter occurring late in the disease. Haemoglobinemia and haemoglobinuria are not present, and this may assist in the differential diagnosis of anaplasmosis from babesiosis, which is often endemic in the same regions. The disease can only be confirmed, however, by identification of the organism.
Chapter 2.4.1. – Bovine anaplasmosis

1. Identification of the agent

Samples from live cattle should include thin blood smears and blood collected into an anticoagulant. Air-dried thin blood smears will keep satisfactorily at room temperature for at least 1 week. The blood sample in anticoagulant should be held and transferred at 4°C, unless it can reach the laboratory within a few hours. This sample is useful for preparing fresh smears if those submitted are not satisfactory. In addition, a low packed cell volume and/or erythrocyte count can help to substantiate the involvement of *Anaplasma* when only small numbers of the parasites are detected in smears, such as may occur in the recovery stage of the disease.

In contrast to *Babesia bovis*, *Anaplasma* do not accumulate in capillaries, so blood drawn from the jugular or other large vessel is satisfactory. Because of the rather indistinctive morphology of *Anaplasma*, it is essential that smears be well prepared and free from foreign matter, as specks of debris can confuse diagnosis. Thick blood films as used for the diagnosis of babesiosis are not appropriate for the diagnosis of anaplasmosis, as *Anaplasma* are difficult to identify once they become dissociated from erythrocytes.

Samples from dead animals should include air-dried thin smears from the liver, kidney, heart and lungs and from a peripheral blood vessel. The latter is particularly recommended should there be a significant delay before post-mortem examination because, under these circumstances, bacterial contamination of organ smears often makes identification of *Anaplasma* equivocal. Brain smears, which are useful for the diagnosis of some forms of babesiosis, are of no direct value for diagnosing anaplasmosis, but should be included for differential diagnosis where appropriate.

Blood from organs, rather than organ tissues *per se*, is required for smear preparation, as the aim is to be able to examine microscopically intact erythrocytes for the presence of *Anaplasma*. Organ-derived blood smears will store satisfactorily at room temperature for several days.

Both blood and organ smears can be stained in 10% Giemsa stain for approximately 30 minutes after fixation in absolute methanol for 1 minute. After staining, the smears are rinsed three or four times with tap water to remove adhering stain, and are then air-dried. Conditions for Giemsa staining vary from laboratory to laboratory. Commercial stains that give very rapid staining of *Anaplasma* are available in some countries. Smears are examined under oil immersion at a magnification of ×700–1000.

*Anaplasma marginale* appear as dense, rounded and deeply stained intraerythrocytic bodies, approximately 0.3–1.0 µm in diameter. Most of these bodies are located on or near the margin of the erythrocyte. This feature distinguishes *A. marginale* from *A. centrale*, as in the latter most of the organisms have a more central location in the erythrocyte. However, particularly at low levels of rickettsemia, differentiation of these two species in smears can be difficult.

The percentage of infected erythrocytes varies with the stage and severity of the disease. Maximum parasitaemias in excess of 50% may occur with *A. marginale*. Multiple infections of individual erythrocytes are common during periods of high parasitaemias.

The infection becomes visible microscopically 2–6 weeks following transmission. During the course of clinical disease, the parasitaemia approximately doubles each day for up to about 10 days, and then decreases at a similar rate. Quite severe anaemia may persist for some weeks after the parasites have become virtually undetectable in blood smears. Following recovery from initial infection, most cattle remain latently infected for life.

An expensive procedure, but one that may occasionally be justified to confirm infection, particularly in latently infected cattle, is the inoculation of blood from the suspect animal into a splenectomised calf. A quantity (up to 500 ml) of the donor’s blood in anticoagulant is inoculated intravenously into the splenectomised calf, which is then tested by blood smear examination at least every 2–3 days. If the donor is infected, *Anaplasma* will be observed in smears from the splenectomised calf generally within 4 weeks, but this period may extend up to 8 weeks.

Nucleic-acid-based tests to detect *A. marginale* infection in carrier cattle have been developed. The analytical sensitivity of polymerase chain reaction (PCR)-based methods has been estimated at 0.0001% infected erythrocytes, but at this level only a proportion of carrier cattle would be detected. A sensitive and potentially specific nested PCR has been used to identify *A. marginale* carrier cattle. This technique is capable of identifying as few as 30 infected erythrocytes per ml of blood, equivalent to a parasitaemia of approximately 0.000001%, well below the lowest levels in carriers. However, nested PCR poses significant quality control problems for routine use.

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1. Commercial stains include Camco-Quik and Diff-Quik, Baxter Scientific Products, McGaw Park, Illinois, USA, and Hema-Quik, Curtin-Matheson, Houston, Texas, USA.
specificity due to nonspecific amplification. An additional step such as restriction enzyme analysis, Southern hybridisation, or sequencing can confirm the specificity of the fragment amplified in nested PCR.

In general, unless animals have been treated or are at a very early stage of infection (<14 days), serology using the competitive enzyme-linked immunosorbent assay (C-ELISA) or card agglutination test (CAT) (see below) is the preferred method of identifying infected animals.

2. Serological tests

*Anaplasma* infections usually persist for the life of the animal. However, except for occasional small recrudescences, *Anaplasma* cannot readily be detected in blood smears after an acute parasitaemic episode. Thus, a number of serological tests have been developed with the aim of detecting persistently infected animals.

A feature of the serological diagnosis of anaplasmosis is the highly variable results with regard to both sensitivity and specificity reported for many of the tests from different laboratories. This is due at least in part to inadequate evaluation of the tests using significant numbers of known positive and negative animals. Importantly, the capacity of several assays to detect known infections of long-standing duration has rarely been adequately addressed. An exception is C-ELISA (see below), which has been validated using true positive and negative animals defined by nested PCR (36), and the card agglutination assay, for which relative sensitivity and specificity in comparison with the C-ELISA has been evaluated (24). Therefore, while most of the tests described in this section are useful for obtaining broad-based epidemiological data, caution is advised on their use for disease certification. Both the C-ELISA and card agglutination test are described in detail below.

It should be noted that there is a high degree of cross-reactivity between *A. marginale* and *A. centrale* in serological tests. While the infecting species can sometimes be identified using antigens from homologous and heterologous species, equivocal results are obtained on many occasions.

a) Competitive enzyme-linked immunosorbent assay

A C-ELISA using a recombinant antigen termed rMSP5 and MSP5-specific monoclonal antibody (MAb) has proven very sensitive and specific for detection of *Anaplasma*-infected animals (18, 27, 35, 37). All *A. marginale* strains tested, *A. ovis* and *A. centrale*, express the MSP5 antigen and induce antibodies against the immunodominant epitope recognised by the MSP5-specific MAb. A recent report suggests that antibodies from cattle experimentally infected with *A. phagocytophilum* will test positive in the C-ELISA (8). However, in another study no cross-reactivity could be demonstrated, and the MAb used in the assay did not react with *A. phagocytophilum* MSP5 in direct binding assays (35). Thus, additional work is necessary to clarify these conflicting results. The test was 100% specific using 261 known negative sera from a non-endemic region, detecting acutely infected cattle as early as 16 days after experimental tick or blood inoculation. An exception was demonstrated to detect cattle that have been experimentally infected as long as 6 years previously (18). In detecting persistently infected cattle from an anaplasmosis-endemic region that were defined as true positive or negative using a nested PCR procedure, the rMSP5 C-ELISA had a sensitivity of 96% and a specificity of 95% (36). An independent study using an indirect ELISA (I-ELISA) validates the use of rMSP5 as a diagnostic antigen (32). However, initial studies suggest that in its current format the indirect rMSP5 ELISA is less sensitive than the C-ELISA (32).

Test results using the rMSP5 C-ELISA are available in less than 2.5 hours. A test kit available commercially contains specific instructions. In general, however, it is conducted as follows.

- **Kit reagents**
  - A 96-well microtitre plate coated with rMSP5 antigen,
  - A 96-well coated adsorption/transfer plate for serum adsorption to reduce background binding,
  - 100 x MAb/peroxidase conjugate,
  - 10 x wash solution and ready-to-use conjugate-diluting buffer,
  - Ready-to-use substrate and stop solutions,
  - Positive and negative controls

- **Test procedure**
  1. Add 70 µl of undiluted serum sample to the coated adsorption/transfer plate and incubate at room temperature for 30 minutes.
  2. Transfer 50 µl per well of the adsorbed serum to the rMSP5-coated plate and incubate at room temperature for 60 minutes.
iii) Discard the serum and wash the plate twice using diluted wash solution.

iv) Add 50 µl per well of the 1 × diluted MAb/peroxidase conjugate to the rMSP5-coated plate, and incubate at room temperature for 20 minutes.

v) Discard the 1 × diluted MAb/peroxidase conjugate and wash the plate four times using diluted wash solution.

vi) Add 50 µl per well of the substrate solution, cover the plate with foil, and incubate for 20 minutes at room temperature.

vii) Add 50 µl per well of stop solution to the substrate solution already in the wells and gently tap the sides of the plate to mix the wells.

viii) Read the plate in the plate reader at 620 nm.

**Test validation**

The mean optical density (OD) of the negative control must range from 0.40 to 2.10. The per cent inhibition of the positive control must be ≥30%.

**Interpretation of the results**

The % inhibition is calculated as follows:

\[
\text{Per cent inhibition} = \left( \frac{100 - \text{Sample OD} \times 100}{\text{Mean negative control OD}} \right)
\]

Samples with <30% inhibition are negative. Samples with ≥30% inhibition are positive.

Specificity of the MSP5 C-ELISA may be increased by using a higher percentage inhibition cut-off value (5); however the effect of this change on sensitivity has not been thoroughly evaluated.

**b) Card agglutination test**

The advantages of the CAT are that it is sensitive, may be undertaken either in the laboratory or in the field, and gives a result within a few minutes. Nonspecific reactions may be a problem, and subjectivity in interpreting assay reactions can result in variability in test interpretation. In addition, the CAT antigen, which is a suspension of *A. marginale* particles, can be difficult to prepare and can vary from batch to batch and laboratory to laboratory. Splenectomised calves are infected by intravenous inoculation with blood containing *Anaplasma*-infected erythrocytes. When the parasitaemia exceeds 50%, the animal is exsanguinated, the infected erythrocytes are washed, lysed, and the erythrocyte ghosts and *Anaplasma* particles are pelleted. The pellets are sonicated, washed, and then resuspended in a stain solution to produce the antigen suspension.

A test procedure that has been slightly modified from that originally described (1, 2) is as follows:

i) Ensure all test components are at a temperature of 25–26°C before use (this constant temperature is critical for the test).

ii) On each circle of the test card (a clear perspex/plastic or glass plate marked with circles that are 18 mm in diameter), place next to each other, but not touching, 10 µl of bovine serum factor (BSF), 10 µl of test serum, and 5 µl of CAT antigen\(^2\). Negative and low positive control sera must be tested on each card.

BSF is serum from a selected animal with high known conglutinin level. If the conglutinin level is unknown, fresh serum from a healthy animal known to be free from *Anaplasma* can be used. The Jersey breed is often suitable. The BSF must be stored at −70°C in small aliquots, a fresh aliquot being used each time the tests are performed. The inclusion of BSF improves the sensitivity of the test.

iii) Mix well with a glass stirrer. After mixing each test, wipe the stirrer with clean tissue to prevent cross-contamination.

iv) Place the test card in a humid chamber and rock at 100–110 rpm for 7 minutes.

v) Read immediately against a backlight. Characteristic clumping of the antigen (graded from +1 to +3) is considered to be a positive result. The test is considered to give a negative result when there is no characteristic clumping.

---

\(^2\) The test as conducted in the USA and Mexico uses larger volumes of reagents: antigen (15 µl), serum (30 µl), and bovine serum factor (30 µl), and a 4-minute reaction time (see step iv).
c) Complement fixation test

The complement fixation (CF) test has been used extensively for many years; however, it shows variable sensitivity (ranging from 20 to 60%), possibly reflecting differences in techniques for antigen production, and poor reproducibility. In addition, it has been demonstrated that the CF assay fails to detect a significant proportion of carrier cattle (5). It is also uncertain as to whether or not the CF test can identify antibodies in acutely infected animals prior to other assays (6, 24). Therefore, the CF test is no longer recommended as a reliable assay for detecting infected animals.

d) Additional ELISAs

Indirect enzyme-linked immunosorbent assay – an I-ELISA based on the use of a normal red blood cell antigen (negative antigen) and an A.-marginale-infected red blood cell antigen (positive antigen) has been found to be reliable for the detection of A.-marginale-positive sera (10). Although more cumbersome than tests using only one antigen, this test eliminates those sera that have high levels of nonspecific activity due to iso-antibodies to normal red blood cell components. The test correctly identified all 100 known positive sera taken from cattle up to 3 years after infection, while 3% of negative sera, 2% of Babesia bovis and 4% of B. bigemina sera gave false-positive results.

Dot enzyme-linked immunosorbent assay – a dot ELISA has also been described. Compared with the I-ELISA, the dot ELISA has the potential advantages of being rapid, inexpensive and simple to perform. The dot ELISA has been reported to have a sensitivity of 93% and a specificity of 96% (25).

e) Indirect fluorescent antibody test

Because of the limitations on the number of indirect fluorescent antibody (IFA) tests that can be performed daily by one operator, other serological tests are generally preferred to the IFA test. The IFA test is performed as described for bovine babesiosis in Chapter 2.4.2, except that A.-marginale-infected blood is used for the preparation of antigen smears. A serious problem encountered with the test is nonspecific fluorescence. Antigen made from blood collected as soon as adequate parasitaemia (5–10%) occurs is most likely to be suitable. Nonspecific fluorescence due to antibodies adhering to infected erythrocytes can be reduced by washing the erythrocytes in an acidic glycine buffer before antigen smears are prepared (26). Infected erythrocytes are washed twice in 0.1 M glycine buffer (pH 3.0, centrifuged at 1000 g for 15 minutes at 4°C) and then once in PBS, pH 7.4.

C. REQUIREMENTS FOR VACCINES AND DIAGNOSTIC BIOLOGICALS

Several immunisation methods have been used to protect cattle against anaplasmosis in countries where the disease is endemic, but none is ideal (22). Recently a review of A. marginale vaccines and antigens has been published (16). Use of the less pathogenic A. centrale, which gives partial cross-protection against A. marginale, is the most widely accepted method, although not used in North America. Another method involves the use of a strain of A. marginale attenuated by passage in nonbovine hosts, such as deer or sheep (34).

In this section, the production of live A. centrale vaccine is described. It involves infection of a susceptible, splenectomised calf and the use of its blood as a vaccine. Detailed accounts of the production procedure are available and reference should be made to these publications for details of the procedures outlined here (3, 7, 29).

Guidelines for the production of veterinary vaccines are given in Chapter 1.1.8 Principles of veterinary vaccine production. The guidelines given here and in Chapter 1.1.8 are intended to be general in nature and may be supplemented by national and regional requirements.

Anaplasma centrale vaccine can be provided in either frozen or chilled form depending on demand, transport networks, and the availability of liquid nitrogen or dry ice supplies. Frozen vaccine is recommended in most instances, as it allows for thorough post-production quality control of each batch. It is, however, more costly to produce and more difficult to transport than chilled vaccine. The risk of contamination makes post-production control essential, but may be prohibitively expensive.

1. Seed management

a) Characteristics of the seed

Anaplasma centrale was isolated in 1911 in South Africa, and has been used as a vaccine in South America, Australia, Africa, the Middle East, and South-East Asia. It affords only partial, but adequate, protection in regions where the challenging strains are of moderate virulence (e.g. Australia) (4). In the
humid tropics where *A. marginale* appears to be a very virulent parasite, the protection afforded by *A. centrale* may be inadequate to prevent disease in some animals.

*Anaplasma centrale* usually causes benign infections, especially if used in calves under 9 months of age. Severe reactions following vaccination have been reported when adult cattle are inoculated.

b) **Preparation and storage of stabilate**

Infective material is readily stored as frozen stabilates of infected blood in liquid nitrogen or dry ice. Dimethyl sulphoxide (DMSO) and polyvinylpyrrolidone M.W. 40,000 (3) are the recommended cryopreservatives, as they allow for intravenous administration after thawing of the stabilate. A detailed account of the freezing technique using DMSO is reported elsewhere (23), but briefly involves the following: infected blood is collected, chilled to 4°C, and cold cryoprotectant (4 M DMSO in PBS) is added slowly with stirring to a final blood:protectant ratio of 1:1, to give a final concentration of 2 M DMSO. The entire dilution procedure is carried out in an ice bath and the diluted blood is dispensed into suitable containers (e.g. 5 ml cryovials), and frozen, as soon as possible, in the vapour phase of a liquid nitrogen container.

c) **Validation as a vaccine**

The suitability of an isolate of *A. centrale* as a vaccine can be determined by inoculating susceptible cattle, monitoring the subsequent reactions, and then challenging the animals and susceptible controls with a virulent local strain of *A. marginale*. Both safety and efficacy can be judged by monitoring parasitaemias in stained blood films and the depression of packed cell volumes of inoculated cattle during the vaccination and challenge reaction periods. Evidence of purity of the isolate can be determined by serological testing of paired sera from the cattle used in the safety test for possible contaminants that may be present (3, 30).

2. **Method of manufacture**

a) **Production of frozen vaccine**

Quantities of the frozen stabilate (5–10 ml) are thawed by immersing the vials in water preheated to 40°C. The thawed material is kept on ice and used as soon as possible (within 30 minutes) to infect a susceptible, splenectomised calf by intravenous inoculation.

The parasitaemia of the donor calf is monitored daily by examining stained films of jugular blood, and the blood is collected for vaccine production when suitable parasitaemias are reached. A parasitaemia of $1 \times 10^9$/ml (approximately 2% parasitaemia in jugular blood) is the minimum required for production of vaccine. If a suitable parasitaemia is not obtained, passage of the strain by subinoculation of 100–200 ml of blood to a second splenectomised calf may be necessary.

Blood from the donor is collected by aseptic jugular or carotid cannulation using heparin as an anticoagulant (5 International Units [IU] heparin/ml blood).

In the laboratory, the parasitised blood is mixed in equal volumes with 3 M glycerol in PBS supplemented with 5 mM glucose at 37°C (final concentration of glycerol 1.5 M). The mixture is then equilibrated at 37°C for 30 minutes and dispensed into suitable containers (e.g. 5 ml cryovials). The vials are cooled at approximately 10°C/minute in the vapour phase of liquid nitrogen and, when frozen, stored in the liquid phase (3).

DMSO can be used as a cryoprotectant in the place of glycerol. This is done in the same way as outlined for the preparation of stabilate (23, 28).

If glycerolised vaccine is to be diluted, the diluent should consist of PBS with 1.5 M glycerol and 5 mM glucose (14). Vaccine cryopreserved with DMSO should be diluted with diluent containing the same concentration of DMSO as in the original cryopreserved blood (31).

b) **Production of chilled vaccine**

Infective material for chilled vaccine is prepared in the same way as for frozen vaccine, but it must be issued and used as soon as possible after collection. The infective blood can be diluted to provide $1 \times 10^9$ parasites per dose of vaccine. A suitable diluent is 10% sterile bovine serum in a glucose/balanced salt solution containing the following quantities per litre: NaCl (7.00 g), MgCl$_2$.6H$_2$O (0.34 g), glucose (1.00 g), Na$_2$HPO$_4$ (2.52 g), KH$_2$PO$_4$ (0.90 g), and NaHCO$_3$ (0.52 g).

If diluent is not available, acid citrate dextrose (20% [v/v]) or citrate phosphate dextrose (20% [v/v]) should be used as anticoagulant to provide the glucose necessary for survival of the organisms.
c) **Use of vaccine**

In the case of frozen vaccine, vials should be thawed by immersion in water, preheated to 37°C to 40°C, and the contents mixed with suitable diluent to the required dilution. If glycerolised vaccine is prepared, it should be kept cool and used within 8 hours (3). If DMSO is used as a cryoprotectant, the prepared vaccine should be kept on ice and used within 15–30 minutes (28). The vaccine is most commonly administered subcutaneously.

Chilled vaccine should be kept refrigerated and used within 4–7 days of preparation.

The strain of *A. centrale* used in vaccine is of reduced virulence, but is not entirely safe. A practical recommendation is, therefore, to limit the use of vaccine to calves, where nonspecific immunity will minimise the risk of vaccine reactions. When older animals have to be vaccinated, there is a risk of severe reactions. These reactions occur infrequently, but valuable breeding stock or pregnant animals obviously warrant close attention, and should be observed daily for 3 weeks post-vaccination. Clinically sick animals should be treated with oxytetracycline or imidocarb at dosages recommended by the manufacturers.

Protective immunity develops in 6–8 weeks and usually lasts for several years.

Anaplasmosis and babesiosis vaccines are often used concurrently, but it is not advisable to use any other vaccines at the same time (3).

3. **In-process control**

a) **Source and maintenance of vaccine donors**

A source of calves free from natural infections of *Anaplasma* and other tick-borne diseases should be identified. If a suitable source is not available, it may be necessary to breed the calves under tick-free conditions specifically for the purpose of vaccine production.

The calves should be maintained under conditions that will prevent exposure to infectious diseases and to ticks and biting insects. In the absence of suitable facilities, the risk of contamination with the agents of infectious diseases present in the country involved should be estimated, and the benefits of local production of vaccine weighed against the possible adverse consequences of spreading disease (3).

b) **Surgery**

Donor calves should be splenectomised to allow maximum yield of organisms for production of vaccine. This is best carried out in young calves and under general anesthesia.

c) **Screening of vaccine donors before inoculation**

Donor calves should be examined for all blood-borne infections prevalent in the vaccine-producing country, including *Babesia*, *Anaplasma*, *Cowdria*, *Theileria* and *Trypanosoma*. This can be done by routine examination of stained blood films after splenectomy, and preferably also by serology. Any calves showing evidence of natural infections of any of these agents should be rejected. The absence of other infective agents should also be confirmed. These may include the agents of enzootic bovine leukosis, mucosal disease, infectious bovine rhinotracheitis, ephemeral fever, Akabane disease, bluetongue, foot and mouth disease, and rinderpest. The testing procedures will depend on the diseases prevalent in the country and the availability of tests, but should involve serology of paired sera at the very least and, in some cases, virus isolation, antigen, or DNA/RNA detection (3, 28, 30).

d) **Monitoring of parasitaemias following inoculation**

It is necessary to determine the concentration of parasites in blood being collected for vaccine. The parasite concentration can be estimated from the erythrocyte count and the parasitaemia (percentage of infected erythrocytes).

e) **Collection of blood for vaccine**

All equipment should be sterilised before use (e.g. by autoclaving). Once the required parasitaemia is reached, the blood is collected in heparin using strict aseptic techniques. This is best done if the calf is sedated and with the use of a closed-circuit collection system.

Up to 3 litres of heavily infected blood can be collected from a 6-month-old calf. If the calf is to live, the transfusion of a similar amount of blood from a suitable donor is indicated. Alternatively, the calf should be killed immediately after collection of the blood.
f) Dispensing of vaccine

All procedures are performed in a suitable environment, such as a laminar flow cabinet, using standard sterile techniques. Use of a mechanical or magnetic stirrer will ensure thorough mixing of blood and diluent throughout the dispensing process.

4. Batch control

The potency, safety and sterility of vaccine batches cannot be determined in the case of chilled vaccine, and specifications for frozen vaccine depend on the country involved. The following are the specifications for frozen vaccine produced in Australia.

a) Sterility and freedom from contaminants

Standard tests for sterility are employed for each batch of vaccine and diluent (see Chapter 1.1.9 Tests for sterility and freedom from contamination of biological materials).

The absence of contaminants is determined by doing appropriate serological testing of donor cattle, by inoculating donor lymphocytes into sheep and then monitoring them for evidence of viral infection, and by inoculating cattle and monitoring them serologically for infectious agents that could potentially contaminate the vaccine. Cattle inoculated during the test for potency (see Section C.4.c) are suitable for the purpose.

Depending on the country of origin of the vaccine, these agents include the causative organisms of enzootic bovine leukosis, infectious bovine rhinotracheitis, mucosal disease, ephemeral fever, Akabane disease, Aino virus, blue tongue, parainfluenza, foot and mouth disease, lumpy skin disease, rabies, Rift Valley fever, rinderpest, contagious bovine pleuropneumonia, Jembrana disease, heartwater, pathogenic *Theileria* and *Trypanosoma* spp., *Brucella abortus*, *Coxiella*, and *Leptospira* (3, 28, 30).

b) Safety

Vaccine reactions of the cattle inoculated in the test for potency (see Section C.4.c) are monitored by measuring parasitaemia and depression of packed cell volume. Only batches with pathogenicity levels equal to or lower than a predetermined standard are released for use.

c) Potency

Vaccine is thawed and diluted 1/5 with a suitable diluent (3). The diluted vaccine is then incubated for 8 hours at 4°C, and five cattle are inoculated subcutaneously with 2 ml doses. The inoculated cattle are monitored for the presence of infections by examination of stained blood smears. All should become infected for a batch to be accepted. A batch proving to be infective is recommended for use at a dilution of 1/5 with isotonic diluent.

d) Duration of immunity

Partial but long-lasting immunity results from one inoculation. There is no evidence that repeated vaccination will have a boosting effect.

e) Stability

The vaccine can be kept for 5 years when stored in liquid nitrogen. Once thawed, it rapidly loses its potency. Thawed vaccine cannot be refrozen.

f) Preservatives

No preservatives are added. Penicillin (500,000 IU/litre) and streptomycin (370,000 µg/litre) are added to the vaccine at the time of dispensing.

g) Precautions (hazards)

The vaccine is not infective for humans. When the product is stored in liquid nitrogen, the usual precautions pertaining to the storage, transportation and handling of deep-frozen material applies.

5. Tests on final product

a) Safety

See Section C.4.b.
b) Potency

See Section C.4.c.

REFERENCES


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SUMMARY

Babesiosis is a tick-borne disease of cattle caused by the protozoan parasites Babesia bovis, B. bigemina, B. divergens and others. Boophilus spp., the principal vectors of B. bovis and B. bigemina, are widespread in tropical and subtropical countries. The major vector of B. divergens is Ixodes ricinus. Other important vectors include Haemaphysalis and Rhipicephalus spp.

Identification of the agent: Demonstration of parasites in dead animals is possible by microscopic examination of smears of blood, brain, kidney, liver and spleen, provided decomposition is not advanced. The smears are fixed with methanol, stained with 10% Giemsa for 20–30 minutes, and examined at ×800–1000 magnification under oil immersion. In the case of live animals, thick and thin films of capillary blood should be taken from, for example, the tip of the tail. Sensitive polymerase chain reaction assays are available that can detect and differentiate Babesia species in cattle.

Serological tests: The indirect fluorescent antibody (IFA) test is the most widely used test for the detection of antibodies to B. bovis and B. divergens, but enzyme-linked immunosorbent assays are gaining popularity. The IFA test has been used for detection of antibodies to B. bigemina, but serological cross-reactions make species diagnosis difficult. The complement fixation test has also been used to detect antibodies against B. bovis and B. bigemina.

Requirements for vaccines and diagnostic biologicals: Vaccines consisting of live, attenuated strains of B. bovis, B. bigemina or B. divergens are produced in several countries from the blood of infected donor animals. The vaccines are provided in frozen or chilled forms. Production of frozen vaccine is usually recommended as it allows thorough post-production control of each batch. The risk of contamination of this blood-derived vaccine makes thorough quality control essential, but it may be prohibitively expensive.

Live Babesia vaccines are not entirely safe. A practical recommendation is to limit their use to calves, preferable less than 1 year old, when nonspecific immunity will minimise the risk of vaccine reactions. When older animals are to be vaccinated, the risk of reaction warrants close surveillance and treatment with a babesiacide if reactions occur.

Protective immunity develops in 3–4 weeks and lasts for several years after a single vaccination.

A. INTRODUCTION

Bovine babesiosis is caused by protozoan parasites of the genus Babesia, order Piroplasmida, phylum Apicomplexa. Of the species affecting cattle, two – Babesia bovis and B. bigemina – are widely distributed and of major importance in Africa, Asia, Australia, and Central and South America. Babesia divergens is economically important in some parts of Europe.

Tick species are the vectors of Babesia (18). Boophilus microplus is the principal vector of B. bigemina and B. bovis and is widespread in the tropics and subtropics. The vector of B. divergens is Ixodes ricinus. Other important vectors include Haemaphysalis, Rhipicephalus and other Boophilus spp.

Babesia bigemina has the widest distribution but generally, B. bovis is more pathogenic than B. bigemina or B. divergens. Infections are characterised by high fever, ataxia, anorexia, general circulatory shock, and sometimes also nervous signs as a result of sequestration of infected erythrocytes in cerebral capillaries. In acute cases, the maximum parasitaemia (percentage of infected erythrocytes) in circulating blood is less than 1%. This is in contrast to B. bigemina infections, where the parasitaemia often exceeds 10% and may be as high
as 30%. In *B. bigemina* infections, the major signs include fever, haemoglobinuria and anaemia. Intravascular sequestration of infected erythrocytes does not occur with *B. bigemina* infections. The parasitaemia and clinical appearance of *B. divergens* infections are somewhat similar to *B. bigemina* infections (41).

Infected animals develop a life-long immunity against reinfection with the same species. There is also evidence of a degree of cross-protection in *B. bigemina*-immune animals against subsequent *B. bovis* infections. Calves rarely show clinical signs of disease after infection regardless of the *Babesia* spp. involved or the immune status of the dams (4, 10).

**B. DIAGNOSTIC TECHNIQUES**

1. **Identification of the agent**

The traditional method of identifying the agent in infected animals is by microscopic examination of thick and thin blood films stained with, for example, Giemsa. The sensitivity of this technique is such that it can detect parasitaemias as low as 1 parasite in 10^6 red blood cells (RBCs) (8). Species differentiation is good in thin films but poor in the more sensitive thick films. This technique is usually adequate for detection of acute infections, but not for detection of carriers where the parasitaemias are mostly very low. Parasite identification and differentiation can be improved by using a fluorescent dye, such as acridine orange instead of Giemsa (8). A quantitative buffy coat method using acridine orange to stain parasites in capillary tubes was developed to demonstrate *Plasmodium* in human blood and could potentially also detect low *Babesia* parasitaemias, but differentiation is likely to be poor (8).

Samples from live animals should preferably be taken from capillaries, such as those in the tip of the ear or tip of the tail, as *B. bovis* is more common in capillary blood. *Babesia bigemina* and *B. divergens* parasites are uniformly distributed through the vasculature. If it is not possible to make fresh smears from capillary blood, sterile jugular blood should be collected into an anticoagulant such as ethylene diamine tetra-acetic acid (EDTA) (e.g. 1 mg/ml). Heparin may affect the colour characteristics of the staining and is not recommended. The sample should be kept cool, preferably at 5°C, until delivery to the laboratory, again preferably within hours of collection. Thin blood films are air-dried, fixed in absolute methanol for 1 minute, and stained in 10% Giemsa stain for 20–30 minutes. It is preferable to stain blood films as soon as possible after preparation to ensure proper stain definition. Thick films are made by placing a small drop (approximately 50 µl) of blood on to a clean glass slide. This droplet is then air-dried, heat-fixed at 80°C for 5 minutes, and stained in 10% Giemsa for 15–20 minutes. Unstained blood smears should not be stored with formalin solutions as it may affect staining quality.

Samples from dead animals should consist of thin blood films, as well as smears from (in order of preference), cerebral cortex, kidney, liver, spleen and bone marrow. Organ smears are made by pressing a clean slide on to a freshly cut surface of the organ or by crushing a small sample of the tissue between two clean microscope slides drawn lengthwise to leave a film of tissue on each slide. The smear is then air-dried (assisted by gentle warming in humid climates), fixed for 5 minutes in absolute methanol, and stained for 20–30 minutes in 10% Giemsa. This method is especially suitable for the diagnosis of *B. bovis* infections, but is unreliable if samples are taken 24 hours or longer after death has occurred. However, parasites can often be detected in blood taken from veins in the lower limb region one or more days after death.

All stained smears are examined under oil immersion using (as a minimum) a ×8 eyepiece and a ×60 objective lens. *Babesia bovis* is a small parasite, usually centrally located in the erythrocyte. It measures approximately 1–1.5 μm long and 0.5–1.0 μm wide, and is often found as pairs that are at an obtuse angle to each other. *Babesia divergens* is also a small parasite and is very similar morphologically to *B. bovis*. However, obtuse-angled pairs are often located at the rim of the erythrocyte. *Babesia bigemina* is a much longer parasite, and is often found as pairs at an acute angle to each other. *Babesia bigemina* is typically pear-shaped, but many diverse single forms are found. It is 3–3.5 μm long and 1–1.5 μm wide, and paired forms often have two discrete red-staining dots in each parasite (*B. bovis* and *B. divergens* always have only one). In acute cases, the parasitaemia of *B. bovis* seldom reaches 1%, but with *B. bigemina* and *B. divergens* much higher parasitaemias are the norm. Thick blood films are especially useful for the diagnosis of low level *B. bovis* infections, as are organ smears (2).

Polymerase chain reaction (PCR) assays have proven to be very sensitive particularly in detecting *B. bovis* and *B. bigemina* in carrier cattle (9, 11, 17, 34, 36, 38). Thanamasirak et al. found their PCR-enzyme-linked immunosorbent assay (ELISA) to be at least 1000 times more sensitive than thin blood smears for detection of *B. bovis* (38), and detection levels as low as three parasitised erythrocytes in 20 µl of packed cells have been claimed (37). A number of PCR techniques have been described that can detect and differentiate species of *Babesia* in carrier infections (9, 11, 17, 36). PCR assays to differentiate isolates of *B. bovis* have also been described (6). The application of the reverse line blot procedure, in which PCR products are hybridised to membrane-bound, species-specific oligonucleotide probes, to *Babesia* (21) has enabled the simultaneous detection of multiple species even in carrier state infections. However, current PCR assays generally do not lend themselves well to large-scale testing and at this time are unlikely to supplant serological tests as the method of
choice for epidemiological studies. PCR assays are useful as confirmatory tests and in some cases for regulatory testing.

In-vitro culture methods have been used to demonstrate the presence of carrier infections of *Babesia* spp. (22), and *B. bovis* has also been cloned in culture. The minimum parasitaemia detectable by this method will depend, to a large extent, on the facilities available and the skills of the operator (8), but could be as low as 10–10 (19), making it a very sensitive method for the demonstration of infection. An added benefit is that it is 100% specific.

Confirmation of infection in a suspected carrier animal can also be made by transfusing approximately 500 ml of jugular blood intravenously into a splenectomised calf known to be *Babesia*-free, and monitoring the calf for the presence of infection. This method is cumbersome and expensive, and obviously not suitable for routine diagnostic use. Mongolian gerbils (*Meriones unguiculatus*) can, however, be used to demonstrate the presence of *B. divergens* (41).

2. Serological tests

The indirect fluorescent antibody (IFA) test is widely used to detect antibodies to *Babesia* spp., but the *B. bigemina* test has poor specificity. Cross-reactions with antibodies to *B. bovis* in the *B. bigemina* IFA test are a particular problem in areas where the two parasites coexist. The IFA test has the disadvantages of low sample throughput and subjectivity. The complement fixation (CF) test has been described as a method to detect antibodies against *B. bovis* and *B. bigemina* (1). This test has been used to qualify animals for importation into some countries. The test is based on the procedure previously described and validated for the detection of antibody against *Babesia caballi* and *Theileria equi* (see Chapter 2.5.8 Equine piroplasmosis). An ELISA for the diagnosis of *B. bovis* infection that uses a whole merozoite antigen has undergone extensive evaluation (12, 29, 39). Competitive ELISAs using recombinant merozoite surface and rhesypt associated antigens of *B. bovis* have recently been developed (7, 14, 20) but have not yet been widely validated. Despite the efforts of several investigators in different laboratories, there is still no well-validated ELISA available for *B. bigemina*. ELISAs for antibodies to *B. bigemina* typically have poor specificity. In one study (16), *B. bigemina* antiseraum appeared to react non-specifically with fibrinogen. To the best of our knowledge a competitive ELISA developed and validated in Australia (30) is the only ELISA in routine use. In the absence of any other workable test for *B. bigemina*, the procedure for that assay has been included here. ELISAs have also been developed for *B. divergens* (9) using antigen derived from culture, *Meriones* or cattle, but there does not appear to be one that has been validated internationally.

a) *Babesia bovis* enzyme-linked immunosorbent assay

Antigen preparation is based on a technique described by Waltisbuhl et al. (39). Infected blood (usually 5–10% parasitaemia) is collected from a splenectomised calf into EDTA. The blood is washed three times in five volumes of phosphate buffered saline (PBS), and then infected cells are concentrated by differential lysis of uninfected cells in hypotonic saline solution. Infected cells are more resistant to lysis in hypotonic saline solutions than are uninfected cells. A series of hypotonic saline solutions are prepared, ranging from 0.35% to 0.50% NaCl, in 0.025% increments. To find the best concentration, five volumes of each saline solution is then added to one volume of packed RBC, which are gently mixed and allowed to stand for 5 minutes.

The mixtures are then centrifuged and the supernatants are aspirated. An equal volume of plasma (retained from the original blood) is added to each tube containing packed RBC, and the contents of the tubes are mixed. Thin blood films are prepared from each of these resuspended blood cell mixtures, fixed in methanol, and stained with Giemsa. These films are examined under a microscope to determine which saline solution lyses most uninfected RBC but leaves infected RBC intact. It should be possible to achieve >95% infection in the remaining intact RBC. The bulk of the packed RBC is then differentially lysed with the optimal saline solution and centrifuged. The sediment (>95% infected RBC) is lysed in distilled water at 4°C, and parasites are pelleted at 12,000 g for 30 minutes. The pellet is washed three times in PBS by resuspension and centrifugation at 4°C. It is then resuspended in one to two volumes of PBS at 4°C, and sonicated in appropriate volumes using medium power for 60–90 seconds. The sonicated material is ultracentrifuged, (105,000 g for 60 minutes at 4°C) and the supernatant is retained. The supernatant is mixed with an equal volume of glycerol and stored in 2–5 ml aliquots at −70°C. Short-term storage at −20°C is acceptable for the working aliquot.

- **Test procedure**
  i) 100 µl of this antigen, diluted from 1/400 to 1/1600 in 0.1 M carbonate buffer, pH 9.6, is added to each well of a polystyrene 96-well microtitre plate. The plate is covered and incubated overnight at 4°C.
  ii) Antigen is removed and the wells are then blocked for 2 hours at room temperature by the addition of 200 µl of a 2% solution of sodium caseinate in carbonate buffer.
  iii) After blocking, the wells are rinsed briefly with PBS containing 0.1% Tween 20 (PBST) and 100 µl of bovine serum diluted 1/100 in PBST containing either 5% normal horse serum or 5% skim milk powder is added, and the plates are incubated for 2 hours at room temperature.
iv) The washing step consists of a brief rinse with PBST, followed by three 5-minute washes with the same buffer (during which the plate is shaken vigorously), and finally the plates are given a further brief rinse.

v) Next, 100 µl of peroxidase-labelled anti-bovine IgG diluted appropriately in PBST containing horse serum or skim milk is added and the plates are shaken for a further 30 minutes at room temperature. (NB: some batches of skim milk powder may contain immunoglobulins that can interfere with anti-bovine IgG conjugates).

vi) Wells are washed as described in step iv above, and 100 µl of peroxidase substrate (ABTS [2,2’-Azino-bis-(3-ethylbenzothiazoline-6-sulphonic acid)]) is added to each well. The substrate reaction is allowed to continue until the absorbance of a strong positive control serum included on each plate approaches 1. At this point the absorbance at 414 nm is read on a microtitre plate reader.

To control for inter-plate variation, known positive and negative sera are included in each plate (29). Test sera are then ranked relative to the positive control. ELISA results are expressed as a percentage of this positive control (percentage positivity). Positive and negative threshold values should be determined in each laboratory by testing as many known positive and negative sera as possible.

Each batch of antigen and conjugate should be titrated using a checkerboard layout. The most suitable enzyme label for the conjugate is horseradish peroxidase. ABTS or tetramethyl benzidine (TMB) are suitable substrates. With this test, it is possible to detect antibodies at least 4 years after a single infection. There should be 95–100% positive reactions with B.-bovis-immune animals, 1–2% false-positive reactions with B.-bigemina-immune animals.

b) Babesia bigemina enzyme-linked immunosorbent assay

This ELISA is based on an immunodominant 58 kDa antigen identified by a number of groups in B. bigemina isolates from Australia, Central America and Texas, United States of America, Egypt and Kenya (30). A monoclonal antibody (MAb) (D6) (Tick Fever Centre, Qld, Australia) directed against this antigen has been used to develop a competitive inhibition ELISA (30). The antigen used in the ELISA is a 26 kDa peptide (Tick Fever Centre, Qld, Australia), encoded by a 360 bp fragment of the p58 gene, expressed in Escherichia coli and affinity purified. This antigen can also be used in an indirect ELISA format, but some cross-reactivity of antibodies to B. bovis should be expected.

• Test procedure
  i) The recombinant 26 kDa antigen is diluted in 0.1 M carbonate buffer, pH 9.6, to a concentration of approximately 2 µg/ml and 100 µl is added to each well of a 96-well microtitre plate. The plates are incubated overnight at 4°C.
  ii) Excess antigen is removed and the wells are then blocked for 1 hour at room temperature by addition of 200 µl per well of a 2% solution of sodium caseinate in carbonate buffer.
  iii) Following a brief rinse (3 × 200 µl) with PBS containing 0.1% Tween 20 (PBST), 100 µl of undiluted serum is added and the plates are incubated for 30 minutes at room temperature with gentle shaking.
  iv) The plates are then washed with PBST (5 × 200 µl rinse, 5-minute soak with shaking), and 100 µl of peroxidase-labelled MAb D6 diluted to a concentration of 0.03 µg/ml in PBST containing 2% skim milk powder is added to each well. The plates are then incubated at room temperature for 30 minutes with gentle shaking.
  v) Plates are washed again, 100 µl TMB peroxidase substrate is added to each well, and the plates are incubated in the dark until the absorbance of the conjugate control wells (no serum) approaches 1. At this point the reaction is stopped by the addition of 50 µl of 1 M sulphuric acid and the absorbance is read at 450 nm. Positive and negative control sera should be included on each test plate.

The per cent inhibition (PI) for test sera is calculated relative to the conjugate control (PI = 100 – [100 × test absorbance/conjugate control absorbance]). Positive and negative threshold values should be determined in each laboratory by testing as many known positive and negative sera as possible.

The specificity of the ELISA has been estimated at 97.0% and the sensitivity for detection of antibodies in experimentally infected cattle is 95.7% (30).

c) Indirect fluorescent antibody test

• Antigen preparation

Antigen slides are made from jugular blood, ideally when the parasitaemia is between 2% and 5%.
Blood is collected into a suitable anticoagulant (sodium citrate or EDTA), and is then washed at least three times in from five to ten volumes of PBS to remove contaminating plasma proteins and, in particular, host immunoglobulins. After washing, the infected RBCs are resuspended in two volumes of PBS to which 1% bovine serum albumin (BSA) has been added. The BSA is used to adhere RBCs to the glass slide. By preference, single-layered blood films are made by placing a drop of blood on to a clean glass slide, which is then spun in a cytocentrifuge. This produces very uniform smears. Alternatively, thin blood films may be made by the conventional technique (dragging with the end of another slide). The films are air-dried and fixed for 5 minutes in an oven at 80°C. Fixed blood films are then covered (e.g. with aluminium foil or brown paper sticking tape) so as to be airtight, and stored at –70°C until required (maximum 5 years).

- **Test procedure**

Test and control sera are diluted 1/30 in PBS. Sera may be used with or without heat inactivation at 56°C for 30 minutes. The slides are marked into 8–10 divisions with an oil pen to produce hydrophobic divisions. To each test square 5–10 µl of each serum dilution is added using a fine pipette. The preparations are then incubated at 37°C for 30 minutes, in a humid chamber. For controls, dilutions of weak positive and negative sera are used on each test slide.

After incubation, the slides are gently rinsed once with PBS, and given two 10-minute washes with PBS followed by water. An appropriate dilution of anti-bovine IgG antibody labelled with fluorescein isothiocyanate (which is available commercially) is then added to each test square. Every new batch of conjugate must be titrated, the working range usually being between 1/400 and 1/1200. Conjugated rabbit and chicken antibodies are usually more suitable for this purpose than goat antibodies. The slides with the conjugate are incubated again at room temperature for 30 minutes, and washed as above. The wet slides are mounted with cover-slips in 1/1 glycerol and PBS, and examined by standard fluorescence microscopy. A competent operator can examine approximately 150 samples per day.

d) **Complement fixation**

The CF test is used by some countries for general diagnosis and to qualify cattle for importation. A brief description is provided here of antigen production and test protocols used by the United States Department of Agriculture (1). The methods are essentially the same as those described in this *Terrestrial Manual* for the microtitration CF test for equine piroplasmosis.

- **Solutions**

_Alsever's solution:_ prepare 1 litre of Alsever’s solution by dissolving 20.5 g glucose; 8.0 g sodium citrate; 4.2 g sodium chloride in sufficient distilled water. Adjust to pH 6.1 using citric acid, and make up the volume to 1 litre with distilled water. Sterilise by filtration.

_Stock veronal buffer (5×):_ dissolve the following in 1 litre of distilled water: 85.0 g sodium chloride; 3.75 g sodium 5,5 diethyl barbituric; 1.68 g magnesium chloride (MgCl₂.6H₂O); 0.28 g calcium chloride. Dissolve 5.75 g of 5,5 diethyl barbituric acid in 0.5 litre hot (near boiling) distilled water. Cool this acid solution and add to the salt solution. Make up to 2 litre with distilled water and store at 4°C. To prepare a working dilution, add one part stock solution to four parts distilled water. The final pH should be from 7.4 to 7.6.

- **Antigen production**

Blood is obtained from cattle with a high parasitaemia (e.g. 30% parasitaemia for *B. bovis* and 60% for *B. bigemina*), and mixed with equal volumes of Alsever’s solution as an anticoagulant. The plasma/Alsever’s supernatant and buffy coat are removed when the RBCs have settled to the bottom of the flask. The RBCs are washed several times with cold veronal buffer and then disrupted. The antigen is recovered from the lysate by centrifugation at 30,900 g for 30 minutes.

The recovered antigen is washed several times in cold veronal buffer by centrifugation at 20,000 g for 15 minutes. Polyvinyl pyrrolidone (5% w/v) is added as a stabiliser and the preparation is mixed on a magnetic stirrer for 30 minutes, strained through two thicknesses of sterile gauze, dispensed into 2 ml volumes and freeze-dried. The antigen can then be stored at below –50°C for several years.

- **Test procedure – Microtitration method**

i) The specificity and potency of each batch of antigen should be checked against standard antisera of known specificity and potency. Optimal antigen dilutions are also determined in a preliminary checkerboard titration.

ii) Test sera are inactivated for 30 minutes at 58°C and tested in dilutions of 1/5 to 1/320. Veronal buffer is used for all dilutions.
iii) Complement is prepared and titrated spectrophotometrically to determine the 50% haemolytic dose (C’H50) and used in the test at five times C’H50. The haemolytic system (sensitised RBCs) consists of equal parts of a 2% sheep RBC suspension and veronal buffer with optimally diluted haemolysin.

iv) The total volume of the test is 0.125 ml, made up of equal portions (0.025 ml) of antigen, complement (five times C’H50) and diluted serum. Incubation is performed for 1 hour at 37°C.

v) A double portion (0.05 ml) of the haemolytic system (sensitised sheep RBCs) is added and the plates are incubated for a further 45 minutes at 37°C with shaking after 20 minutes.

vi) The plates are centrifuged for 5 minutes at 300 g before being read over a mirror.

vii) The reaction in each well is recorded as follows: 100% lysis = 0 or negative, 75% lysis = 1+, 50% lysis = 2+, 25% lysis = 3+, 0% lysis = 4+. A 2+ reaction (50% lysis) or stronger at the 1/5 dilution is recorded as positive, with titre results reported as the reaction, if any, at the next dilution higher than the greatest serum dilution with a 4+ reaction (e.g. 1+ at 1:10, for a sample with a 4+ reaction at 1/5 and a 1+ reaction at 1/10). A full set of controls must be included in each test, including positive and negative sera, as well as control antigen prepared from normal (uninfected) horse RBCs.

Anticomplementary samples are examined by the IFA test.

e) Other tests

Other serological tests have been described in recent years, and include a dot ELISA (31), a slide ELISA (25), and latex and card agglutination tests (3, 26). These tests show acceptable levels of sensitivity and specificity for *B. bovis* and, in the case of the dot ELISA, also for *B. bigemina*. However, none of these tests appears to have been adopted for routine diagnostic use in laboratories other than those in which the original development and validation took place. Adaptability of these tests to routine diagnostic laboratories is therefore unknown.

C. REQUIREMENTS FOR VACCINES AND DIAGNOSTIC BIOLOGICALS

Cattle develop a durable, long-lasting immunity after a single infection with *B. bovis*, *B. divergens* or *B. bigemina*. This feature has been exploited in some countries to immunise cattle against babesiosis (4, 27, 35). Most of these live vaccines contain specially selected strains of *Babesia*, mainly *B. bovis* and *B. bigemina*, and are produced in government-supported production facilities as a service to the livestock industries, in particular in Australia, Argentina, South Africa, Israel and Uruguay. An experimental *B. divergens* vaccine prepared from the blood of infected *Meriones* has also been used successfully in Ireland (41).

A killed *B. divergens* vaccine is prepared in Austria from the blood of infected calves (15), but little information is available on the level and duration of the conferred immunity. Experimental vaccines containing antigens produced *in vitro* have also been developed (3, 32), but the level and duration of protection against heterologous challenge are unclear. Parasite proteins have been characterised and there has been some progress towards the development of subunit vaccines (11, 33). No effective subunit vaccine is available commercially.

Guidelines for the production of veterinary vaccines are given in Chapter 1.1.8 Principles of veterinary vaccine production. The guidelines given here in Chapter 1.1.8 are intended to be general in nature and may be supplemented by national and regional requirements.

This section will deal with the production of live babesiosis vaccines, mainly those against *B. bovis* and *B. bigemina* infections in cattle. Production involves infection of calves with selected strains, and use of the blood as vaccine (4). Calves used for infection with these strains must be free of infectious agents that can be transmitted by products derived from their blood. In the case of *B. divergens*, blood of infected gerbils (*Meriones unguiculatus*) can be used instead of bovine blood. *In-vitro* culture methods have also been used to produce parasites for vaccine (24, 27). However, the relatively high cost of production from culture and evidence of possible antigenic drift during long-term maintenance in culture, make mass culture of *Babesia* impractical in most laboratories at present.

*Babesia bovis* and *B. bigemina* vaccines can be prepared in either frozen or chilled form depending on demand, transport networks and the availability of liquid nitrogen or dry ice supplies. Preparation of frozen vaccine is preferred (4, 27, 35), as it allows for thorough post-production control of each batch. However, it is more costly to produce and more difficult to transport than chilled vaccine. The potential risk of contamination of this blood-derived vaccine makes post-production control essential, but may put production beyond the financial means of some countries in endemic regions (13). A production facility supplying an annual market of fewer than 50,000 doses is unlikely to operate without financial support.
1. Seed management

a) Characteristics of the seed

- Internationally available strains
  
  Attenuated Australian strains of *B. bovis* and *B. bigemina* have been used effectively to immunise cattle in Africa, South America and South-East Asia (4). Tick-transmissible and nontransmissible strains are available. A strain of *B. divergens* with reduced virulence for *Meriones* has also been developed (40).

- Isolation and purification of local strains
  
  Strains of *B. bovis*, *B. divergens* and *B. bigemina* that are free of contaminants, such as *Anaplasma*, *Eperythrozoon*, *Theileria*, *Trypanosoma* and various viral and bacterial agents, are most readily isolated by feeding infected ticks on susceptible splenectomised cattle. The vectors and modes of transmission of the species differ, and these features can be used to separate the species (19).

*Babesia* spp. can also be isolated from infected cattle by subinoculation of blood into susceptible splenectomised calves. A major disadvantage of this method is the difficulty of separating the *Babesia* spp. from contaminants such as *Anaplasma* and *Eperythrozoon*. Isolation of *B. divergens* is a relatively simple process because of the susceptibility of *Meriones* (41). Maintenance of isolated strains in vitro (23) can be used to eliminate most contaminants, but not to separate *Babesia* spp. Selective chemotherapy, for example 1% trypan blue to eliminate *B. bigemina*, can be used to obtain pure *B. bovis* from a mixed *Babesia* infection, while rapid passaging in susceptible calves will allow isolation of *B. bigemina* (2).

- Attenuation of strains
  
  Various ways of attenuating *Babesia* spp. have been reported. The most reliable method of reducing the virulence of *B. bovis* involves rapid passage of the strain through susceptible splenectomised calves. Attenuation is not guaranteed, but usually follows after 8 to 20 calf passages (4).

The virulence of *B. bigemina* decreases during prolonged residence of the parasite in latently infected animals. This feature has been used to obtain avirulent strains by infecting calves, splenectomising them after 3 months and then using the ensuing relapse parasites to repeat the procedure (4).

Attenuation of *B. divergens* for *Meriones* followed long-term maintenance in vitro (40).

Attenuation of *Babesia* spp. with irradiation has been attempted, but the results were variable. Similarly, maintenance in vitro in modified media has been used experimentally.

Avirulent strains should be stored as stabilate for safety testing and for future use as master seed in the production of vaccine.

b) Preparation and storage of master seed

Avirulent strains are readily stored as frozen infected blood in liquid nitrogen or dry ice. Dimethyl sulphoxide (DMSO) and polyvinylpyrrolidone MW 40,000 (4) are the recommended cryopreservatives, as they allow for intravenous administration after thawing of the master seed. A detailed account of the freezing technique using DMSO is reported elsewhere (28). Briefly, it involves the following:

Infected blood is collected and chilled to 4°C. Cold cryoprotectant (4 M DMSO in PBS) is then added, while stirring slowly, to a final blood:protectant ratio of 1:1 with the final concentration of DMSO being 2 M. This dilution procedure is carried out in an ice bath, and the diluted blood is dispensed into suitable containers (e.g. 5 ml cryovials), and frozen, as soon as possible, in the vapour phase of a liquid nitrogen container. The vials are stored in the liquid phase in a designated tank to prevent loss of viability and contamination. Stored in this way, master seed lots of *Babesia* have been known to remain viable for 20 years.

c) Preparation and storage of working seed

Working seed is prepared in the same way as master seed (Section C.1.b) using master seed as starting material.

d) Validation of safety and efficacy of working seed

The suitability of a working seed is determined by inoculating suitable numbers of susceptible cattle with vaccine prepared from it and then challenging them and susceptible controls with a virulent, heterologous strain. Both safety and efficacy can be judged by monitoring fever, parasitaemias in stained blood films, and
depression of packed cell volumes. The purity of the working seed is tested by monitoring the cattle used in the safety test for evidence of possible contaminants as mentioned in Section C.4.b.

2. Method of manufacture

a) Production of frozen vaccine concentrate

First, 5–10 ml quantities of working seed are rapidly thawed by immersing the vials in water preheated to 40°C. The thawed material is kept on ice and used as soon as possible (within 30 minutes if DMSO is used) to infect a susceptible, splenectomised calf (free of potential vaccine contaminants) by intravenous inoculation.

Blood suitable for vaccine is obtained by monitoring films of jugular blood and collecting the required volume of blood when a suitable parasitaemia is reached. A parasitaemia of $1 \times 10^8$/ml (approximately 2% parasitaemia in jugular blood) is usually adequate for production of vaccine. If a suitable $B. bovis$ parasitaemia is not obtained, passage of the strain by subinoculation of 100–800 ml of blood into a second splenectomised calf may be necessary. Passage of $B. bigemina$ is not recommended.

Blood from the infected donor calf is collected by jugular cannulation using preservative-free heparin as anticoagulant (5 International Units [IU] heparin/ml blood).

In the laboratory, the parasitised blood is mixed in equal volumes with 3 M glycerol in PBS supplemented with 5 mM glucose (final concentration of glycerol 1.5 M) at 37°C. The mixture is then equilibrated at 37°C for 30 minutes, and dispensed into suitable containers (e.g. 5 ml cryovials). The vials are cooled at approximately 10°C/minute in the vapour phase of liquid nitrogen and, when frozen, stored in the liquid phase (4).

DMSO can be used as cryoprotectant in the place of glycerol. This is carried out in the same way as outlined for the preparation of master seed (35).

If glycerolised frozen vaccine is to be diluted, the diluent should be iso-osmotic and consist of PBS containing 1.5 M glycerol and 5 mM glucose. Similarly, the diluent used in vaccine cryopreserved with DMSO should be iso-osmotic, and should contain the same concentration of DMSO in PBS.

Frozen vaccine containing both $B. bovis$ and $B. bigemina$ can be prepared (27) by mixing equal numbers of the parasites obtained from different donors. A 3 in 1 vaccine containing packed red cells infected with $B. bovis$, $B. bigemina$ and $Anaplasma Centrale$ is also made in Australia. Packed cells from 3 donors are concentrated and mixed to produce the trivalent concentrate which on thawing is mixed with a diluent before use (4).

The recommended dose of vaccine after reconstitution and dilution ranges from 1 to 2 ml depending on local practices and requirements.

b) Production of chilled vaccine

Infective material used in the production of chilled vaccine is obtained in the same way as for frozen vaccine, but should be issued and used as soon as possible after collection. If it is necessary to obtain the maximum number of doses per calf, the infective material can be diluted to provide the required number of parasites per dose (usually from 2.5 to 10 $\times 10^7$). A suitable diluent is 10% sterile bovine serum in a balanced salt solution containing the following ingredients per litre: NaCl (7.00 g), MgCl$_2$.6H$_2$O (0.34 g), glucose (1.00 g), Na$_2$HPO$_4$ (2.52 g), KH$_2$PO$_4$ (0.90 g), and NaHCO$_3$ (0.52 g).

Blood containing $B. divergens$ may be diluted in Hanks’ solution. If diluent is not required, sterile acid citrate dextrose or citrate phosphate dextrose should be used as the anticoagulant, at a rate of one part to four parts blood, to provide the glucose necessary for parasite survival.

3. In-process control

a) Sources and maintenance of vaccine donors

A source of donors free of natural infections with Babesia, other tick-borne diseases, and other infectious agents transmissible with blood, should be identified. If a suitable source is not available, it may be necessary to breed donor calves under tick-free conditions specifically for the purpose.

Donor calves should be maintained under conditions that will prevent exposure to infectious diseases and to ticks and biting insects. In the absence of suitable facilities, the risk of contamination with the agents of infectious diseases present in the country involved should be estimated, and the benefits of local production
of vaccine (as opposed to importation of a suitable product) should be weighed against the possible adverse consequences of spreading disease (4).

b) Surgery
Donor calves should be splenectomised to allow maximum yield of parasites for production of vaccine. This is easier in calves less than 3 months of age and best done under general anaesthesia.

c) Screening of vaccine donors before inoculation
Donor calves should be examined for agents of all blood-borne infections prevalent in the country, including Babesia, Anaplasma, Theileria, and Trypanosoma. This can be done by routine examination of stained blood films after splenectomy, and preferably also by serological testing pre- and post-quarantine. Calves showing evidence of natural infections with any of these agents should be rejected. The absence of other infective agents endemic in the country should also be confirmed; these may include the agents of enzootic bovine leukosis, bovine immunodeficiency virus, bovine pestivirus, infectious bovine rhinotracheitis, Akabane disease, ephemeral fever, bluetongue, foot and mouth disease, and rinderpest. The test procedures will depend on the diseases prevalent in the country and the availability of tests, but should involve serology of paired sera and, in some cases, virus isolation, antigen or DNA detection (4, 35).

d) Monitoring of parasitaemias following inoculation
It is necessary to determine the concentration of parasites in blood collected for vaccine. There are accurate techniques for determining the parasite count (2), but the parasite concentration can be estimated from the RBC count and the parasitaemia (% infected RBCs).

e) Collection of blood for vaccine
All equipment should be sterilised before use (e.g. by autoclaving). The blood is collected in heparin using strict aseptic techniques when the required parasitaemia is reached. This is best done if the calf is sedated with, for example, xylazine and with the use of a closed-circuit collection system.

Up to 3 litres of heavily infected blood can be collected from a 6-month-old calf. If the calf is to live, the transfusion of a similar amount of blood from a suitable donor is indicated. Alternatively, the calf should be killed immediately after collection of the blood.

f) Dispensing of vaccine
All procedures are performed in a suitable environment, such as a laminar flow cabinet, using standard sterile techniques. Use of a mechanical or magnetic stirrer will ensure thorough mixing of blood and diluent throughout the dispensing process.

4. Batch control

The potency, safety and sterility of vaccine batches cannot be determined in the case of chilled vaccine, and specifications of frozen vaccine depend on the code of practice of the country involved. The following are the specifications for frozen vaccine produced in Australia.

a) Sterility and freedom from contaminants
Standard tests for sterility are employed for each batch of vaccine and diluent. The absence of contaminants is determined by doing appropriate serological testing of donor cattle and by inoculating donor lymphocytes into sheep and then monitoring them for evidence of viral infection. Potential contaminants include the agents of enzootic bovine leukosis, infectious bovine rhinotracheitis, bovine pestivirus, ephemeral fever, Akabane disease, Aino virus, bluetongue, Brucella abortus and Leptospira, foot and mouth disease, lumpy skin disease, rabies, Rift Valley fever, rinderpest, contagious bovine pleuropneumonia, heartwater, Jembrana disease, and pathogenic Theileria and Trypanosoma spp. (4, 35).

b) Safety
Vaccine reactions of the cattle inoculated in the test for potency (see Section C.4.c) are monitored by measuring parasitaemia, fever and depression of packed cell volume. Only batches with pathogenicity levels equal to or lower than a predetermined standard are released for use.

c) Potency
Frozen, glycerolised vaccine concentrate is thawed and diluted 1/10 with isotonic diluent (4, 35). The prepared vaccine is then incubated for 8 hours at 4°C, and five cattle are inoculated subcutaneously with
2 ml doses each. The inoculated cattle are then monitored for the presence of infections by examination of stained blood smears. Only fully infective batches are released for use at a working dilution of 1/10.

d) Duration of immunity
Long-lasting immunity usually results from one inoculation. Evidence of *B. bovis* vaccine failures have been reported and are related to the choice of vaccine strains, the presence of heterologous field strains, and are related to choice of vaccine strain, the presence of heterologous field strains and host factors (4). There is little evidence of time-related waning of immunity (5).

e) Stability
When stored in liquid nitrogen, the vaccine can be kept for 5 years. Sterile diluent can be kept for 2 years in a refrigerator. Thawed vaccine rapidly loses potency and cannot be refrozen.

f) Preservatives
Penicillin (500,000 IU/litre) and streptomycin (370,000 µg/litre) are added to the vaccine at the time of dispensing.

g) Use of vaccine
In the case of frozen vaccine, vials should be thawed by immersion in water preheated to 37–40°C. Glycerolised vaccine should be kept cool and used within 8 hours (4), while vaccine with DMSO as cryoprotectant should be kept on ice and used within 15–30 minutes of thawing (35).

Chilled vaccine should be kept refrigerated and used within 4–7 days of preparation, depending on the viability of the parasites.

The strains of *B. bovis*, *B. divergens* and *B. bigemina* used in the vaccine may be of reduced virulence, but will not be entirely safe. A practical recommendation is, therefore, to limit the use of vaccine to calves, when nonspecific immunity will minimise the risk of vaccine reactions. If older animals are to be vaccinated, there is a risk of severe reactions. These reactions occur infrequently, but valuable breeding stock or pregnant animals warrant due attention and should be observed daily for 3 weeks after vaccination. Ideally, rectal temperatures of vaccinated cattle should be taken and the animals should be treated if significant fever develops. Reactions to *B. bigemina* and *B. divergens* are usually seen by day 6–8 and those to *B. bovis* by day 10–16 (4).

Protective immunity develops in 3–4 weeks, and lasts at least 4 years in most cases (4).

Babesiosis and anaplasmosis vaccines are often used concurrently, but it is not advisable to use any other vaccines at the same time (4).

h) Precautions
*Babesia bovis* and *B. bigemina* vaccines are not infective for humans. However, cases of *B. divergens* have been reported in splenectomised individuals. When the vaccine is stored in liquid nitrogen, the usual precautions pertaining to the storage, transportation and handling of deep-frozen material applies.

5. Tests on the final product

a) Safety
See Section C.4.b.

b) Potency
See Section C.4.c.

REFERENCES

Chapter 2.4.2. — Bovine babesiosis


* * *

NB: There is an OIE Reference Laboratories for Bovine babesiosis (see Table in Part 3 of this Terrestrial Manual or consult the OIE Web site for the most up-to-date list: www.oie.int).
CHAPTER 2.4.3.

BOVINE BRUCELLOSIS

SUMMARY

Bovine brucellosis is usually caused by Brucella abortus, less frequently by B. melitensis, and by B. suis. Infection is widespread globally. Several countries in Northern and Central Europe, Canada, Japan, Australia and New Zealand are believed to be free from the agent.

Clinically, the disease is characterised by one or more of the following signs: abortion, retained placenta, orchitis, epididymitis and, rarely, arthritis, with excretion of the organisms in uterine discharges and in milk. Diagnosis depends on the isolation of Brucella from abortion material, udder secretions or from tissues removed at post-mortem. Presumptive diagnosis can be made by assessing specific cell-mediated or serological responses to Brucella antigens.

Brucella abortus, B. melitensis and B. suis are highly pathogenic for humans, and all infected tissues, cultures and potentially contaminated materials must be handled under appropriate containment conditions.

Identification of the agent: Presumptive evidence of Brucella is provided by the demonstration, by modified acid-fast staining of organisms, of Brucella morphology in abortion material or vaginal discharge, especially if supported by serological tests. The recently developed polymerase chain reaction methods provide additional means of detection. Whenever possible, Brucella spp. should be isolated using plain or selective media by culture from uterine discharges, aborted fetuses, udder secretions or selected tissues, such as lymph nodes and male and female reproductive organs. Species and biovars should be identified by phage lysis, and by cultural, biochemical and serological criteria. Polymerase chain reaction (PCR) can provide both a complementary and biotyping method based on specific genomic sequences.

Serological and allergic skin tests: The buffered Brucella antigen tests, i.e. rose bengal test and buffered plate agglutination test, the complement fixation test, the enzyme-linked immunosorbent assay (ELISA) or the fluorescence polarization assay, are suitable tests for screening herds and individual animals. However, no single serological test is appropriate in each and all epidemiological situations. Therefore, the reactivity of samples that are positive in screening tests should be confirmed using an established confirmatory strategy. The indirect ELISA or milk ring test performed on bulk milk samples are effective for screening and monitoring dairy cattle for brucellosis, but the milk ring test is less reliable in large herds. Another immunological test is the brucellin skin test, which can be used as a screening or as a confirmatory herd test when positive serological reactors occur in the absence of obvious risk factors in unvaccinated herds. Interferon gamma tests, precipitin tests using native hapten antigen and indirect ELISA using rough lipopolysaccharide antigen have shown promise in differentiating brucellosis from exposure to cross-reacting microorganisms.

Requirements for vaccines and diagnostic biologicals: Brucella abortus strain 19 remains the reference vaccine to which any other vaccines are compared. It should be prepared from US-derived seed cultures, and each batch must conform to minimum standards for viability, smoothness, residual virulence and ability to immunise mice against challenge with a virulent strain of B. abortus or designated (colony-forming units) CFU per dose. Brucella abortus strain RB51 vaccine was produced from a laboratory-derived rough mutant of smooth B. abortus strain 2308. Cattle efficacy tests of the RB-51 vaccine have been completed and it is licensed in the United States of America. It has also become the official vaccine for prevention of brucellosis in cattle in some other countries. Brucellin preparations for the intradermal test must be free of smooth
The World Health Organization (WHO) laboratory of brucellosis in these animals are similar to those in cattle. and also occurs in the African buffalo (*Syncerus caffer*). The first three of these are subdivided into biovars based on published in the Approved Lists of Bacterial Names, 1980, and the designated type strains are attached to these nomenspecies with recognised biovars. The classical names related to the six *Brucella* genospecies are closely related to some plant pathogens and symbionts of the genera *Rhizobium* and *Bartonella* (and opportunistic or soil bacteria (*Ochrobactrum*).

**A. INTRODUCTION**

Brucellosis in cattle is usually caused by biovars of *Brucella abortus*. In some countries, particularly in southern Europe and western Asia, where cattle are kept in close association with sheep or goats, infection can also be caused by *B. melitensis* (41). Occasionally, *B. suis* may cause a chronic infection in the mammary gland of cattle, but it has not been reported to cause abortion or spread to other animals (29). The disease is usually asymptomatic in nonpregnant females. Following infection with *B. abortus* or *B. melitensis*, pregnant adult females develop a placentitis usually resulting in abortion between the fifth and ninth month of pregnancy. Even in the absence of abortion, profuse excretion of the organism occurs in the placenta, fetal fluids and vaginal discharges. The mammary gland and associated lymph nodes may also be infected, and organisms may be excreted in the milk. Subsequent pregnancies are usually carried to term, but uterine and mammary infection recurs, with reduced numbers of organisms in cyetic products and milk. In acute infections, the organism is present in most major body lymph nodes. Adult male cattle may develop orchitis and brucellosis may be a cause of infertility in both sexes. Hygromas, usually involving leg joints, are a common manifestation of brucellosis in some tropical countries and may be the only obvious indicator of infection; the hygroma fluid is often infected with *Brucella*.

Brucellosis has been reported in the one-humped camel (*Camelus dromedarius*) and in the two-humped camel (*C. bactrianus*), and in the South American camelids, llama (*Lama glama*), alpaca (*Lama pacos*), guanaco (*Lama guinicoe*), and vicuna (*Vicugne vicugne*) related to contact with large and small ruminants infected with *B. abortus* or *B. melitensis*. In addition, brucellosis has been observed in the domestic buffalo (*Bubalus bubalis*), American and European bison (*Bison bison*, *Bison bonasus*), yak (*Bos grunniens*), elk/wapiti (*Cervus elaphus*) and also occurs in the African buffalo (*Syncerus caffer*) and various African antelope species. The manifestations of brucellosis in these animals are similar to those in cattle.

The World Health Organization (WHO) laboratory biosafety manual classifies *Brucella* in Risk group III. Brucellosis is readily transmissible to humans, causing acute febrile illness – undulant fever – which may progress to a more chronic form and can also produce serious complications affecting the musculo–skeletal, cardiovascular, and central nervous systems. Where the disease is endemic, precautions should be taken to prevent human infection. Infection is often due to occupational exposure and is essentially acquired by the oral, respiratory, or conjunctival routes, but ingestion of dairy products constitutes the main risk to the general public where the disease is endemic. There is an occupational risk to veterinarians and farmers who handle infected animals and aborted fetuses or placentae. Brucellosis is one of the most easily acquired laboratory infections, and strict safety precautions should be observed when handling cultures and heavily infected samples, such as products of abortion. Specific recommendations have been made for the safety precautions to be observed with *Brucella*-infected materials (for further details see refs 2, 42, 95 and Chapter 1.1.2 Biosafety and biosecurity in the veterinary microbiological laboratory and animal facilities). Laboratory manipulation of live cultures or contaminated material from infected animals is hazardous and must be done under containment level 3 or higher, as outlined in Chapter 1.1.2, to minimise occupational exposure. Where large-scale culture of *Brucella* is carried out (e.g. for antigen or vaccine production) then biosafety level 3 is essential.

Genetic and immunological evidence indicates that all members of the *Brucella* genus are closely related. Nevertheless, based on relevant differences in host preference and epidemiology displayed by the major variants, as well as molecular evidence of genomic variation, the International Committee on Systematics of Prokaryotes, Subcommittee on the Taxonomy of *Brucella* took a clear position in 2005 on a return to pre-1986 *Brucella* taxonomic opinion; the consequences of this statement imply the re-approval of the six *Brucella* nomenspecies with recognised biovars. The classical names related to the six *Brucella* nomenspecies are validly published in the Approved Lists of Bacterial Names, 1980, and the designated type strains are attached to these validly published names: *Brucella abortus*, *B. melitensis*, *B. suis*, *B. neotomae*, *B. ovis* and *B. canis* (http://www.the-icsp.org/subcoms/Brucella.htm). The first three of these are subdivided into biovars based on cultural and serological properties (see Tables 1 and 2). Strains of *Brucella* have been isolated in the last decade from marine mammals that cannot be ascribed to any of the above-recognised species (28, 31). Investigations are continuing to establish their correct position in the taxonomy of the genus and it is proposed that they could be classified into two new species, *B. ceti* and *B. pinnipedialis* (18, 31, 40). Finally, *Brucella* shows close genetic relatedness to some plant pathogens and symbionts of the genera *Agrobacterium* and *Rhizobium*, as well as, animal pathogens (*Bartonella*) and
Table 1. Differential characteristics of species of the genus Brucella

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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Biovar 2: swine, hare</td>
</tr>
<tr>
<td><em>B. suis</em></td>
<td>S</td>
<td>–</td>
<td>+</td>
<td>+g</td>
<td>+g</td>
<td>–</td>
<td>+</td>
<td>+h</td>
<td>Biovar 3: swine</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Biovar 4: reindeer</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Biovar 5: wild rodents</td>
</tr>
<tr>
<td><em>B. melitensis</em></td>
<td>S</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>+i</td>
<td>Sheep and goats</td>
<td></td>
</tr>
<tr>
<td><em>B. neotomae</em></td>
<td>S</td>
<td>–</td>
<td>–</td>
<td>+k</td>
<td>+</td>
<td>–</td>
<td>+h</td>
<td>Desert wood rat</td>
<td></td>
</tr>
<tr>
<td><em>B. ovis</em></td>
<td>R</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>Rams</td>
<td></td>
</tr>
<tr>
<td><em>B. canis</em></td>
<td>R</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>+h</td>
<td>Dogs</td>
<td></td>
</tr>
</tbody>
</table>

From refs 2, 42.

a Phages: Tbilisi (Tb), Weybridge (Wb), Izatnagar1(Iz 1) and R/C
b Normally occurring phase: S: smooth, R: rough
c RTD: routine test dilution
d *Brucella abortus* biovar 2 generally requires serum for growth on primary isolation
e Some African isolates of *B. abortus* biovar 3 are negative
f Intermediate rate, except strain 544 and some field strains that are negative
g Some isolates of *B. suis* biovar 2 are not or partially lysed by phage Wb or Iz 1
h Rapid rate
i Some isolates are lysed by phage Wb
j Slow rate, except some strains that are rapid
k Minute plaques
l Neotoma lepida
### Table 2. Differential characteristics of the biovars of *Brucella* species

<table>
<thead>
<tr>
<th>Species</th>
<th>Biovar</th>
<th>CO₂ requirement</th>
<th>H₂S production</th>
<th>Growth on dyes&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Agglutination with monospecific sera</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Thionin</td>
<td>Basic fuchsin</td>
<td>A</td>
<td>M</td>
<td>R</td>
</tr>
<tr>
<td><em>B. melitensis</em></td>
<td>1</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td><em>B. abortus</em></td>
<td>1</td>
<td>+ or &lt;sup&gt;b&lt;/sup&gt;</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td><em>B. suis</em></td>
<td>4</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>–&lt;sup&gt;c&lt;/sup&gt;</td>
<td>–</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>9</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td><em>B. neotomae</em></td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>–&lt;sup&gt;d&lt;/sup&gt;</td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td><em>B. ovis</em></td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>–&lt;sup&gt;f&lt;/sup&gt;</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td><em>B. canis</em></td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–&lt;sup&gt;f&lt;/sup&gt;</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>+</td>
</tr>
</tbody>
</table>

From refs 2, 42.

<sup>a</sup> Dye concentration in serum dextrose medium: 20 µg/ml
<sup>b</sup> Usually positive on primary isolation
<sup>c</sup> Some basic fuchsinsensitive strains have been isolated
<sup>d</sup> Some strains are inhibited by dyes
<sup>e</sup> Some basic fuchsinsensitive strains have been isolated
<sup>f</sup> Negative for most strains
<sup>g</sup> Growth at a concentration of 10 µg/ml thionin

### B. DIAGNOSTIC TECHNIQUES

All abortions in cattle should be treated as suspected brucellosis and should be investigated. The clinical picture is not pathognomonic, although the herd history may be helpful. Unequivocal diagnosis of *Brucella* infections can be made only by the isolation and identification of *Brucella*, but in situations where bacteriological examination is not practicable, diagnosis must be based on serological methods. There is no single test by which a bacterium can be identified as *Brucella*. A combination of growth characteristics, serological, bacteriological methods and/or molecular methods is usually needed.
1. **Identification of the agent (2, 20, 21, 42)**

a) **Staining methods**

*Brucella* are coccobacilli or short rods measuring from 0.6 to 1.5 µm long and from 0.5 to 0.7 µm wide. They are usually arranged singly, and less frequently in pairs or small groups. The morphology of *Brucella* is fairly constant, except in old cultures where pleomorphic forms may be evident. *Brucella* are nonmotile. They do not form spores, and flagella, pili, or true capsules are not produced. *Brucella* are Gram negative and usually do not show bipolar staining. They are not truly acid-fast, but are resistant to decolorisation by weak acids and thus stain red by the Stamp’s modification of the Ziehl–Neelsen method. This is the usual procedure for the examination of smears of organs or biological fluids that have been previously fixed with heat or ethanol, and by this method, *Brucella* organisms stain red against a blue background. A fluorochrome or peroxidase-labelled antibody conjugate based technique could also be used (77). The presence of intracellular, weakly acid-fast organisms of *Brucella* morphology or immuno-specifically stained organisms is presumptive evidence of brucellosis. However, these methods have a low sensitivity in milk and dairy products where *Brucella* are often present in small numbers, and interpretation is frequently impeded by the presence of fat globules. Care must be taken as well in the interpretation of positive results in the Stamps’s method because other organisms that cause abortions, e.g. *Chlamydia abortus* (formerly *Chlamydia psittaci*) or *Coxiella burnetii* are difficult to differentiate from *Brucella* organisms. The results, whether positive or negative, should be confirmed by culture.

DNA probes or polymerase chain reaction (PCR) methods can be used to demonstrate the agent in various biological samples (11).

b) **Culture**

i) **Basal media**

Direct isolation and culture of *Brucella* are usually performed on solid media. This is generally the most satisfactory method as it enables the developing colonies to be isolated and recognised clearly. Such media also limit the establishment of nonsmooth mutants and excessive development of contaminants. However, the use of liquid media may be recommended for voluminous samples or for enrichment purpose. A wide range of commercial dehydrated basal media is available, e.g. *Brucella* medium base, tryptose (or trypticase)—soy agar (TSA). The addition of 2–5% bovine or equine serum is necessary for the growth of strains such as *B. abortus* biovar 2, and many laboratories systematically add serum to basal media, such as blood agar base (Oxoid) or Columbia agar (BioMérieux), with excellent results. Other satisfactory media, such as serum–dextrose agar (SDA) or glycerol dextrose agar, can be used (2). SDA is usually preferred for observation of colonial morphology. A nonselective, biphasic medium, known as Castañeda’s medium, is recommended for the isolation of *Brucella* from blood and other body fluids or milk, where enrichment culture is usually advised. Castañeda’s medium is used because brucellae tend to disassociate in broth medium, and this interferes with biotyping by conventional bacteriological techniques.

ii) **Selective media**

All the basal media mentioned above can be used for the preparation of selective media. Appropriate antibiotics are added to suppress the growth of organisms other than *Brucella*. The most widely used selective medium is the Farrell’s medium (30), which is prepared by the addition of six antibiotics to a basal medium. The following quantities are added to 1 litre of agar: polymyxin B sulphate (5000 units = 5 mg); bacitracin (25,000 units = 25 mg); natamycin (50 mg); nalidixic acid (5 mg); nystatin (100,000 units); vancomycin (20 mg).

A freeze-dried antibiotic supplement is available commercially (Oxoid). However, nalidixic acid and bacitracin, at the concentration used in Farrell’s medium, have inhibitory effects on some *B. abortus* and *B. melitensis* strains (50). Therefore the sensitivity for *B. melitensis* isolation increases significantly by the simultaneous use of both Farrell’s and the modified Thayer–Martin medium. Briefly, the modified Thayer–Martin’s medium can be prepared with GC medium base (38 g/litre; Biolife Laboratories, Milan, Italy) supplemented with haemoglobin (10 g/litre; Difco) and colistin methanesulphonate (7.5 mg/litre), vancomycin (3 mg/litre), nitrofurantoin (10 mg/litre), nystatin (100,000 International Units [IU]/litre = 17.7 mg) and amphotericin B (2.5 mg/litre) (all products from Sigma Chemical, St Louis, United States of America [USA]) (50). Contrary to several biovars of *B. abortus*, growth of *B. melitensis* is not dependent on an atmosphere of 5–10% CO₂ (Table 2).

As the number of *Brucella* organisms is likely to be lower in milk, colostrum and some tissue samples than in abortion material, enrichment is advisable. In the case of milk, results are also improved by centrifugation and culture from the cream and the pellet. Enrichment can be carried out in liquid medium consisting of serum–dextrose broth, tryptose broth (or trypticase)—soy broth (TSA) or *Brucella* broth supplemented with an antibiotic mixture of at least amphotericin B (1 µg/ml), and vancomycin (20 µg/ml) (all final concentrations). The enrichment medium should be incubated at 37°C in air supplemented with 5–10% (v/v) CO₂ for up to 6 weeks, with weekly subcultures on to solid selective
iii) Collection and culture of samples

For the diagnosis of animal brucellosis by cultural examination, the choice of samples usually depends on the clinical signs observed. The most valuable samples include aborted fetuses (stomach contents, spleen and lung), fetal membranes, vaginal secretions (swabs), milk, semen and arthritis or hygroma fluids. From animal carcasses, the preferred tissues for culture are those of the reticulo-endothelial system (i.e. head, mammary and genital lymph nodes and spleen), the late pregnant or early post-parturient uterus, and the udder. Growth normally appears after 3–4 days, but cultures should not be discarded as negative until 8–10 days have elapsed.

**Tissues:** Samples are removed aseptically with sterile instruments. The tissue samples are prepared by removal of extraneous material (e.g. fat), cut into small pieces, and macerated using a ‘Stomacher’ or tissue grinder with a small amount of sterile phosphate buffered saline (PBS), before being inoculated onto solid media.

**Vaginal discharge:** A vaginal swab taken after abortion or parturition is an excellent source for the recovery of *Brucella* and far less risky for the personnel than abortion material. The swab is then streaked on to solid media.

**Milk:** Samples of milk must be collected cleanly after washing and drying the whole udder and disinfecting the teats. It is essential that samples should contain milk from all quarters, and 10–20 ml of milk should be taken from each teat. The first streams are discarded and the sample is milked directly into a sterile vessel. Care must be taken to avoid contact between the milk and the milker’s hands. The milk is centrifuged at 6000–7000 g for 15 minutes or 2000 g for 30 minutes in sealed tubes (to avoid the risk of aerosol contamination of personnel), and the cream and deposit are spread on solid selective medium, either separately or mixed. If brucellae are present in bulk milk samples, their numbers are usually low, and isolation from such samples is very unlikely.

**Dairy products:** Dairy products, such as cheeses, should be cultured on the media described above. As these materials are likely to contain small numbers of organisms, enrichment culture is advised. Samples need to be carefully homogenised before culture, after they have been ground in a tissue grinder or macerated and pounded in a ‘Stomacher’ or an electric blender with an appropriate volume of sterile PBS. Superficial strata (rind and underlying parts) and the core of the product should be cultured. As brucellae grow, survive or disappear quite rapidly, their distribution throughout the different parts of the product varies according to the local physico-chemical conditions linked to specific process technologies.

All samples should be cooled immediately after they are taken, and transported to the laboratory in the most rapid way. On arrival at the laboratory, milk and tissue samples should be frozen if they are not to be cultured immediately.

Use of laboratory animals should be avoided unless absolutely necessary, but may sometimes provide the only means of detecting the presence of *Brucella*, especially when samples have been shown to
be heavily contaminated or likely to contain a low number of *Brucella* organisms. Animal inoculation may be either subcutaneously or through abraded skin in guinea-pigs or, preferably, intravenously in mice. This work must be carried out under appropriate biosecurity conditions as outlined in Chapter 1.1.2. The spleens of mice are cultured 7 days after inoculation and, for guinea-pigs, a serum sample is subjected to specific tests 3 and 6 weeks after inoculation, then the spleens are cultured.

c) **Identification and typing**

Any colonies of *Brucella* morphology should be checked using a Gram-stained (or a Stamp-stained) smear. As the serological properties, dyes and phage sensitivity are usually altered in the nonsmooth phases, attention to the colonial morphology is essential in the typing tests described below. The recommended methods for observing colonial morphology are Henry’s method by obliquely reflected light, the acriflavine test described by Braun & Bonestell, or White & Wilson’s crystal violet method of staining colonies (2).

Identification of *Brucella* organisms can be carried out by a combination of the following tests: organism morphology and Gram or Stamp’s staining, colonial morphology, growth characteristics, urease, oxidase and catalase tests, and the slide agglutination test with an anti-*Brucella* polyclonal serum. Species and biovar identification requires elaborate tests (such as phage lysis and agglutination with A-, M- or R-specific antisera), the performance of which is left to reference laboratories with expertise in these methods. The simultaneous use of several phages e.g. Tbilissi (Tb), Weybridge (Wb), Izatnagar (Iz) and R/C provides a phage-typing system that, in experienced hands, allows a practical identification of smooth and rough species of *Brucella*. However, several characteristics, for example added CO\(_2\) requirement for growth, production of H\(_2\)S (detected by lead acetate papers), and growth in the presence of basic fuchsin and thionin at final concentrations of 20 µg/ml, are revealed by routine tests that can be performed in moderately equipped nonspecialised laboratories (see Tables 1 and 2).

When sending *Brucella* strains to a reference laboratory for typing, it is essential that smooth colonies be selected. Cultures should be lyophilised and sealed in ampoules packed in screw-capped canisters or subcultured on to appropriate nutrient agar slopes contained in screw-capped bottles. The strains could also be sent suspended in transport media (e.g. Amies), but this could provide an opportunity for the establishment of rough mutants.

i) *Brucella* organisms are among the most dangerous bacteria with which to work in terms of the risk of producing laboratory-acquired infections. For transporting *Brucella* cultures, the caps of the bottles or canisters should be screwed tightly down and sealed with PVC tapes. Bottles should be wrapped in absorbent paper or cotton wool, sealed in polyethylene bags and packed into a rigid container in accordance with the requirements of the International Air Transport Association (IATA) for shipping dangerous goods (39). These regulations are summarised in Chapter 1.1.1 Collection and shipment of diagnostic specimens, and they must be followed. As *Brucella* cultures are infectious agents, they are designated UN2814 and a Declaration of Dangerous Goods must be completed. There are also restrictions on submitting samples from suspected cases of brucellosis and the IATA regulations should be reviewed before sending samples (39). Other international and national guidelines should also be followed (96).

ii) Before dispatching cultures or diagnostic samples for culture, the receiving laboratory should be contacted to determine if a special permit is needed and if the laboratory has the capability to do the testing requested. If samples are to be sent across national boundaries, an import licence will probably be needed and should be obtained before the samples are dispatched (Chapter 1.1.2).

d) **Nucleic acid recognition methods**

The recently developed PCR provides an additional means of detection and identification of *Brucella* sp. (11, 14–16, 69). Despite the high degree of DNA homology within the genus *Brucella*, several molecular methods, including PCR, PCR restriction fragment length polymorphism (RFLP) and Southern blot, have been developed that allow, to a certain extent, differentiation between *Brucella* species and some of their biovars (for a review see refs 11 and 53). Pulse-field gel electrophoresis has been developed that allows the differentiation of several *Brucella* species (40, 51). *Brucella* biotyping and distinguishing vaccine strains by PCR can be accomplished satisfactorily but there has been limited validation of the PCR for primary diagnosis.

The first species-specific multiplex PCR assay for the differentiation of *Brucella* was described by Bricker & Halling (15). The assay, named AMOS-PCR, was based on the polymorphism arising from species-specific localisation of the insertion sequence IS711 in the *Brucella* chromosome, and comprised five oligonucleotide primers that can identify and differentiate *B. abortus*, biovars 1, 2 and 4 but could not differentiate *B. abortus* biovars 3, 5, 6 and 9. Modifications to the assay, designated BaSS PCR, have been introduced over time to improve performance, and additional strain-specific primers were incorporated for in identification of the *B. abortus* vaccine strains, S19 and RB51 (14, 16, 26, 27). The AMOS PCR and BaSS PCR are both single-tube multiplex PCR assays. The AMOS PCR differentiates *B. abortus*, *B. melitensis*, and
Chapter 2.4.3. — Bovine brucellosis

*B. ovis*, and *B. suis* (only biovar 1 although the other biovars will be detected from the ery primers) to the species level. The BaSS PCR allows differentiation of further strains, specifically the vaccines, S10 and RB51. The procedures for the two tests are the same with the only difference being the primers in the master mix. However, other species and biovars (such as *B. abortus* biovars 3, 5, 6 and 9, *B. suis* biovars 2, 3, 4, and 5, *B. canis*, *B. neotomae*, *B. ceti* and *B. pinnipedialis*) cannot be detected by the AMOS-PCR or BaSS PCR.

There has recently been a report that these PCR procedures have been further modified to also identify *B. abortus* biovars 3, 5, 6 and 9 (69).

- **BaSS PCR procedure** is provided as an example of one molecular procedure (see references 16 and 27 for detailed instructions).
- **Bacterial preparation**
  
  i) Any accepted method for DNA purification would be appropriate. A simple and effective method is to select a single colony and, using a sterile inoculating loop, transfer the bacteria to 100 µl of PCR-grade water. The bacterial suspension should be boiled for a minimum of 5 minutes to kill the bacteria and to facilitate the lysis of most of the bacteria as a template for the reaction. Adjust the concentration of bacteria to a density of 1.5 to 2.0 units of Absorbance at 600 nm with saline. Immediately before use, re-mix the culture suspension and dilute an aliquot 1/10 in PCR-grade water (e.g. 5 µl suspension in 45 µl water). Mix gently but thoroughly. The diluted material should be appropriately discarded after use.

- **PCR primer sequences and stock concentrations**

<table>
<thead>
<tr>
<th>Primer</th>
<th>Nucleotide sequence 5' to 3'</th>
<th>Concentration of 100× stock</th>
</tr>
</thead>
<tbody>
<tr>
<td>IS711-specific</td>
<td>TGC-CGA-TCA-CTT-AAG-GGC-CTT-CAT-TGC-CAG</td>
<td>1.90 µg/µl</td>
</tr>
<tr>
<td>Abortus-specific</td>
<td>GAC-GAA-CGG-AAT-TTT-TTC-AAT-CCC</td>
<td>1.55 µg/µl</td>
</tr>
<tr>
<td>16S-universal-F</td>
<td>GTG-CCA-GCA-GCC-GCC-GTA-ATA-C</td>
<td>1.40 µg/µl</td>
</tr>
<tr>
<td>16S-universal-R</td>
<td>TGG-TGT-GAC-GGG-CGG-TGG-GTA-AA-G</td>
<td>1.60 µg/µl</td>
</tr>
<tr>
<td>eri-F</td>
<td>GCG-CCG-CGA-AGA-CT-TAT-TAA</td>
<td>1.35 µg/µl</td>
</tr>
<tr>
<td>eri-R</td>
<td>CGC-CAT-GTT-AGG-GGC-GGT-GA</td>
<td>1.30 µg/µl</td>
</tr>
<tr>
<td>RB51-3</td>
<td>GCC-AAC-CAA-CCC-AAA-TGC-TCA-CAA</td>
<td>1.55 µg/µl</td>
</tr>
</tbody>
</table>

- **PCR amplification**

  - **Preparation of the master mix (100 assays)**
    
    i) Synthetic oligonucleotides should be dissolved in TE buffer to a concentration of 100× (see Table above). The 100× stock is stable at 4°C for at least 2 years as long as care is taken not to contaminate the solution.
    
    ii) Prepare the primer cocktail by dispensing the following 100× concentrates into a 1.5-ml microfuge tube:

    - 233 µl PCR-grade water
    - 2.5 µl IS711-specific primer
    - 2.5 µl *B. abortus* specific primer
    - 2.5 µl 16S universal primer-F (optional control for inhibitors)
    - 2.5 µl 16S universal primer-R (optional control for inhibitors)
    - 2.5 µl eri primer-F
    - 2.5 µl eri primer-R
    - 2.5 µl RB51-primer

    iii) Prepare the master mix by dispensing the following into a 3 or 5 ml disposable tube:

    - 1130 µl PCR-grade water
    - 250 µl 10× reaction buffer *without* MgCl₂ (see Note 6)
    - 150 µl 25 mM MgCl₂
    - 200 µl 10 mM dNTP mix
    - 250 µl Primer Cocktail from Step 2
    - 500 µl GC Rich Enhancer. If an enhancer is not used, then 500 µl PCR-grade water should be substituted.
    - 20 µl FastStart™ Taq DNA Polymerase
iv) Mix the solution thoroughly but gently by pipetting up and down.

v) Aliquot the master mix in 25 µl quantities into 0.2 µl thin-walled PCR tubes (or alternatively a PCR-certified 96-well plate). Store the assay tubes at -20°C ±2°C.

vi) Prior to use, thaw enough master mix tubes for unknowns and controls, and mix thoroughly but gently by finger tapping.

- Amplification of products by PCR

i) Add between 1.0 and 2.5 µl of unknown sample or control to each assay tube. Be sure to mix each sample thoroughly just before removing the aliquot since Brucella tends to settle out quickly.

ii) Amplify the PCR products by using the following parameters:

\[
\begin{align*}
95^\circ C & \quad 5.0 \text{ minutes} & \quad 1 \text{ cycle} \\
95^\circ C & \quad 15 \text{ seconds} \\
52^\circ C & \quad 30 \text{ seconds} & \quad 40 \text{ cycles} \\
72^\circ C & \quad 90 \text{ seconds} \\
4^\circ C & \quad \text{indefinitely}
\end{align*}
\]

The choice of ramp-time does not appear to be critical.

iii) After amplification, the unopened samples can be stored indefinitely at 4°C until ready for detection.

- Detection of amplified products

i) Prepare a 5 mm thick, 2.0% agarose gel (in 0.5× TBE) with an appropriate number of wells.

ii) Combine 1 µl of 6× loading dye with 8 µl amplified sample and mix well before loading into the gel well.

iii) Run the gel in 0.5× TBE until the bromophenol blue marker is at least 5 cm from the well to achieve good separation of the bands. For the equipment described here, we use 80–85 V for 2.5 hours to maximise resolution without significant diffusion of the bands but adjustments for other equipment may be needed.

iv) Stain the gel for 45 minutes in ethidium bromide solution (250 µg/500 ml of 0.5× TBE). Alternatively, the gel can be stained before electrophoresis or during electrophoresis by adding ethidium bromide to the running buffer. CAUTION: ethidium bromide is a mutagen and potential carcinogen.

- Interpretation of data

Identification is based on the number and the sizes of the products amplified by PCR (see Figures A & B). All samples except the negative controls should amplify at least 1 product, the 800-bp 16S sequence. If this band is not present then the sample may contain PCR inhibitors, the DNA was degraded, or the sample was not dispensed into the master mix. It may be necessary to dilute the original sample to decrease the level of inhibitors in the reaction, repeat the assay with a fresh sample, or simply repeat the assay with the original sample.

All Brucella abortus (biovar 1, 2, and 4) isolates including the vaccine strains will also amplify a 500-bp product from the IS711 alk8 locus. Other Brucella species or bacteria will not amplify this product. Only B. abortus vaccine strain RB51 will amplify a 300-bp product from the IS711 wboA locus. All Brucella species and strains except B. abortus vaccine strain S19 will amplify the 180-bp product from the ervA gene, but other bacteria will not. Sample results are shown in section B of figure.
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Figure Legends

**Figure A.** Predicted amplified loci (rows) for various categories of unknowns (columns). A: The four loci for each category are shown with their hybridising primers; B: the predicted products resulting from successful amplification (fs refers to field strain of B. abortus).

**Figure B.** Typical patterns amplified from bacterial bovine isolates as detected by agarose gel electrophoresis. Lane 1: B. abortus field strain; Lane 2: B. abortus s19; Lane 3: B. abortus RB51; Lane 4: Brucella spp. (not B. abortus); Lane 5: non-Brucella bacteria. A 2% agarose gel was loaded with 8 μl amplified product and 1 μl loading dye per well, electrophoresed for 2.5 hours at 70 V, stained with ethidium bromide, and visualised with UV light.

Another new test that has been developed is HOOF-Prints. This fingerprinting test has recently been developed and is showing great potential as an epidemiology tool (12, 13). This test, also known as multiple locus variable number tandem repeats analysis (MLVA), could be a complement to classical biotyping methods in accordance with the established taxonomy (45).

The use of molecular procedures for the identification of Brucella species has increased and the test procedures improved since the 1990s. Other tests such as as omp 25, 2a and 2b PCR/RFLP for B. abortus are now available and may be used to identify Brucella species (17, 18).

e) Identification of vaccine strains

Identification of the vaccine strains B. abortus S19, B. abortus RB51 and B. melitensis strain Rev.1, depends on further tests.

*Brucella abortus* S19 has the normal properties of a biovar 1 strain of B. abortus, but does not require CO₂ for growth, does not grow in the presence of benzylpenicillin (3 μg/ml = 5 IU/ml), thionin blue (2 μg/ml), and i-erythritol (1 mg/ml) (all final concentrations), and presents a high L-glutamate use (2). In some cases strain 19 will grow in the presence of i-erythritol, but does not use it.

*Brucella melitensis* strain Rev.1 has the normal properties of a biovar 1 strain of B. melitensis, but grows much more slowly on ordinary media, does not grow in the presence of basic fuchsin, thionin (20 μg/ml) or
benzylpenicillin (3 µg/ml) (final concentrations), but does grow in the presence of streptomycin at 2.5 or 5 µg/ml (5 IU/ml) (2, 20, 21, 24).

*Brucella abortus* strain RB51 is identified by several characteristics; these are: rough morphology, growth in the presence of rifampicin (250 µg per ml of media), and inability to produce O-polysaccharide (OPS) (81). The inability to produce OPS can be demonstrated by reacting RB51 colonies with OPS-specific monoclonal antibodies (MAbs), in dot-blot assays or Western blots (77, 81). An indirect way of demonstrating lack of OPS is by injecting $4 \times 10^8$ viable RB51 organisms into BALB/c mice and testing for the induction of OPS-antibodies; the serology will be negative (81).

Vaccine strains S19, Rev.1 and RB51 may be identified using specific PCRs (16, 80, 89, 91).

### 2. Serological tests

No single serological test is appropriate in all epidemiological situations; all have limitations especially when it comes to screening individual animals (36, 68). Consideration should be given to all factors that impact on the relevance of the test method and test results to a specific diagnostic interpretation or application. For the purposes of this chapter, the serological methods described represent standardised and validated methods with suitable performance characteristics to be designated as either prescribed or alternative tests for international trade. This does not preclude the use of modified or similar test methods or the use of different biological reagents. However, the methods and reagents described in this chapter represent a standard of comparison with respect to expected diagnostic performance.

It should be stressed that the serum agglutination test (SAT) is generally regarded as being unsatisfactory for the purposes of international trade. The complement fixation test (CFT) is diagnostically more specific than the SAT, and also has a standardised system of unitage. The diagnostic performance characteristics of some enzyme-linked immunosorbent assays (ELISAs) and the fluorescence polarisation assay (FPA) are comparable with or better than that of the CFT, and as they are technically simpler to perform and more robust, their use may be preferred (64, 98). The performances of several of these tests have been compared.

For the control of brucellosis at the national or local level, the buffered *Brucella* antigen tests (BBATs), i.e. the rose bengal test (RBT) and the buffered plate agglutination test (BPAT), as well as the ELISA and the FPA, are suitable screening tests. Positive reactions should be retested using a suitable confirmatory strategy.

In other species, for example, buffaloes (*Bubalus bubalis*), American and European bison (*Bison bison, Bison bonasus*), yak (*Bos grunniens*), elk/wapiti (*Cervus elaphus*), and camels (*Camelus bactrianus* and *C. dromedarius*), South-American camelids, *Brucella* sp. infection follows a course similar to that in cattle. The same serological procedures may be used for these animals (59,33, 34).

• **Reference sera**

  Primary bovine reference standards are those against which all other standards are compared and calibrated. These reference standards are all available to national reference laboratories and should be used to establish secondary or national standards against which working standards can be prepared and used in the diagnostic laboratory for daily routine use.

  These sera have been developed and designated by the OIE as International Standard Sera\(^1\). The use of these promotes international harmonisation of diagnostic testing and antigen standardisation (98):

  • For RBT and CFT, the OIE International Standard Serum (OIEISS, previously the WHO Second International anti-*Brucella abortus* Serum) that contains 1000 IU and ICFTU (international complement fixation test units) is used.

  • In addition, three OIE ELISA Standard Sera are available for use. These are also of bovine origin and consist of a strong positive (OIEELISASPSS), a weak positive (OIEELISAWPSS) and a negative (OIEELISANSS) standard.

• **Production of cells**

  *Brucella abortus* strain 99 (Weybridge) (S99) (see footnote 1 for address) or *B. abortus* strain 1119-3 (USDA) (S1119-3)\(^2\) should always be used for diagnostic antigen production. It should be emphasised that antigen made with one of these *B. abortus* strains is also used to test for *B. melitensis* or *B. suis* infection. The strains must be completely smooth and should not autoagglutinate in saline and 0.1% (w/v) acriflavine.

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1 Obtainable from the OIE Reference Laboratory for Brucellosis at Veterinary Laboratories Agency (VLA) Weybridge, New Haw, Addlestone, Surrey KT15 3NB, United Kingdom.

2 Obtainable from the United States Department of Agriculture (USDA), National Veterinary Services Laboratories (NVSL), 1800 Dayton Road, Ames, Iowa 50010, United States of America.
They must be pure cultures and conform to the characteristics of CO₂-independent strains of *B. abortus* biovar 1. The original seed cultures should be propagated to produce a seed lot that must conform to the properties of these strains, and should be preserved by lyophilisation or by freezing in liquid nitrogen.

For antigen production, the seed culture is used to inoculate a number of potato-infusion agar slopes that are then incubated at 37°C for 48 hours. SDA and TSA, to which 5% equine or newborn calf serum and/or 0.1% yeast extract may be added, are satisfactory solid media provided a suitable seed is used as recommended above. The growth is checked for purity, resuspended in sterile PBS, pH 6.4, and used to seed layers of potato-infusion agar or glycerol–dextrose agar in Roux flasks. These are then incubated at 37°C for 72 hours with the inoculated surface facing down. Each flask is checked for purity by Gram staining samples of the growth, and the organisms are harvested by adding 50–60 ml of phenol saline (0.5% phenol in 0.85% sodium chloride solution) to each flask. The flasks are gently agitated, the suspension is decanted, and the organisms are killed by heating at 80°C for 90 minutes. Following a viability check, the antigen is stored at 4°C.

Alternatively, the cells may be produced by batch or continuous culture in a fermenter (38), using a liquid medium containing (per litre of distilled water) D-glucose (30 g), a high-grade peptone (30 g), yeast extract (Difco) (10 g), sodium dihydrogen phosphate (9 g) and disodium hydrogen phosphate (3.3 g). The initial pH is 6.6, but this tends to rise to pH 7.2–7.4 during the growth cycle. Care should be taken to check batches of peptone and yeast extract for capacity to produce good growth without formation of abnormal or dissociated cells. Vigorous aeration and stirring is required during growth, and adjustment to pH 7.2–7.4 by the addition of sterile 0.1 M HCl may be necessary. The seed inoculum is prepared as described above. The culture is incubated at 37°C for 48 hours. Continuous culture runs can be operated for much longer periods, but more skill is required to maintain them. In-process checks should be made on the growth from either solid or liquid medium to ensure purity, an adequate viable count and freedom from dissociation to rough forms. Cells for use in the preparation of all antigens should be checked for purity and smoothness at the harvesting stage.

The culture is harvested by centrifugation to deposit the organisms, which are resuspended in phenol saline. The organisms are killed by heating at 80°C for 90 minutes and are stored at 4°C. They must form stable suspensions in physiological saline solutions and show no evidence of autoagglutination. A viability check must be performed on the suspensions and no growth must be evident after 10 days' incubation at 37°C. The packed cell volume (PCV) of the killed suspensions can be determined by centrifuging 1 ml volumes in Wintrobe tubes at 3000 g for 75 minutes.

a) **Buffered *Brucella* antigen tests (prescribed tests for international trade)**

- **Rose bengal test**
  
  This test is a simple spot agglutination test using antigen stained with rose bengal and buffered to a low pH, usually 3.65 ± 0.05 (54).

  - **Antigen production**

    Antigen for the RBT is prepared by depositing killed *B. abortus* S99 or S1119-3 cells by centrifugation at 23,000 g for 10 minutes at 4°C, and uniformly resuspending in sterile phenol saline (0.5%) at the rate of 1 g to 22.5 ml. (Note: if sodium carboxymethyl cellulose is used as the sedimenting agent during preparation of the cell concentrate, insoluble residues must be removed by filtering the suspension through an AMF-CUNO Zeta-plus prefilter [Type CPR 01A] before staining.) To every 35 ml of this suspension, 1 ml of 1% (w/v) rose bengal (Cl No. 45440) in sterile distilled water is added, and the mixture is stirred for 2 hours at room temperature. The mixture is filtered through sterile cotton wool, and centrifuged at 10,000 g to deposit the stained cells, which are then uniformly resuspended at the rate of 1 g cells to 7 ml of diluent (21.1 g of sodium hydroxide dissolved in 353 ml of sterile phenol saline, followed by 95 ml of lactic acid, and adjusted to 1056 ml with sterile phenol saline). The colour of this suspension should be an intense pink and the supernatant of a centrifuged sample should be free of stain; the pH should be 3.65 ± 0.05. After filtration through cotton wool, the suspension is filtered twice through a Sartorius No. 13430 glass fibre prefilter, adjusted to a PCV of approximately 8%, pending final standardisation against serum calibrated against the OIEISS, and stored at 4°C in the dark. The antigen should be stored as recommended by the manufacturer but usually should not be frozen.

    When used in the standard test procedure, the RBT antigen should give a clearly positive reaction with 1/45 dilution, but not 1/55 dilution, of the OIEISS diluted in 0.5% phenol saline. It may also be advisable to compare the reactivity of new and previously standardised batches of antigen using a panel of defined sera.

- **Test procedure**

  i) Bring the serum samples and antigen to room temperature (22 ± 4°C); only sufficient antigen for the day’s tests should be removed from the refrigerator.
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ii) Place 25–30 µl of each serum sample on a white tile, enamel or plastic plate, or in a WHO haemagglutination plate.

iii) Shake the antigen bottle well, but gently, and place an equal volume of antigen near each serum spot.

iv) Immediately after the last drop of antigen has been added to the plate, mix the serum and antigen thoroughly (using a clean glass or plastic rod for each test) to produce a circular or oval zone approximately 2 cm in diameter.

v) The mixture is agitated gently for 4 minutes at ambient temperature on a rocker or three-directional agitator (if the reaction zone is oval or round, respectively).

vi) Read for agglutination immediately after the 4-minute period is completed. Any visible reaction is considered to be positive. A control serum that gives a minimum positive reaction should be tested before each day’s tests are begun to verify the sensitivity of test conditions.

The RBT is very sensitive. However, like all other serological tests, it could sometimes give a positive result due to S19 vaccination or due to false-positive serological reactions (FPSR). Therefore positive reactions should be investigated using suitable confirmatory strategies (including the performance of other tests and epidemiological investigation). False-negative reactions occur rarely, mostly due to prozoning and can sometimes be detected by diluting the serum sample or retesting after 4–6 weeks. Nevertheless RBT appears to be adequate as a screening test for detecting infected herds or to guarantee the absence of infection in brucellosis-free herds.

• Buffered plate agglutination test

• Antigen production

Antigen for the BPAT is prepared from *B. abortus* S1119-3 according to the procedure described by Angus & Barton (3).

Two staining solutions are required: brilliant green (2 g/100 ml) and crystal violet (1 g/100 ml) both certified stains dissolved in distilled water. Once prepared, the two solutions should be stored separately for a period of 24 hours, and then mixed together in equal volumes in a dark bottle and stored in a refrigerator for a period of not less than 6 months before use. The mixed stain may only be used between 6 and 12 months after initial preparation.

Buffered diluent is prepared by slowly dissolving sodium hydroxide (150 g) in 3–4 litres of sterile phenol saline. Lactic acid (675 ml) is added to this solution, and the final volume is adjusted to 6 litres by adding sterile phenol saline. The pH of the solution should be between 3.63 and 3.67.

*Brucella abortus* S1119-3 packed cells are diluted to a concentration of 250 g/litre in phenol saline; 6 ml of stain is added per litre of cell suspension, and the mixture is shaken thoroughly before being filtered through sterile absorbent cotton. The cells are centrifuged at 10,000 g at 4°C, and the packed cells are then resuspended at a concentration of 50 g/100 ml in buffered diluent (as described above). This mixture is shaken thoroughly for 2 hours, and is then further diluted by the addition of 300 ml of buffered diluent per 100 ml of suspended cells (i.e. final concentration of 50 g packed cells/400 ml buffered diluent). The mixture is stirred at room temperature for 20–24 hours before the cell concentration is adjusted to 11% (w/v) in buffered diluent. This suspension is stirred overnight before testing. Pending final quality control tests, the antigen is stored at 4°C until required for use. The antigen has a shelf life of 1 year and should not be frozen.

The pH of the buffered plate antigen should be 3.70 ± 0.03 and the pH of a serum:antigen mixture at a ratio of 8:3 should be 4.02 ± 0.04. The 11% stained-cell suspension should appear blue-green. Each batch of buffered plate antigen should be checked by testing at least 10 weakly reactive sera and comparing the results with one or more previous batches of antigen. If possible, the antigen batches should be compared with the standard antigen prepared by the NVSL, USDA (see footnote 2 for address). There is, however, no international standardisation procedure established for use with the OIEISS.

• Test procedure

i) Bring the serum samples and antigen to room temperature (22 ± 4°C); only sufficient antigen for the day’s tests should be removed from the refrigerator.

ii) Shake the sample well. Place 80 µl of each serum sample on a glass plate marked in 4 × 4 cm squares.

iii) Shake the antigen bottle well, but gently, and place 30 µl of antigen near each serum spot.

iv) Immediately after the last drop of antigen has been added to the plate, mix the serum and antigen thoroughly (using a clean glass or plastic rod for each test) to produce a circular zone approximately 3 cm in diameter.
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v) After the initial mixing, the plate should be rotated three times in a tilting motion to ensure even dispersion of the reagents, and then incubated for 4 minutes in a humid chamber at ambient temperature.

vi) The plate should be removed and rotated as above, and then returned for a second 4-minute incubation.

vii) Read for agglutination immediately after the 8-minute period is completed. Any visible reaction is considered to be positive. A control serum that gives a minimum positive reaction should be tested before each day’s tests are begun to verify the sensitivity of test conditions.

Like the RBT, the test is very sensitive, especially for detection of vaccine-induced antibody, and positive samples should be retested using a confirmatory test(s). False-negative reactions may occur, usually due to prozoning, which may be overcome by diluting the serum or retesting after a given time.

b) Complement fixation test (a prescribed test for international trade)

The CFT is a widely used and accepted confirmatory test although it is complex to perform, requiring good laboratory facilities and adequately trained staff to accurately titrate and maintain the reagents. There are numerous variations of the CFT in use, but this test is most conveniently carried out in a microtitre format. Either warm or cold fixation may be used for the incubation of serum, antigen and complement: either 37°C for 30 minutes or 4°C for 14–18 hours. A number of factors affect the choice of the method: anti-complementary activity in serum samples of poor quality is more evident with cold fixation, while fixation at 37°C increases the frequency and intensity of prozones, and a number of dilutions must be tested for each sample.

Several methods have been proposed for the CFT using different concentrations of fresh or preserved sheep red blood cells (SRBCs) (a 2%, 2.5% or 3% suspension is usually recommended) sensitised with an equal volume of rabbit anti-SRBC serum diluted to contain several times (usually from two to five times) the minimum concentration required to produce 100% lysis of SRBCs in the presence of a titrated solution of guinea-pig complement. The latter is independently titrated (in the presence or absence of antigen according to the method) to determine the amount of complement required to produce either 50% or 100% lysis of sensitised SRBCs in a unit volume of a standardised suspension; these are defined as the 50% or 100% haemolytic unit of complement/minimum haemolytic dose (C'H or MHD_{50} or C'H or MHD_{100}); respectively. It is generally recommended to titrate the complement before each set of tests, a macromethod being preferred for an optimal determination of C'H_{50}. Usually, 1.25–2 C'H_{100} or 5–6 C'H_{50} are used in the test.

Barbital (veronal) buffered saline is the standard diluent for the CFT. This is prepared from tablets available commercially; otherwise it may be prepared from a stock solution of sodium chloride (42.5 g), barbituric acid (2.875 g), sodium diethyl barbiturate (1.875 g), magnesium sulphate (1.018 g), and calcium chloride (0.147 g) in 1 litre of distilled water and diluted by the addition of four volumes of 0.04% gelatin solution before use.

• Antigen production

Numerous variations of the test exist but, whichever procedure is selected, the test must use an antigen that has been prepared from an approved smooth strain of *B. abortus*, such as S99 or S1119-3, and standardised against the OIEISS. Antigen for the CFT can be prepared by special procedures (2, 38) or a whole cell antigen can be used after diluting the stock suspension such that the PCV of the concentrated antigen suspension for CFT should be approximately 2% before standardisation against the OIEISS. The antigen should be standardised to give 50% fixation at a dilution of 1/200 of the OIEISS and must also show complete fixation at the lower serum dilutions, because too weak (or too strong) a concentration of antigen may not produce 100% fixation at the lower dilutions of serum. When two dilutions of antigen are suitable, the more concentrated antigen suspension must be chosen in order to avoid prozone occurrence.

The appearance of the antigen when diluted 1/10 must be that of a uniform, dense, white suspension with no visible aggregation or deposit after incubation at 37°C for 18 hours. It must not produce anti-complementary effects at the working strength for the test. The antigen is stored at 4°C and should not be frozen.

• Test procedure (example)

The undiluted test sera and appropriate working standards should be inactivated for 30 minutes in a water bath at 60°C ± 2°C. If previously diluted with an equal volume of veronal buffered saline these sera could be inactivated at 58°C ± 2°C for 50 minutes. Usually, only one serum dilution is tested routinely (generally 1/4 or 1/5 depending on the CF procedure chosen), but serial dilutions are recommended for trade purposes in order to detect prozone.
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Using standard 96-well microtitre plates with round (U) bottoms, the technique is usually performed as follows:

i) Volumes of 25 µl of diluted inactivated test serum are placed in the well of the first, second and third rows. The first row is an anti-complementary control for each serum. Volumes of 25 µl of CFT buffer are added to the wells of the first row (anti-complementary controls) to compensate for lack of antigen. Volumes of 25 µl of CFT buffer are added to all other wells except those of the second row. Serial doubling dilutions are then made by transferring 25 µl volumes of serum from the third row onwards; 25 µl of the resulting mixture in the last row are discarded.

ii) Volumes of 25 µl of antigen, diluted to working strength, are added to each well except in the first row.

iii) Volumes of 25 µl of complement, diluted to the number of units required, are added to each well.

iv) Control wells containing diluent only, complement + diluent, antigen + complement + diluent, are set up to contain 75 µl total volume in each case. A control serum that gives a minimum positive reaction should be tested in each set of tests to verify the sensitivity of test conditions.

v) The plates are incubated at 37°C for 30 minutes or at 4°C overnight, and a volume (25 or 50 µl according to the technique) of sensitised SRBCs is added to each well. The plates are re-incubated at 37°C for 30 minutes.

vi) The results are read after the plates have been centrifuged at 1000 g for 10 minutes at 4°C or left to stand at 4°C for 2–3 hours to allow unlysed cells to settle. The degree of haemolysis is compared with standards corresponding to 0, 25, 50, 75 and 100% lysis. The absence of anti-complementary activity is checked for each serum in the first row.

vii) Standardisation of results of the CFT:

There is a unit system that is based on the OIEISS. This serum contains 1000 ICFTU (international complement fixation test units) per ml. If this serum is tested in a given method and gives a titre of, for example 200 (50% haemolysis), then the factor for an unknown serum tested by that method can be found from the formula: 1000 × 1/200 × titre of test serum = number of ICFTU of antibody in the test serum per ml. The OIEISS contains specific IgG; national standard sera should also depend on this isotype for their specific complement-fixing activity. Difficulties in standardisation arise because different techniques selectively favour CF by different immunoglobulin isotypes. It is recommended that any country using the CFT on a national scale should obtain agreement among the different laboratories performing the test to use the same method in order to obtain the same level of sensitivity. To facilitate comparison between countries, results should always be expressed in ICFTUs, calculated in relation to those obtained in a parallel titration with a standard serum, which in turn may be calibrated against the OIEISS.

vii) Interpretation of the results: Sera giving a titre equivalent to 20 ICFTU/ml or more are considered to be positive.

This procedure is an example, other volumes and quantities of reagents could be chosen provided that the test is standardised against the OIEISS as described above and the results expressed in ICFTU/ml.

The CFT is very specific. However, like all other serological tests, it could sometimes give a positive result due to S19 vaccination or due to FPSR. Therefore positive reactions should be investigated using suitable confirmatory strategies. Females that have been vaccinated with Brucella abortus S19 between 3 and 6 months are usually considered to be positive if the sera give positive fixation at a titre of 30 or greater ICFTU/ml when the animals are tested at an age of 18 months or older.

c) Enzyme-linked immunosorbent assays (prescribed tests for international trade)

- Indirect ELISA

Numerous variations of the indirect ELISA (I-ELISA) have been described employing different antigen preparations, antiglobulin-enzyme conjugates, and substrate/chromogens. Several commercial I-ELISAs are available that have been validated in extensive field trials and are in wide use. In the interests of international harmonisation, the three OIE ELISA Standard Sera should be used by national reference laboratories to check or calibrate the particular test method in question.

The assay should be calibrated such that the optical density (OD) of the strong positive OIE ELISA Standard Serum should represent a point on the linear portion of a typical dose−response curve just below the plateau. The weak positive OIE ELISA Standard Serum should consistently give a positive reaction that lies on the linear portion of the same dose−response curve just above the positive/negative threshold. The negative serum and the buffer control should give reactions that are always less than the positive/negative threshold (97). The threshold should be established in the test population using appropriate validation techniques (see Chapter 1.1.4 Principles of validation of diagnostic assays for infectious diseases).
The I-ELISA is a highly sensitive test but it is sometimes not capable of differentiating between antibody resulting from S19 vaccination or other FPSR problems and that induced by pathogenic *Brucella* strains (60). The I-ELISA should therefore be considered more as a screening test than a confirmatory test in the testing of vaccinated cattle or herds affected by FPSR problems.

The problem with FPSR may be partly overcome by performing an I-ELISA using rLPS as the antigen, cytosol antigens or chaotropic chemicals such as potassium thiocyanate. Most FPSR are a result of cross reaction with the O-polysaccharide portion of the sLPS molecule, however, cross reaction among core regions of LPS are less frequent (57).

For the screening I-ELISA, preparations rich in smooth lipopolysaccharide (sLPS) should be used as the antigen. There are several protocols for preparing a suitable antigen.

Monoclonal, polyclonal antiglobulin or protein A/G enzyme conjugates may be used depending on availability and performance requirements. An MAb specific for the heavy chain of bovine IgG₁ may provide some improvement in specificity at the possible cost of some loss of sensitivity while a protein A/G enzyme conjugate may provide a reagent useful for testing a variety of mammalian species (57, 67).

The test method described below is an example of a test that has been internationally validated and has been used extensively in internationally sponsored, technical cooperation and research collaboration projects world-wide.

The antigen-coating buffer is 0.05 M carbonate/bicarbonate buffer, pH 9.6, composed of sodium hydrogen carbonate (2.93 g) and sodium carbonate (1.59 g) (sodium azide [0.20 g/litre] is optional) in 1 litre of distilled water. The conjugate and test sera diluent buffer is 0.01 M PBS, pH 7.2, composed of disodium hydrogen orthophosphate (1.4 g), potassium dihydrogen phosphate (0.20 g), sodium chloride (8.50 g) and 0.05% Tween 20 dissolved in 1 litre of distilled water (PBST). This buffer is also used as wash buffer.

The conjugate used in this example is an MAb specific for the heavy chain of bovine IgG, and conjugated to horseradish peroxidase (HRPO). The substrate stock solution is 3% hydrogen peroxide. The chromogen stock solution is 0.16 M 2,2'-azino-bis-(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS) in distilled water. Substrate buffer is citrate buffer, pH 4.5, composed of trisodium citrate dihydrate (7.6 g) and citric acid (4.6 g) dissolved in 1 litre of distilled water. The enzymatic reaction-stopping solution is 4% sodium dodecyl sulphate (SDS).

- **Antigen production (example)**

  sLPS from *B. abortus* S1119-3 or S99 is prepared by heating 5 g dry weight (or 50 g wet weight) of cells suspended in 170 ml distilled water to 66°C followed by the addition of 190 ml of 90% (v/v) phenol at 66°C. The mixture is stirred continuously at 66°C for 15 minutes, cooled and centrifuged at 10,000 g for 15 minutes at 4°C. The brownish phenol in the bottom layer is removed with a long cannula and large cell debris may be removed by filtration (using a Whatman No. 1 filter) if necessary.

  The sLPS is precipitated by the addition of 500 ml cold methanol containing 5 ml methanol saturated with sodium acetate. After 2 hours' incubation at 4°C, the precipitate is removed by centrifugation at 10,000 g for 10 minutes. The precipitate is dispersed by addition of 80 ml of distilled water for 18 hours and centrifuged at 10,000 g for 10 minutes. The supernatant solution is kept at 4°C. The precipitate is resuspended in 80 ml distilled water and stirred for an additional 2 hours at 4°C. The supernatant solution is recovered by centrifugation as above and pooled with the previously recovered supernatant.

  Next, 8 g of trichloroacetic acid is added to the 160 ml of crude LPS. After stirring for 10 minutes, the precipitate is removed by centrifugation and the translucent supernatant solution is dialysed against distilled water (two changes of at least 4000 ml each) and then freeze dried.

  The freeze-dried LPS is weighed and reconstituted to 1 mg/ml in 0.05 M carbonate buffer, pH 9.6, and sonicated in an ice bath using approximately 6 watts three times for 1 minute each. The LPS is then freeze dried in 1 ml amounts and stored at room temperature.

- **Test procedure (example)**

  i) The freeze-dried sLPS is reconstituted to 1 ml with distilled water and is further diluted 1/1000 (or to a dilution predetermined by titration against the OIE ELISA Standard Sera) in 0.05 M carbonate buffer, pH 9.6. To coat the microplates, 100 µl volumes of the diluted sLPS solution are added to all wells, and the plates are covered and incubated for 18 hours at 4°C. After incubation, the plates may be used or sealed, frozen and stored at −20°C for up to a year. Frozen plates are thawed for 30–45 minutes at 37°C before use.

  ii) Unbound antigen is removed by washing all microplate wells with PBST four times. Volumes (100 µl) of serum diluted in the range of 1/50 to 1/200 in PBST, pH 6.3, containing 7.5 mM each of ethylene
diamino tetra-acetic acid (EDTA) and ethylene glycol tetra-acetic acid (EGTA) (PBST/EDTA) are added to specified wells and incubated at ambient temperature for 30 minutes.

iii) Test sera are added to the plates and may be tested singly or in duplicate. The controls, calibrated against the OIE ELISA Standard Sera, are set up in duplicate wells and include a strong positive, a weak positive, a negative control serum, and a buffer control.

iv) Unbound serum is removed by washing four times with PBST (PBST containing EDTA/EGTA must not be used with HRPO as it inactivates the enzyme). Volumes (100 µl) of conjugate (MAb M23) specific for a heavy chain epitope of bovine IgG1 conjugated with HRPO and diluted in PBST (predetermined by titration) are added to each well and the plates are incubated at ambient temperature for 30 minutes.

v) Unbound conjugate is removed by four washing steps. Volumes (100 µl) of substrate/chromogen (1.0 mM H2O2 [100 µl/20 ml citrate buffer] and 4 mM ABTS [500 µl/20 ml citrate buffer]) are added to each well; the plate is shaken for 10 minutes and colour development is assessed in a spectrophotometer at 414 or 405 nm. If required, 100 µl volumes of 4% SDS may be added directly to all wells as a stopping reagent.

vi) The control wells containing the strong positive serum are considered to be 100% positive and all data are calculated from these absorbance readings (between 1.000 and 1.800) using the equation:

\[
\text{Per cent positivity (}%P\text{) = absorbance (test sample)/absorbance (strong positive control) } \times 100
\]

The sLPS antigen, small amounts of the MAb specific for the heavy chain of bovine IgG\(_1\), software for generation of data using particular spectrophotometers and a standard test protocol for the I-ELISA are available for research and standardisation purposes\(^3\).

Using this or another similar I-ELISA protocol calibrated against the OIE ELISA Standard Sera described above, the diagnostic sensitivity should be equal to or greater than the BBATs in the testing of infected cattle, and the diagnostic specificity should be equivalent to the CFT in the testing of unvaccinated cattle (66, 67). It can be expected that the diagnostic specificity in the testing of S19 vaccinated cattle or in the case of FPSR will be significantly lower than for the CFT depending on where the I-ELISA positive/negative threshold is set.

### Competitive ELISA

The competitive ELISA (C-ELISA) using an MAb specific for one of the epitopes of the \textit{Brucella} sp. OPS has been shown to have higher specificity than the I-ELISA (49, 64, 83, 92). This is accomplished by selecting an MAb that has higher affinity than cross-reacting antibody. However, it has been shown that the C-ELISA eliminates some but not all reactions (FPSR) due to cross-reacting bacteria (61.). The C-ELISA is also capable of eliminating most reactions due to residual antibody produced in response to vaccination with S19. The choice of MAb and its unique specificity and affinity will have a distinct influence on the diagnostic performance characteristics of the assay. As with any MAb-based assay, the universal availability of the MAb or the hybridoma must also be considered with respect to international acceptance and widespread use.

Several variations of the C-ELISA have been described. The C-ELISA is also commercially available. In the interests of international harmonisation, the three OIE ELISA Standard Sera should be used by national reference laboratories to check or calibrate the test method in question.

The assay should be calibrated such that the OD of the strong positive OIE ELISA Standard Serum should represent a point on the linear portion of a typical dose–response curve just above the plateau (i.e. close to maximal inhibition). The weak positive OIE ELISA Standard Serum should give a reaction that lies on the linear portion of the same dose–response curve just above the positive/negative threshold (i.e. moderate inhibition). The negative serum and the buffer/MAb control should give reactions that are always less than the positive/negative threshold (i.e. minimal inhibition).

The test method described below is an example of a test, which has been internationally validated and has been used extensively in internationally sponsored, technical cooperation and research collaboration projects world-wide.

The buffer systems are the same as those described for the I-ELISA.

#### Antigen production (example)

sLPS from \textit{B. abortus} S1119-3 is prepared and used as for the I-ELISA.

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3 Obtainable from the OIE Reference Laboratory for Brucellosis at the Animal Diseases Research Institute, 3851 Fallowfield Road, Nepean, Ontario K2H 8P9, Canada.
Test procedure

i) The freeze-dried sLPS is reconstituted to 1 ml with distilled water and further diluted 1/1000 with 0.05 M carbonate buffer, pH 9.6. To coat the microplates, 100 µl volumes of LPS solution are added to all wells and the plates are covered and incubated for 18 hours at 4°C. After incubation, the plates may be used or sealed, frozen and stored at –20°C for up to 1 year. Frozen plates are thawed for 30–45 minutes at 37°C before use.

ii) Unbound antigen is removed by washing all microplate wells four times with PBST. Volumes (50 µl) of MAb (M84 in this example) diluted appropriately in PBST/EDTA are added to each well, followed immediately by 50 µl volumes of serum diluted 1/10 in PBST/EDTA. Plates are incubated for 30 minutes at ambient temperature with shaking for at least the initial 3 minutes.

iii) Test sera are added to the plates and may be tested as singly or in duplicate. The controls, calibrated against the OIE ELISA Standard Sera, are set up in duplicate wells and include a strong positive, a weak positive, a negative control serum, and a buffer control.

iv) Unbound serum and MAb are removed by washing the microplate four times with PBST. Volumes (100 µl) of commercial goat anti-mouse IgG (H and L chain) HRPO conjugate diluted in PBST (predetermined by titration) are added to each well and the plates are incubated at ambient temperature for 30 minutes.

v) Unbound conjugate is removed by four washing steps. Volumes (100 µl) of substrate/chromogen (1.0 mM H2O2 and 4 mM ABTS) are added to each well, the plates are shaken for 10 minutes and colour development is assessed in a spectrophotometer at 414 or 405 nm. If required, 100 µl volumes of 4% SDS may be added directly as a stopping reagent.

vi) The control wells containing MAb and buffer (no serum) are considered to give 0% inhibition and all data are calculated from these absorbance readings (between 1.000 and 1.800) using the equation:

\[
\text{Per cent inhibition} (\%I) = 100 - \left( \frac{\text{absorbance [test sample]}}{\text{absorbance [buffer control]}} \times 100 \right)
\]

The sLPS antigen, small amounts of the MAb, software for generation of data using particular spectrophotometers and a standard operating procedure for the C-ELISA are available for research and standardisation (see footnote 3 for address).

Using this or a similar C-ELISA protocol calibrated against the OIE ELISA Standard Sera, the diagnostic sensitivity should be equivalent to the BBATs and the I-ELISAs in the testing of infected cattle (63, 64, 66). The diagnostic specificity of the C-ELISA should be equivalent to or greater than the CFT and the I-ELISA in the testing of unvaccinated cattle or in the case of FPSR. The diagnostic specificity of C-ELISA is higher than that of the CFT and I-ELISA especially when testing S19 vaccinated animals.

d) Fluorescence polarisation assay (a prescribed test for international trade)

The FPA is a simple technique for measuring antigen/antibody interaction and may be performed in a laboratory setting or in the field. It is a homogeneous assay in which analytes are not separated and it is therefore very rapid.

The mechanism of the assay is based on random rotation of molecules in solution. Molecular size is the main factor influencing the rate of rotation, which is inversely related. Thus a small molecule rotates faster than a large molecule. If a molecule is labelled with a fluorochrome, the time of rotation through an angle of 68.5° can be determined by measuring polarised light intensity in vertical and horizontal planes. A large molecule emits more light in a single plane (more polarised) than a small molecule rotating faster and emitting more depolarised light.

For most FPAs, an antigen of small molecular weight, less than 50 kD, is labelled with a fluorochrome and added to serum or other fluid to be tested for the presence of antibody. If antibody is present, attachment to the labelled antigen will cause its rotational rate to decrease and this decrease can be measured.

For the diagnosis of brucellosis, a small molecular weight fragment (average 22 kD) of the OPS of B. abortus strain 1119-3 sLPS is labelled with fluorescein isothiocyanate (FITC) and used as the antigen. This antigen is added to diluted serum or whole blood and a measure of the antibody content is obtained in about 2 minutes (for serum) or 15 seconds (for blood) after the addition of antigen using a fluorescence polarisation analyser (62, 66).

The FPA can be performed in glass tubes or a 96-well plate format. The bovine serum is diluted 1/10 for the plate test or 1/100 for the tube test; if EDTA-treated blood is used the dilution for the tube test is 1/50 and 1/5 for the plate test (heparin-treated blood tends to increase assay variability). The diluent used is 0.01 M Tris (1.21 g), containing 0.15 M sodium chloride (8.5 g), 0.05% Igepal CA630 (500 µl) (formerly NP40), 10 mM EDTA (3.73 g) per litre of distilled water, pH 7.2 (Tris buffer). An initial reading to assess light scatter is obtained with the fluorescence polarisation analyser (FPM) after mixing. Suitably labelled titrated antigen (usually giving an intensity of 250,000–300,000) is added, mixed, and a second reading is obtained in the FPM about 2 minutes later for serum and 15 seconds for blood. A reading (in millipolarisation units, mP)
over the established threshold level is indicative of a positive reaction. A typical threshold level is 90–100 mP units, however, the test should be calibrated locally against International Standard reference sera. Control sera of strong positive, weak positive and negative, as well as S19 vaccinate serum, should be included.

**Antigen production (example)**

OPS from 5 g dry weight (or 50 g wet weight) of *B. abortus* S1119-3 is prepared by adding 400 ml of 2% (v/v) acetic acid, autoclaving the suspension for 15 minutes at 121°C and removing the cellular debris by centrifugation at 10,000 *g* for 10 minutes at 4°C. The supernatant solution is then treated with 20 g of trichloroacetic acid to precipitate any proteins and nucleic acids. The precipitate is again removed by centrifugation at 10,000 *g* for 10 minutes at 4°C. The supernatant fluid is dialysed against at least 100 volumes of distilled water and freeze dried.

3 mg of OPS are dissolved in 0.6 ml of 0.1 M sodium hydroxide (4 g NaOH/litre) and incubated at 37°C for 1 hour, followed by the addition of 0.3 ml of FITC isomer 1 at a concentration of 100 mg/ml in dimethyl sulphoxide and a further incubation at 37°C for 1 hour. The conjugated OPS is applied to a 1 × 10 cm column packed with DEAE (diethylaminoethyl) Sephadex A 25 equilibrated in 0.01 M phosphate buffer, pH 7.4. The first fraction (after 10–15 ml of buffer) is bright green, after which the buffer is switched to 0.1 M phosphate, pH 7.4. This results in the elution of 10–15 ml of buffer followed by 25–40 ml of green fluorescent material. The latter material is the antigen used in the FPA. Antigen preparation may be scaled up proportionally.

The amount of antigen used per test is determined by diluting the material derived above until a total fluorescence intensity of 250,000–300,000 is achieved using the FPM.

The antigen can be stored as a liquid for several years at 4°C in a dark bottle or it may be freeze dried in dark bottles.

Small quantities of labelled antigen for research and standardisation purposes and standard operating procedures for antigen preparation and the FPA may be obtained (see footnote 3 for address).

**Test procedure**

i) 1 ml of Tris buffer is added to a 10 × 75 mm borosilicate glass tube followed by 10 µl of serum or 20 µl of EDTA-treated blood. For the 96-well format, 20 µl of serum is added to 180 µl of buffer. It is important to mix well. A reading is obtained on the FPM to determine light scatter.

ii) A volume of antigen, which results in a total fluorescence intensity of 250–300 × 10^3, is added to the tube and mixed well. This volume will vary from batch to batch, but is generally in the range of about 10 µl. A second reading is obtained on the FPM after incubation at ambient temperature for approximately 2 minutes for serum and 15 seconds for EDTA-treated blood.

iii) A reading above the predetermined threshold is indicative of a positive reaction.

iv) The following are included in each batch of tests: a strong positive, a weak positive, a negative working standard serum (calibrated against the OIE ELISA Standard Sera).

The diagnostic sensitivity and specificity of the FPA for bovine brucellosis are almost identical to those of the C-ELISA. The diagnostic specificity for cattle recently vaccinated with S19 is over 99% (62). However the specificity of FPA in FPSR conditions is currently unknown. The FPA should be standardised such that the OIE strong positive and weak positive sera consistently give positive results.

3. Other tests

a) Brucellin skin test

An alternative immunological test is the brucellin skin test, which can be used for screening unvaccinated herds, provided that a purified (free of sLPS) and standardised antigen preparation (e.g. brucellin INRA) is used.

The brucellin skin test has a very high specificity, such that serologically negative unvaccinated animals that are positive reactors to the brucellin test should be regarded as infected animals (78). Also, results of this test may aid the interpretation of serological reactions thought to be FPSR due to infection with cross-reacting bacteria, especially in brucellosis-free areas (23, 74, 78).

Not all infected animals react, therefore this test alone cannot be recommended as the sole diagnostic test or for the purposes of international trade.

It is essential to use a standardised, defined brucellin preparation that does not contain sLPS antigen, as this may provoke nonspecific inflammatory reactions or interfere with subsequent serological tests. One...
such preparation is brucellin INRA prepared from a rough strain of *B. melitensis* that is commercially available⁴.

- **Test procedure**
  
  i) A volume of 0.1 ml of brucellin is injected intradermally into the caudal fold, the skin of the flank, or the side of the neck.
  
  ii) The test is read after 48–72 hours.
  
  iii) The skin thickness at the injection site is measured with vernier callipers before injection and at re-examination.
  
  iv) A strong positive reaction is easily recognised by local swelling and induration. However, borderline reactions require careful interpretation. Skin thickening of 1.5–2 mm would be considered as a positive reaction.

Although the brucellin intradermal test is one of the most specific tests in brucellosis (in unvaccinated animals), diagnosis should not be made solely on the basis of positive intradermal reactions given by a few animals in the herd, but should be supported by a reliable serological test. The intradermal inoculation of brucellin might induce a temporary anergy in the cellular immune response. Therefore an interval of 6 weeks is generally recommended between two tests on the same animal.

The skin test, performed with the homologous RB51 brucellin after calfhood vaccination with RB51, produces an anamnestic humoral response. Thus, the association between the RB51 skin test and the RB51-CFT could represent a diagnostic system to identify single animals vaccinated with RB51 (23).

**b) Serum agglutination test**

While not recognised as a prescribed or alternative test, the SAT has been used with success for many years in surveillance and control programmes for bovine brucellosis. Its specificity is significantly improved with the addition of EDTA to the antigen (35, 48, 65).

The antigen represents a bacterial suspension in phenol saline (NaCl 0.85 % [w/v] and phenol at 0.5% [v/v]). Formaldehyde must not be used. Antigens may be delivered in the concentrated state provided the dilution factor to be used is indicated on the bottle label. EDTA may be added to the antigen suspension to 5 mM final test dilution to reduce the level of false-positive results. Subsequently the pH of 7.2 must be readjusted in the antigen suspension.

The OIEISS contains 1000 IUs of agglutination. The antigen should be prepared without reference to the cell concentration, but its sensitivity must be standardised in relation to the OIEISS in such a way that the antigen produces either 50% agglutination with a final serum dilution of 1/600 to 1/1000 or 75% agglutination with a final serum dilution of 1/500 to 1/750. It may also be advisable to compare the reactivity of new and previously standardised batches of antigen using a panel of defined sera.

The test is performed either in tubes or in microplates. The mixture of antigen and serum dilutions should be incubated for 16–24 hours at 37°C. If the test is carried out in microplates, the incubation time can be shortened to 6 hours. At least three dilutions must be prepared for each serum in order to refute prozone negative responders. Dilutions of suspect serum must be made in such a way that the reading of the reaction at the positivity limit is made in the median tube (or well for the microplate method).

**Interpretation of results:** The degree of *Brucella* agglutination in a serum must be expressed in IU per ml. A serum containing 30 or more IU per ml is considered to be positive.

**c) Native hapten and polyB tests**

Native hapten and polyB tests are confirmatory tests⁵ that have been used successfully in an eradication programme in combination with the RBT as a screening test (4). The optimal sensitivity is obtained in a reverse radial immunodiffusion (RID) system in which the serum diffuses into a hypertonic gel containing the polysaccharide (24, 43). However, the double gel diffusion procedure is also useful (46, 47). Calves vaccinated subcutaneously with the standard dose of S19 at 3–5 months of age are negative 2 months after vaccination, and adult cattle vaccinated subcutaneously 4–5 months previously with the reduced dose of S19 do not give positive reactions unless the animals become infected and shed the vaccine in their milk (43). The conjunctival vaccination (both in young and adults) reduces the time to obtain a negative response in native hapten and polyB tests. A remarkable characteristic of the RID test is that a positive result correlates with *Brucella* shedding as shown in experimentally infected cattle and in naturally infected cattle undergoing antibiotic treatment (42). Precipitin tests using native hapten antigen or cytosol proteins

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⁴ Brucellergène OCB®, Symbiotics Europe, 2 rue Alexander Fleming, 69007 Lyon, France.

⁵ The detailed procedure can be obtained from the Brucellosis Laboratory, Centro de Investigacion y Tecnologia Agroalimentaria/Gobierno de Aragon, Apartado 727, 50080 Zaragoza, Spain.
have also been shown to eliminate most FPSR reactions caused by *Yersinia enterocolitica* O:9 and FPSR of unknown origin (57).

d) **Milk tests**

An efficient means of screening dairy herds is by testing milk from the bulk tank. Milk from these sources can be obtained cheaply and more frequently than blood samples and is often available centrally at dairies. When a positive test result is obtained, all cows contributing milk should be blood tested. The milk I-ELISA is a sensitive and specific test, and is particularly valuable for testing large herds. The milk ring test (MRT) is a suitable alternative if the ELISA is not available.

- **Milk I-ELISA**

As with the serum I-ELISA numerous variations of the milk I-ELISA are in use. Several commercial I-ELISAs are available that have been validated in extensive field trials and are in wide use. In the interests of international harmonisation, the three OIE ELISA Standard Sera should be used by national reference laboratories to check or calibrate the particular test method in question. The I-ELISA should be standardised such that the OIE ELISA strong positive standard when diluted 1/125 in negative serum and further diluted 1/10 in negative milk consistently tests positive. Bulk milk samples are generally tested at much lower dilutions than sera, i.e. undiluted to 1/2 to 1/10 in diluent buffer, with the remainder of the assay being similar to that described for serum. The C-ELISA should not be used to test whole milk but may be used with whey samples.

- **Milk ring test**

In lactating animals, the MRT can be used for screening herds for brucellosis. In large herds (> 100 lactating cows), the sensitivity of the test becomes less reliable. The MRT may be adjusted to compensate for the dilution factor from bulk milk samples from large herds. The samples are adjusted according to the following formula: herd size < 150 animals use 1 ml bulk milk, 150–450 use 2 ml milk sample, 451–700 use 3 ml milk sample. False-positive reactions may occur in cattle vaccinated less than 4 months prior to testing, in samples containing abnormal milk (such as colostrum) or in cases of mastitis. Therefore, it is not recommended to use this test in very small farms where these problems have a greater impact on the test results.

- **Antigen production**

MRT antigen is prepared from concentrated, killed *B. abortus* S99 or S1119-3 cell suspension, grown as described previously. It is centrifuged at, for example, 23,000 *g* for 10 minutes at 4°C, followed by resuspension in haematoxylin-staining solution. Various satisfactory methods are in use; one example is as follows: 100 ml of 4% (w/v) haematoxylin (Cl No. 75290) dissolved in 95% ethanol is added to a solution of ammonium aluminium sulphate (5 g) in 100 ml of distilled water and 48 ml of glycerol. 2 ml of freshly prepared 10% (w/v) sodium iodate is added to the solution. After standing for 30 minutes at room temperature, the deep purple solution is added to 940 ml of 10% (w/v) ammonium aluminium sulphate in distilled water. The pH of this mixture is adjusted to 3.1, and the solution must be aged by storage at room temperature in the dark for 45–90 days.

Before use, the staining solution is shaken and filtered through cotton wool. The packed cells are suspended in the staining solution at the rate of 1 g per 30 ml stain, and held at room temperature for 48 hours (some laboratories prefer to heat at 80°C for 10 minutes instead). The stained cells are then deposited by centrifugation, and washed three times in a solution of sodium chloride (6.4 g), 85% lactic acid (1.5 ml) and 10% sodium hydroxide (4.4 ml) in 1.6 litres of distilled water, final pH 3.0. The washed cells are resuspended at the rate of 1 g in 27 ml of a diluent consisting of 0.5% phenol saline, adjusted to pH 4.0 by the addition of 0.1 M citric acid (approximately 2.5 ml) and 0.5 M disodium hydrogen phosphate (approximately 1 ml) and maintained at 4°C for 24 hours. The mixture is filtered through cotton wool, the pH is checked, and the PCV is determined and adjusted to approximately 4%.

The sensitivity of the new batch should be compared with a previously standardised batch using a panel of samples of varying degrees of reaction prepared by diluting a positive serum in milk. The antigen should be standardised against the OIE ISS so that a 1/500 dilution is positive and 1/1000 dilution is negative. The antigen should be stored as recommended by the manufacturer but usually should be stored at 4°C.

The pH of the antigen should be between 3.3 and 3.7 and its colour should be dark blue. A little free stain in the supernatant of a centrifuged sample is permissible. When diluted in milk from a brucellosis-free animal, the antigen must produce a uniform coloration of the milk layer with no deposit and no coloration of the cream layer.

- **Test procedure**

The test is performed on bulk tank milk samples. If necessary, samples could be pretreated with preservative (0.1% formalin or 0.02% bronopol) for 2–3 days at 4°C prior to use.
i) Bring the milk samples and antigen to room temperature (20 ± 3°C); only sufficient antigen for the day’s tests should be removed from the refrigerator.

ii) Gently shake the antigen bottle well.

iii) The test is performed by adding 30–50 µl of antigen to a 1–2 ml volume of whole milk (the volume of milk may be increased for bulk samples from larger herds – see above “Milk ring test”).

iv) The height of the milk column in the tube must be at least 25 mm. The milk samples must not have been frozen, heated, subjected to violent shaking or stored for more than 72 hours.

v) The milk/antigen mixtures are normally incubated at 37°C for 1 hour, together with positive and negative working standards. However, overnight incubation at 4°C increases the sensitivity of the test and allows for easier reading.

vi) A strongly positive reaction is indicated by formation of a dark blue ring above a white milk column. Any blue layer at the interface of milk and cream should be considered to be positive as it might be significant, especially in large herds.

vii) The test is considered to be negative if the colour of the underlying milk exceeds that of the cream layer.

viii) When the MRT is adjusted for large herd sizes (2 or 3 ml of milk used), 0.1 ml of pooled negative cream is added to the test tube and is followed by 30–50 µl of the ring test antigen. After mixing, the test is incubated and read in the same manner as the unadjusted MRT. The negative pooled cream is collected from the separation of composite, unpasteurised milk from a brucellosis negative herd of 25 or more cows.

e) Interferon gamma test

As the prevalence of brucellosis decreases, accuracy of serological tests becomes more important. False-positive reactions result in trace-backs and epidemiological investigations that are expensive and time consuming. Therefore, assays that eliminate FPSR will become more and more useful. In general, the interferon gamma test involves stimulation of lymphocytes in whole blood with a suitable antigen, in this case, Brucellin has been shown to work well and then measuring the resulting gamma interferon production by a capture ELISA (44, 93, 94). This test protocol has also been found useful for detection of FPSR in sheep (25) and pigs (76).

f) Detection of antibody to rough Brucella

The use of rough Brucella (RB51) as a vaccine and in some cases of atypical Brucella infection has led to the need for serological tests for detection of antibody against the core of sLPS. Although *B. abortus* RB51 sLPS has been shown to contain small amounts of OPS (19), generally the antibody response to OPS is negligible and a more suitable antigen is rLPS. rLPS is readily extracted from *B. abortus* RB51 by the method of Galanos et al. (32). This antigen may then be used in IELISA (66, 67). The complement fixation test has also been shown to detect antibody to rLPS using a whole cell antigen (1).

C. REQUIREMENTS FOR VACCINES AND DIAGNOSTIC BIOLOGICALS

Brucellosis is one of the most easily acquired laboratory infections, and strict safety precautions should be observed. Laboratory manipulation of live cultures of *Brucella*, including vaccine strains, is hazardous and must be done under containment level 3 or higher, as outlined in Chapter 1.1.2, to minimise occupational exposure.

C1. Brucellin

Brucellin–INRA is an LPS-free extract from rough *B. melitensis* B115. This preparation does not provoke formation of antibodies reactive in BBAT, CFT or ELISA.

1. Seed management

a) Characteristics of the seed

Production of brucellin-INRA is based on a seed-lot system as described for antigens and vaccines. The original seed *B. melitensis* strain B115 for brucellin production6 should be propagated to produce a seed lot,

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6 Obtainable from Institut National de la Recherche Agronomique (INRA), Laboratoire de Pathologie Infectieuse et Immunologie, 37380 Nouzilly, France.
which should be preserved by lyophilisation or freezing at liquid nitrogen temperature. It should conform to the properties of a pure culture of a rough strain of *B. melitensis* and must not produce smooth *Brucella* LPS. It should produce reasonable yields of a mixture of protein antigens reactive with antisera to smooth and rough *Brucella* strains.

b) **Method of culture (2)**

*Brucella melitensis* strain B115 is best grown in the liquid medium described above for fermenter culture. It may be grown by the batch or continuous method in a fermenter or in flasks agitated on a shaker. Purity checks should be made on each single harvest, and the organisms must be in the rough phase.

c) **Validation as an in-vivo diagnostic reagent**

Laboratory and field studies in France have confirmed that brucellin-INRA is safe, non-toxic and specific in action. The preparation contains 50–75% proteins, mainly of low molecular weight and 15–30% carbohydrate. It does not contain LPS antigens. Brucellin-INRA does not provoke inflammatory responses in unsensitised animals, and it is not itself a sensitising agent. It does not provoke antibodies reactive in the standard serological tests for brucellosis. More than 90% of small ruminants infected with *B. melitensis* manifest delayed hypersensitivity to brucellin-NRA at some stage. The preparation is not recommended as a diagnostic agent for individual animals, but can be useful when used for screening herds. It is given to small ruminants in 100-µg doses by the intradermal route, and provokes a local delayed hypersensitivity reaction visible at 48–72 hours in sensitised animals. Positive reactions can be given by vaccinated as well as by infected animals (78).

2. **Method of manufacture (2)**

*Brucella melitensis* B115 cells are killed after culture by raising the temperature to 70°C for 90 minutes, cooled to 4°C, and harvested by centrifugation at 9000 g for 15 minutes at 4°C. The cells are washed in cold sterile distilled water and dehydrated by precipitating with three volumes of acetone at –20°C, and then allowed to stand at –20°C for 24–48 hours. After repeated washing in cold acetone, followed by a final rinse in diethyl ether, the cells are dried over calcium chloride and held at 4°C. The dried cells are subjected to a viability check. They are resuspended in sterile 2.5% sodium chloride to a final concentration of 5% (w/v) and agitated for 3 days at 4°C. Bacterial cells are removed by centrifugation as above, and the supernatant is concentrated to one-fourth the volume by ultrafiltration on a Diaflo PM10 membrane (Amicon) and precipitated by the addition of three volumes of ice-cold ethanol. The mixture is held at 4°C for 24 hours and the precipitate is recovered by centrifugation, redissolved in sterile water, and dialysed to remove ethanol. After centrifugation at 105,000 g for 6 hours at 4°C, the supernatant material, comprising the unstandardised brucellin, is subjected to assays for protein and carbohydrate. It may be freeze-dried either as bulk material or after it has been dispensed into its final containers.

3. **In-process control**

The crude brucellin extract should be checked for sterility after acetone extraction, to ensure killing of *Brucella* cells, and again at the end of the process to check possible contamination. The pH and protein concentration should be determined, and identity tests should be performed on the bulk material before filling the final containers.

4. **Batch control**

a) **Sterility**

Allergen preparations should be checked for sterility as described in Chapter 1.1.9 Tests for sterility and freedom from contamination of biological materials.

b) **Safety**

Samples of brucellin from the final containers should be subjected to the standard sterility test. Brucellin preparations should also be checked for abnormal toxicity. Doses equivalent to 20 cattle doses (2 ml) should be injected intraperitoneally into a pair of normal guinea-pigs that have not been exposed previously to *Brucella* organisms or their antigens. Five normal mice are also inoculated subcutaneously with 0.5 ml of the brucellin to be examined. Animals are observed for 7 days, and there should be no local or generalised reaction to the injection.

Dermo-necrotic capacity is examined by intradermal inoculation of 0.1 ml of the product to be examined into the previously shaved and disinfected flank of three normal albino guinea-pigs that have not been exposed previously to *Brucella* organisms or their antigens. No cutaneous reaction should be observed. Absence of allergic and serological sensitisation is checked by intradermal inoculation of three normal albino guinea-
pigs, three times every 5 days, with 0.1 ml of a 1/10 dilution of the preparation to be examined. A fourth similar injection is given, 15 days later, to the same three animals and to a control lot of three guinea-pigs of the same weight that have not been injected previously. The animals should not become seropositive to the standard tests for brucellosis (RBT, CFT) when sampled 24 hours after the last injection, and should not develop delayed hypersensitivity responses.

c) **Potency**

The potency of brucellin preparations is determined by intradermal injection of graded doses of brucellin into guinea-pigs that have been sensitised by subcutaneous inoculation of 0.5 ml of reference brucellin\(^7\) in Freund’s complete adjuvant from 1 to 6 months previously. The erythematous reactions are read and measured at 24 hours and the titre is calculated by comparison with a reference brucellin\(^8\). This method is only valid for comparing brucellin preparations made according to the same protocol as the sensitising allergen. Initial standardisation of a batch of allergen and the sensitisation and titration in ruminants is described (2).

d) **Duration of sensitivity**

Duration of sensitivity is uncertain. Individual animals vary considerably in the degree of hypersensitivity manifested to brucellin. Animals in the very early stages of infection, or with long-standing infection, may not manifest hypersensitivity to intradermal injection.

e) **Stability**

The freeze-dried preparation retains full potency for several years. The liquid commercial preparation should retain potency for the recommended shelf-life.

f) **Preservatives**

The use of preservatives is not recommended when the preparation is freeze-dried. In the liquid form, sodium merthiolate (at most 0.1 mg/ml) may be used as a preservative. If freeze-dried, the preparation should not be reconstituted until immediately before use.

g) **Precautions (hazards)**

Brucellin is not toxic. Nevertheless it may provoke severe hypersensitivity reactions in sensitised individuals who are accidentally exposed to it. Care should be taken to avoid accidental injection or mucosal contamination. Used containers and injection equipment should be carefully decontaminated or disposed of by incineration in a suitable disposable container.

5. **Tests on final product**

a) **Safety**

A sterility test should be performed by the recommended method. The in-vivo safety tests are as those described for batch control (see Section C1.4.b). These tests on the batch may be omitted if the full test is performed on the final filling lots.

b) **Potency**

This is performed by injection of a single dose into guinea-pigs using the procedure described in Section C1.4.c.

C2. **Vaccines**

*Brucella abortus* strain 19 vaccine

The most widely used vaccine for the prevention of brucellosis in cattle is the *Brucella abortus* S19 vaccine, which remains the reference vaccine to which any other vaccines are compared. It is used as a live vaccine and is normally given to female calves aged between 3 and 6 months as a single subcutaneous dose of 5–8 ×

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7 A national French reference brucellin has been produced by INRA-PIII (F-37380 Nouzilly, France) and is obtainable from the OIE Reference Laboratory for Brucellosis, AFSSA, 23 avenue du Général-de-Gaulle, 94706 Maisons-Alfort Cedex, France.

8 The statistical procedure can be obtained from the OIE Reference Laboratory for Brucellosis, AFSSA, BP67, 94703 Maisons-Alfort Cedex, France.
10^{10} viable organisms. A reduced dose of from 3 × 10^8 to 3 × 10^9 organisms can be administered subcutaneously to adult cattle, but some animals will develop persistent antibody titres and may abort and excrete the vaccine strain in the milk (84). Alternatively, it can be administered to cattle of any age as two doses of 5–10 × 10^9 viable organisms, given by the conjunctival route; this produces protection without a persistent antibody response and reduces the risks of abortion and excretion in milk.

_Brucella abortus_ S19 vaccine induces good immunity to moderate challenge by virulent organisms. The vaccine must be prepared from USDA-derived seed (see footnote 2 for address) and each batch must be checked for purity (absence of extraneous microorganisms), viability (live bacteria per dose) and smoothness (determination of dissociation phase). Seed lots for S19 vaccine production should be regularly tested for residual virulence and immunogenicity in mice.

Control procedures for this vaccine follow.

**Brucella abortus strain RB51 vaccine**

Since 1996, _B. abortus_ strain RB51 has become the official vaccine for prevention of brucellosis in cattle in several countries (82). However there is disagreement in regards to how the efficiency of strain RB51 compares to protection induced by S19 in cattle (55, 56, 84, 85, 87). Each country uses slightly different methods to administer the vaccine. In the USA, calves are vaccinated subcutaneously between the ages of 4 and 12 months with 1–3.4 × 10^{10} viable strain RB51 organisms. Vaccination of cattle over 12 months of age is carried out only under authorisation from the State or Federal Animal Health Officials, and the recommended dose is 1–3 × 10^9 viable strain RB51 organisms (70, 86). In other countries, it is recommended to vaccinate cattle as calves (4–12 months of age) with a 1–3.4 × 10^{10} dose, with revaccination from 12 months of age onwards with a similar dose to elicit a booster effect and increase immunity (79, 82).

Abortions may occur when S19 is used in pregnant animals (52, 58). As demonstrated in a study done in the United States, adult vaccination with the 10^8 CFU (colony-forming units) of RB51 has not been associated with reports of significant numbers of abortions under field conditions (71). Both RB51 and S19 have been isolated from milk of vaccinates after adult vaccination (58, 72, 73, 79). In large comparative S19 and RB51 vaccine studies, when S19 calfhood vaccinates were adult vaccinated with RB51 or S19, a greater percentage of S19 vaccinates shed the vaccine strain in milk and for a longer period of time than cattle vaccinated with RB51 (71, 73, 79). Use of S19 as an adult vaccine in _Brucella-_infected herds has facilitated reductions in abortions in acutely infected, but not chronically infected cattle herds, and contributed to eradication efforts in difficult herds (6). Similar epidemiologic data for adult vaccination with RB51 has not been reported. Due to these observations, vaccination of pregnant cattle with S19 or RB51 should be used judiciously (72).

It should be emphasised that, that S19, can infect humans and cause undulant fever if not treated (95). There have been limited studies with RB-51 in humans but it appears, as compared to S19 that the risk of developing undulant fever after exposure is minimal (5, 86, 91). The diagnosis of the infection produced by RB51 requires special tests not available in most hospitals. The Centers for Disease Control, Department of Health and Human Services, Atlanta, Georgia, USA (CDC) established passive surveillance for accidental inoculation with the RB51 vaccine in the USA to determine if this vaccine is associated with human disease. This study included 26 participants that had been exposed to the vaccine during animal vaccination. The number of reported adverse event case-patients in this study (twenty-six) is small compared to the number of vaccination events (several million calves vaccinated), and estimated inadvertent RB51 inoculations predicted (8 per 11,000). The report indicated that appropriate antibiotic use should protect against infection, but it remains undetermined to what degree the organism versus other vaccine components contribute to the adverse events (5). This is in contrast to Strain 19 where development of undulant fever caused by accidental exposure is well documented to occur without preventive treatment. Physicians making decisions on prophylactic treatment for accidental exposure to RB51 should be informed that this vaccine strain is highly resistant to rifampicin, one of the antibiotics of choice for treating human brucellosis.

Control procedures for this vaccine follow.

**Brucella melitensis strain Rev.1 vaccine**

It is not infrequent to isolate _B. melitensis_ in cattle in countries with a high prevalence of this infection in small ruminants (90). There has been some debate on the protective efficacy of S19 against _B. melitensis_ infection in cattle and it has been hypothesised that Rev.1 should be a more effective vaccine in these conditions, however there is only one report related to this issue that demonstrated that S19 is able to control _B. melitensis_ at the field level (42, 88). By contrast, no experiments have been conducted showing the efficacy of Rev.1 against _B. melitensis_ infection in cattle. Moreover, the safety of this vaccine is practically unknown in cattle (8, 90).

Until safety of Rev.1 in cattle of different physiological status and efficacy studies against _B. melitensis_ under strictly controlled conditions are performed, this vaccine should not be recommended for cattle.
1. Seed management

a) Characteristics of the seed

*Brucella abortus* S19 original seed for vaccine production must be obtained from the USDA (see footnote 2 for address), and used to produce a seed lot that is preserved by lyophilisation or by freezing at liquid nitrogen temperature. The properties of this seed lot must conform to those of a pure culture of a CO₂-independent *B. abortus* biovar 1 that is also sensitive to benzylpenicillin, thionin blue and i-erythritol at recommended concentrations, and that displays minimal pathogenicity for guinea-pigs.

*Brucella abortus* RB51 original seed for vaccine production is available commercially 9. These companies have legal rights to the vaccine.

b) Method of culture

*Brucella abortus* S19 for vaccine production is grown on medium free from serum or other animal products, under conditions similar to those described above for *B. abortus* S99 or S1119-3 (2).

*Brucella abortus* strain RB51 follows similar culture methods.

c) Validation as a vaccine

Numerous independent studies have confirmed the value of S19 as a vaccine for protecting cattle from brucellosis. The organism behaves as an attenuated strain when given to sexually immature cattle. In rare cases, it may produce localised infection in the genital tract. Antibody responses persisting for 6 months or longer are likely to occur in a substantial proportion of cattle that have been vaccinated subcutaneously with the standard dose as adults. Some of the cattle vaccinated as calves may later develop arthropathy, particularly of the femoro-tibial joints (10, 22). The vaccine is safe for most animals if administered to calves between 3 and 8 months of age. It may also be used in adult animals at a reduced dose. It produces lasting immunity to moderate challenge with virulent *B. abortus* strains, but the precise duration of this is unknown. The length of protection against *B. melitensis* is unknown. The vaccine strain is stable and reversion to virulence is extremely rare. It has been associated with the emergence of i-erythritol-using strains when inadvertently administered to pregnant animals. The organism behaves as an attenuated strain in mice, and even large inocula are rapidly cleared from the tissues.

Reports from both experimental challenge studies and field studies concluded the value of *B. abortus* strain RB51 in protecting cattle from brucellosis. The organism is attenuated in calves and adults. As *B. abortus* strain RB51 contains minimally expressed sLPS and there is no serological conversion against sLPS in vaccinated animals. In addition, RB51 does not induce detectable antibodies, using current testing procedures, to the OPS antigen (86). It produces immunity to moderate challenge with virulent strains, but the precise duration of this is unknown. The vaccine is very stable and no reversion to smoothness has been described in *vivo* or *in vitro*. The organism behaves as an attenuated strain in a variety of animals including mice where it is rapidly cleared from the tissues.

S19 and RB51 vaccines have some virulence for humans, and infections may follow accidental inoculation with the vaccine. Care should be taken in its preparation and handling, and a hazard warning should be included on the label of the final containers. In any case, accidental inoculations should be treated with appropriate antibiotics (see Section C2.4.g).

2. Method of manufacture

For production of S19 vaccine, the procedures described above can be used, except that the cells are collected in PBS, pH 6.3, and deposited by centrifugation or by the addition of sodium carboxymethyl cellulose at a final concentration of 1.5 g/litre. The yield from one fermenter run or the pooled cells from a batch of Roux flask cultures that have been inoculated at the same time from the same seed lot constitutes a single harvest. More than one single harvest may be pooled to form a final bulk, which is used to fill the final containers of a batch of vaccine. Before pooling, each single harvest must be checked for purity, cell concentration, dissociation and identity. A similar range of tests must be done on the final bulk, which should have a viable count of between 8 and 24 × 10⁹ CFU/ml. Adjustments in concentration are made by the addition of PBS for vaccine to be dispensed in liquid form, or by the addition of stabiliser for lyophilised vaccine. If stabiliser is to be used, loss of viability on lyophilisation should be taken into account, and should not be in excess of 50%. The final dried product should not be exposed to a temperature exceeding 35°C during drying, and the residual moisture content should be 1–2%. The contents must be sealed under vacuum or dry nitrogen immediately after drying, and stored at 4°C.

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9 Colorado Serum Company, 4950 York Street, P.O. Box 16428, Denver, Colorado 80216-0428, USA; or Veterinary Technologies Corporation, 1872 Pratt Drive, Suite 1100B, Blacksburg, Virginia 24060, USA.
The production process for *B. abortus* strain RB51 is very similar to the one used for S19.

### 3. In-process control

*Brucella abortus* S19 vaccine should be checked for purity and smoothness during preparation of the single harvests. The cell concentration of the bulks should also be checked. This can be done by opacity measurement, but a viable count must be performed on the final filling lots. The identity of these should also be checked by agglutination tests with antiserum to *Brucella* A antigen. The viable count of the final containers should not be less than $5 \times 10^9$ per standard dose after lyophilisation, if this is to be done, and at least 95% of the cells must be in the smooth phase.

*Brucella abortus* strain RB51 vaccine should be checked for purity and roughness during preparation of the single harvests. The cell concentration of the bulks should also be checked. A viable count must be performed on the final filling lots. The viable count of the final containers should be 1–3.4 $\times 10^{10}$ viable CFU of RB51 per dose (dose of 2 ml to be applied subcutaneously) and 100% of the cells must be in the rough phase. All colonies should be negative on dot-blot assays with MAbs specific for the OPS antigen.

### 4. Batch control

#### a) Sterility

Tests for sterility and freedom from contamination of biological materials may be found in Chapter 1.1.9.

#### b) Safety

The S19 vaccine is a virulent product per se, and it should keep a minimal virulence to be efficient (see Section C2.4.c). However a safety test is not routinely done. If desired, when a new manufacturing process is started and when a modification in the innocuousness of the vaccine preparation is expected, it may be performed on cattle. This control should be done as follows: the test uses 12 female calves, aged 4–6 months. Six young females are injected with one or three recommended doses. Each lot of six young females are kept separately. All animals are observed for 21 days. No significant local or systemic reaction should occur. If, for a given dose and route of administration, this test gives good results on a representative batch of the vaccine, it does not have to be repeated routinely on seed lots or vaccine lots prepared with the same original seed and with the same manufacturing process. A safety test on S19 vaccine may also be performed in guinea-pigs. Groups of at least ten animals are given intramuscular injections of doses of vaccine diluted in PBS, pH 7.2, to contain $5 \times 10^9$ viable organisms. The animals should show no obvious adverse effects and there must be no mortality.

A safety test on *B. abortus* strain RB51 vaccine is not routinely done. If desired, 8–10-week-old female Balb/c mice can be injected intraperitoneally with $1 \times 10^8$ CFUs and the spleens cultured at 6 weeks post-inoculation. Spleens should be free from RB51 and the mice should not develop anti-OPS antibodies.

#### c) Potency

- **S19 vaccine**

An S19 vaccine is efficient if it possesses the characteristics of the S19 original strain, i.e. if it is satisfactory with respect to identity, smoothness, immunogenicity and residual virulence (9). Batches should also be checked for the number of viable organisms.

- **Identity**

  The reconstituted S19 vaccine should not contain extraneous microorganisms. *Brucella abortus* present in the vaccine is identified by suitable morphological, serological and biochemical tests and by culture: *Brucella abortus* S19 has the normal properties of a biovar 1 strain of *B. abortus*, but does not require CO$_2$ for growth, does not grow in the presence of benzylpenicillin (3 μg/ml = 5 IU/ml), thionin blue (2 μg/ml), and i-erythritol (1 mg/ml) (all final concentrations).

- **Smoothness (determination of dissociation phase)**

  The S19 vaccine reconstituted in distilled water is streaked across six agar plates (serum–dextrose agar or trypticase–soy agar (TSA) with added serum 5% [v/v] or yeast extract 0.1 % [w/v]) in such a manner that the colonies will be close together in certain areas, while semi-separated and separated in others. Slight differences in appearance are more obvious in adjacent than widely separated colonies. Plates are incubated at 37°C for 5 days and examined by obliquely reflected light (Henry’s method) before and after staining (three plates) with crystal violet (White & Wilson’s staining method). In addition, S19 is sensitive to rifampicin.
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Appearance of colonies before staining: S colonies appear round, glistening and blue to blue-green in colour. R colonies have a dry, granular appearance and are dull yellowish-white in colour. Mucoid colonies (M) are transparent and greyish in colour and can be distinguished by their slimy consistency when touched with a loop. Intermediate colonies (I), which are the most difficult to classify, have an appearance intermediate between S and R forms: they are slightly opaque and more granular than S colonies.

Appearance of colonies after staining with crystal violet: S colonies do not take up the dye. Dissociated colonies (I, M, or R) are stained various shades of red and purple and the surface may show radial cracks. Sometimes a stained surface film slips off a dissociated colony and is seen adjacent to it.

The colony phase can be confirmed by the acriflavine agglutination test (2). S colonies remain in suspension, whereas R colonies are agglutinated immediately and, if mucoid, will form threads. Intermediate colonies may remain in suspension or a very fine agglutination may occur.

- Enumeration of live bacteria

Inoculate each of at least five plates of tryptose, serum–dextrose or other suitable agar medium with 0.1 ml of adequate dilutions of the vaccine spread with a sterile glass, wire or plastic spreader. CFU per vaccine volume unit are enumerated.

- Residual virulence (50% persistence time or 50% recovery time) (9, 24, 37, 75)

  i) Prepare adequate suspensions of both the B. abortus S19 seed lot or batch to be tested (test vaccine) and the S19 original seed culture (as a reference strain). For this, harvest a 24–48 hours growth of each strain in sterile buffered saline solution (BSS: NaCl 8.5 g; KH₂PO₄ 1.0 g; K₂HPO₄ 2.0 g; distilled water 1000 ml; pH 6.8) and adjust the suspension in BSS to 10⁹ CFU/ml using a spectrophotometer (0.170 OD when read at 600 nm). The exact number of CFU/ml should be checked afterwards by plating serial tenfold dilutions on to adequate culture medium (blood agar base or TSA are recommended).

  ii) Inject subcutaneously 0.1 ml (10⁸ CFU/mouse) of the suspension containing the test vaccine into each of 32 female CD1 mice, aged 5–6 weeks. Carry out, in parallel, a similar inoculation in another 32 mice using the suspension containing the S19 reference strain. The original seed S19 strain, which has been shown satisfactory with respect to immunogenicity and/or residual virulence, can be obtained from USDA (see footnote 2 for address).

  iii) Kill the mice by cervical dislocation, in groups of eight selected at random 3, 6, 9 and 12 weeks later.

  iv) Remove the spleens and homogenise individually and aseptically with a glass grinder (or in adequate sterile bags with the Stomacher) in 1 ml of sterile BSS.

  v) Spread each whole spleen suspension in toto on to several plates containing a suitable culture medium and incubate in standard Brucella conditions for 5–7 days (lower limit of detection: 1 bacterium per spleen). An animal is considered infected when at least 1 CFU is isolated from the spleen.

  vi) Calculate the 50% persistence time or 50% recovery time (RT₅₀) by the SAS® statistical method specifically developed for RT₅₀ calculations (to obtain the specific SAS® file see footnote 5 for address) . For this, determine the number of cured mice (no colonies isolated in the spleen) at each slaughtering point time (eight mice per point) and calculate the percentage of cured accumulated mice over time, by the Reed and Muench method (described in ref. 7). The function of distribution of this percentage describes a sigmoid curve, which must be linearised for calculating the RT₅₀ values, using the computerised PROBIT procedure of the SAS® statistical package.

  vii) Compare statistically the parallelism (intercept and slope) between the distribution lines obtained for both tested and reference S19 strains using the SAS® file specifically designed for this purpose. Two RT₅₀ values can be statistically compared exclusively when they come from parallel distribution lines. If parallelism does not exist, the residual virulence of the tested strain should be considered inadequate, and discarded for vaccine production.

  viii) If the parallelism is confirmed, compare statistically the RT₅₀ values obtained for both tested and reference S19 strains using a SAS® file specifically designed for this purpose. To be accepted for vaccine production, the RT₅₀ obtained with the tested strain should not differ significantly from that obtained with the reference S19 strain (RT₅₀ and confidence limits are usually around 7.0 ± 1.3 weeks).

The underlying basis of the statistical procedure for performing the above residual virulence calculations have been recently described in detail (7–9). Alternatively, the statistical calculations described in steps vi) to viii) can be avoided by an easy-to-use specific HTML-JAVA script program (Rev2) recently developed and available free at: http://www.afssa.fr/interne/Rev2.html.
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If this test has been done with good results on a representative seed lot or batch of the test vaccine, it does not have to be repeated routinely on other vaccine lots prepared from the same seed lot and using the same manufacturing process.

- **Immunogenicity in mice (7, 8)**

This test uses three groups of six female CD1 mice, aged 5–7 weeks, that have been selected at random.

i) Prepare and adjust spectrophotometrically the vaccine suspensions as indicated above.

ii) Inject subcutaneously a suspension containing \(10^5\) CFU (in a volume of 0.1 ml/mouse) of the vaccine to be examined (test vaccine) into each of six mice of the first group.

iii) Inject subcutaneously a suspension containing \(10^5\) CFU of live bacteria of a reference S19 vaccine into each of six mice of the second group. The third group will serve as the unvaccinated control group and should be inoculated subcutaneously with 0.1 ml of BSS.

iv) The exact number of CFU inoculated should be checked afterwards by plating serial tenfold dilutions on to adequate culture medium (blood agar base or TSA are recommended).

v) All the mice are challenged 30 days after vaccination (and immediately following 16 hours’ starvation), intraperitoneally with a suspension (0.1 ml/mouse) containing \(2 \times 10^5\) CFU of \(B.\ abortus\) strain 544 (CO\(_2\)-dependent), prepared, adjusted and retrospectively checked as above.

vi) Kill the mice by cervical dislocation 15 days later.

vii) Each spleen is excised aseptically, the fat is removed, and the spleen is weighed and homogenised. Alternatively, the spleens can be frozen and kept at -20°C for from 24 hours to 7 weeks.

viii) Each spleen is homogenised aseptically with a glass grinder (or in adequate sterile bags in Stomacher) in nine times its weight of BSS, pH 6.8 and three serial tenfold dilutions (1/10, 1/100 and 1/1000) of each homogenate made in the same diluent. Spread 0.2 ml of each dilution by quadruplicate in agar plates and incubate two of the plates in a 10% CO\(_2\) atmosphere (allows the growth of both vaccine and challenge strains) and the other two plates in air (inhibits the growth of the \(B.\ abortus\) 544 CO\(_2\)-dependent challenge strain), both at 37°C for 5 days.

ix) Colonies of \(Brucella\) should be enumerated on the dilutions corresponding to plates showing fewer than 300 CFU. When no colony is seen in the plates corresponding to the 1/10 dilution, the spleen is considered to be infected with five bacteria. These numbers of \(Brucella\) per spleen are first recorded as X and expressed as Y, after the following transformation: \(Y = \log(X/\log X)\). Mean and standard deviation, which are the response of each group of six mice, are then calculated.

x) The conditions of the control experiment are satisfactory when: i) the response of unvaccinated mice (mean of \(Y\)) is at least of 4.5; ii) the response of mice vaccinated with the reference S19 vaccine is lower than 2.5; and iii) the standard deviation calculated on each lot of six mice is lower than 0.8.

xi) Carry out the statistical comparisons (the least significant differences [LSD] test is recommended) of the immunogenicity values obtained in mice vaccinated with the S19 strain to be tested with respect to those obtained in mice vaccinated with the reference vaccine and in the unvaccinated control group. The test vaccine would be satisfactory if the immunogenicity value obtained in mice vaccinated with this vaccine is significantly lower than that obtained in the unvaccinated controls and, moreover, does not differ significantly from that obtained in mice vaccinated with the reference vaccine. (For detailed information on this procedure, see footnote 5 for contact address.)

If this test has been done with good results on a representative batch of the test vaccine, it does not have to be repeated routinely on other vaccine lots prepared from the same seed lot and with the same manufacturing process.

- **RB51 vaccine**

As dosage (CFU) of the master seed was correlated to protection as part of licensure of RB-51 for cattle in the USA, \textit{in vivo} potency tests are not routinely conducted for serials of the RB-51 vaccine. In the USA, plate counts of viable organisms have been approved and used as a measure of potency (this approach is identical to the potency test for S19 vaccine in the USA). A test in Balb/c female mice using \(1 \times 10^6\) \(B.\ abortus\) strain 2308 organisms as the challenge strain has been proposed, but the correlation of this test to vaccine protection in cattle has not been completely determined. In the USA plate counts of viable organisms have been approved and used (85). Rough vaccines for brucellosis have been discussed in some detail (55).
d) Duration of immunity

Vaccinating calves with a full dose of S19 vaccine is considered to give long-lasting immunity, and subsequent doses are not recommended. However, there is no proven evidence for this and revaccination could be advisable in endemic areas.

e) Stability

Brucella abortus S19 vaccine prepared from seed stock from appropriate sources is stable in characteristics, provided that the in-process and batch control requirements described above are fulfilled, and shows no tendency to reversion to virulence. The lyophilised vaccine shows a gradual loss of viable count, but should retain its potency for the recommended shelf life. Allowance for this phenomenon is normally made by ensuring that the viable count immediately following lyophilisation is well in excess of the minimum requirement. Maintenance of a cold chain during distribution of the vaccine will ensure its viability.

Brucella abortus strain RB51 has shown no tendency to revert to virulent smooth organisms after many passages in vitro or in vivo. This is probably due to the nature and place of the mutations found in this strain. Brucella abortus strain RB51 has its wboA gene disrupted by an IS711 element impeding synthesis of OPS. Unpublished data indicate that it also contains a second mutation affecting the export of OPS to the bacterial surface or the coupling of OPS to the core of the LPS, or both.

f) Preservatives

Antimicrobial preservatives must not be used in live S19 or B. abortus strain RB51 vaccines. For preparation of the lyophilised vaccine, a stabiliser containing 2.5% casein digest, e.g. Tryptone (Oxoid), 5% sucrose and 1% sodium glutamate, dissolved in distilled water and sterilised by filtration is recommended.

g) Precautions (hazards)

Brucella abortus S19 and RB51, although attenuated strains, are still capable of causing disease in humans. The cell cultures and suspensions must be handled under appropriate conditions of biohazard containment. Reconstitution and subsequent handling of the reconstituted vaccine should be done with care to avoid accidental injection or eye or skin contamination. Vaccine residues and injection equipment should be decontaminated with a suitable disinfectant (phenolic, iodophor or aldehyde formulation) at recommended concentration. Medical advice should be sought in the event of accidental exposure. The efficacy of the antibiotic treatment of infections caused by S19 and RB51 in humans has not been adequately established; however, the CDC will provide treatment recommendations. If S19 contamination occurs, a combined treatment with doxycycline plus rifampicin could be recommended. In the case of contamination with RB51 (a rifampicin-resistant strain), the treatment with rifampicin should be avoided and a regimen of doxycycline and streptomycin or gentamycin should be used except in pregnant women, which should be treated with trimethoprim sulfa-methoxazole. However, there have been limited studies on treatment of humans exposed to RB-51 (5) and there has been at least one report of human infection with RB-51. The RB-51 strain is highly susceptible to tetracycline and treatment with doxycycline alone maybe satisfactory (5).

5. Tests of the final product

a) Safety

See Section C2.4.b.

b) Potency

For the lyophilised vaccine, potency must be determined on the final product. The procedure is as described in Section C2.4.c.

REFERENCES


75. POURILLOT R., GRILLO M.J., ALABART J.L., GARN-BASTUJI B. & BLASCO J.M. (2004). Statistical procedures for calculating the residual virulence of Brucella abortus strain 19 (S19) and Brucella melitensis strain Rev.1


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NB: There are OIE Reference Laboratories for Bovine brucellosis (see Table in Part 3 of this Terrestrial Manual or consult the OIE Web site for the most up-to-date list: www.oie.int).
CHAPTER 2.4.4.

BOVINE CYSTICERCOSIS

See Chapter 2.9.5. Cysticercosis

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CHAPTER 2.4.5.

BOVINE GENITAL CAMPYLOBACTERIOSIS

SUMMARY

Definition of the disease: Bovine genital campylobacteriosis (BGC) is a venereal disease also known as bovine venereal campylobacteriosis (BVC). The causal agent of this sexually transmissible disease is Campylobacter fetus subsp. venerealis. The species is divided into two closely related subspecies: C. fetus subsp. venerealis and C. fetus subsp. fetus. By definition C. fetus subsp. venerealis is associated with BGC, causing fertility problems with considerable economic losses, particularly in endemic regions. Bovine infections with C. fetus subsp. fetus are associated with abortion and have a more sporadic occurrence.

Description of the disease: BGC is a venereal disease that is characterised by infertility, early embryonic death, and abortion. The disease is caused by C. fetus subsp. venerealis, a bacterium with pronounced tropism for the genital system of cattle. Transmission of the causal agent takes place mainly during natural mating, and the presence of C. fetus subsp. venerealis in the semen of bulls creates the risk of spread of the disease through artificial insemination.

Identification of the agent: Samples taken from bulls, cows or aborted fetuses can be analysed for the presence of the causal organism. The organism is a thin Gram-negative curved rod that may form S-shapes, seagull-shapes and spirals, and can be cultured at 37°C for at least 3 days in a microaerobic atmosphere. Confirmation of the isolate and discrimination between the subspecies of C. fetus can be performed by biochemical or molecular methods. Immunofluorescence may also be used to identify the organism, but it will not differentiate between different subspecies.

Serological tests: Enzyme-linked immunosorbent (ELISA) can be used for testing herd immunity, but is not suitable for diagnosis of the infection in individual animals. This test can not differentiate between infections caused by the two subspecies.

Requirements for vaccines and diagnostic biologicals: A vaccine may be prepared from C. fetus subsp. venerealis and/or C. fetus subsp. fetus that shares antigens with C. fetus subsp. venerealis. This vaccine is inactivated with formalin, and may be administered in an oil-emulsion adjuvant.

A. INTRODUCTION

1. Disease

Bovine genital campylobacteriosis (BGC, also known as bovine venereal campylobacteriosis [BVC]) is a venereal disease characterised by infertility, early embryonic death, and abortion in cattle. The causal agent of this sexually transmissible disease is Campylobacter fetus subsp. venerealis. It can be isolated from the genital tract of cattle (e.g. preputial smegma, vaginal mucus) or internal organs of aborted fetuses.

Campylobacter fetus is divided into the two closely related subspecies: C. fetus subsp. venerealis and C. fetus subsp. fetus (28). An intermediate biovar of C. fetus subsp. venerealis has been described. Whether this variant has specific clinical features is unclear. By definition C. fetus subsp. venerealis is associated with BGC, causing fertility problems with considerable economic losses particularly in endemic regions. Campylobacter fetus subsp. fetus can be recovered from the intestinal tract of cattle and other animal species (6). Campylobacter fetus subsp. fetus can be isolated from aborted bovine fetuses showing its clinical relevance in cattle. However, C. fetus subsp. fetus is associated with sporadic cases of abortion in bovine whereas C. fetus subsp. venerealis is associated with endemic abortion and fertility problems in certain areas.
Although *C. fetus* is primarily recognised as a veterinary pathogen, *C. fetus* subsp. *fetus* is occasionally diagnosed as an opportunistic emerging pathogen in humans. Infections usually occur in pregnant or immunocompromised individuals and are often systemic with a variety of neurological and vascular complications (21).

2. **Taxonomy**

In 1991 a revision of the taxonomy and nomenclature of the genus *Campylobacter* was proposed. According to the Bergey’s Manual, the genus *Campylobacter* comprises sixteen species and six subspecies. More recently, two additional species have been proposed. Two subspecies of *C. fetus* have been recognised. Although the clinical signs of two subspecies overlap, they were originally defined by the differences in clinical presentation (19, 28). The two subspecies can be differentiated in the laboratory by one biochemical trait: glycine tolerance. Subspecies *venerealis* is considered as glycine sensitive and subspecies *fetus* as glycine tolerant. *Campylobacter fetus* subsp. *venerealis* biovar *intermedius* strains have been described (18), yet their taxonomic position needs to be clarified. On the basis of protein-banding patterns using polyacrylamide gel electrophoresis (PAGE) of whole cell proteins, no discrimination can be made between the two *C. fetus* subspecies (27). Studies of DNA–DNA hybridisation have failed to reveal any major difference between the *venerealis* and *fetus* subspecies (10). However, several molecular methods have been shown to be able to differentiate the two subspecies, including polymerase chain reaction (PCR) (12, 22, 25, 30), PFGE (pulsed-field gel electrophoresis) (17), multilocus sequence typing (MLST) (23) and amplified fragment length polymorphism (AFLP) (29) (see also Section B.1.h).

### B. DIAGNOSTIC TECHNIQUES

1. **Isolation and identification of the agent (the prescribed test for international trade)**

   a) **Collection of samples**

      i) **Male: preputial smegma and semen**

      In bulls, smegma may be obtained by different methods: scraping (20), aspiration (3), and washing (4). Smegma is commonly collected by scraping and can be used for isolation of the bacteria, or is rinsed into a tube with approximately 5 ml of phosphate buffered saline (PBS) with 1% of formalin for immunofluorescence (I FAT) diagnosis. Smegma can also be collected from the artificial vagina after semen collection, by washing the artificial vagina with 20–30 ml of PBS.

      For preputial washing, 20–30 ml of PBS is introduced into the preputial sac. After vigorous massage for 15–20 seconds, the infused liquid is collected.

      Semen is collected under conditions that are as aseptic as possible. Semen samples must be diluted with PBS and are sown directly onto culture medium or transport and enrichment medium.

      ii) **Female: (cervico) vaginal mucus (CVM)**

      Samples may be obtained by aspiration, or washing the vaginal cavity.

      For aspiration, the vulva region is cleaned with a tissue paper, and an artificial insemination (AI) pipette or Cassou pipette (blue sheath type) is inserted into the vaginal cavity so that the anterior reaches the cervix (3). Gentle suctioning is applied while moving the pipette gently backwards and forwards. The pipette is removed, and the collected mucus is sown directly onto culture medium or transport and enrichment medium.

      CVM may also be collected by washing the vaginal cavity: 20–30 ml of PBS is infused into the cavity through a syringe attached to an AI pipette. The fluid is sucked out and re-infused four to five times before being collected and spread directly on to culture medium or added to transport and enrichment medium. Washing fluid in the vaginal cavity may also be collected by a tampon or gauze held inside the vagina for 5–10 minutes after PBS infusion. Samples of CVM obtained by suction may be diluted with PBS, or sown directly onto culture medium or transport and enrichment medium.

      CVM is transferred into approximately 5 ml of PBS with 1% of formalin.

      iii) **Aborted fetuses, placentas**

      The placenta as well as the liver, lungs and stomach contents of the fetus provide the best samples for isolation of the causative bacteria. Samples are inoculated directly in transport and enrichment medium, or into PBS with 1% formalin for IFA testing.
b) Transport of samples

The use of a transport medium is essential if the samples are not processed in the laboratory within the same day after collection. For dispatch to the laboratory, if the samples are not in transport medium, the samples must be placed in an insulated container (within the temperature range 4–10°C), and protected from light.

Various transport and enrichment media are available, such as Clark’s, Lander’s, SBL, Foley’s and Clark’s, Weybridge’s, Cary-Blair’s (7, 11, 15).

Some of the transport and enrichment media mentioned above contain cycloheximide. Because of its potential toxicity, amphotericin B can be used as an alternative.

c) Treatment of samples

On arrival at the laboratory, samples should be inoculated directly onto culture medium, or processed further if required.

i) Genital tract samples

Preputial washings may be centrifuged (3500 \(g\)) to concentrate the sample. The final sample (reduced to 250 µl) may be inoculated onto the culture medium (directly and/or using the filter method).

If the CVM is not very viscous it can be inoculated directly or diluted with an equal volume of PBS. When the CVM is very viscous, it may be necessary to liquefy it by adding an equal volume of cysteine solution (aqueous solution of cysteine hydrochloride at 0.25 g/100 ml, pH 7.2, sterilised by membrane filtration). After 15–20 minutes, the diluted and liquefied mucus can then be inoculated onto isolation medium.

ii) Aborted fetuses, placentas

Fetal stomach contents are inoculated directly onto culture medium. Internal organs or pieces of organs are flamed to disinfect the surface, and are subsequently homogenised. The homogenate is inoculated on to culture medium.

After washing placental membranes with PBS to eliminate the majority of the surface contamination, the chorionic villi are scraped and the scrapings are transferred to culture medium.

d) Isolation of Campylobacter fetus

i) Culture media for isolation

Many media are currently in use for the bacteriological diagnosis of BGC. It should be noted that several media used for the isolation of Campylobacter spp. are not suitable for the isolation of \(C.\) fetus due to antimicrobials (e.g. cephalosporins) that may inhibit \(C.\) fetus growth (24). Most culture media contain cycloheximide. Because of its potential toxicity, this antifungal agent can be replaced by amphotericin B. The recommended selective medium for isolation of \(C.\) fetus is Skirrow’s. Skirrow’s medium is a blood-based medium with 5–7% (lysed) defibrinated blood and contains the selective agents: polymyxin B sulphate (2.5 IU/ml), trimethoprim (5 µg/ml), vancomycin (10 µg/ml), and cycloheximide (50 µg/ml).

Alternatively, a non-selective blood-based (5–7% blood) medium in combination with filtration (0.65 µm) can be used; however, it may be less sensitive when compared with a selective medium.

Quality control of each batch of media should be performed using control strains.

ii) Incubation conditions

Plates are incubated at 37°C and under microaerobic atmosphere of 5–10% oxygen, 5–10% carbon dioxide and preferably 5–9% hydrogen for optimal growth (26). Appropriate microaerobic conditions may be produced by a variety of methods. In some laboratories the suitable atmosphere is created by a gas replacement in a jar. Gas generator kits are also available from commercial sources. Variable atmosphere incubators can also be used.

Conditions of culture and incubation are systematically verified by using control strains of \(C.\) fetus subsp. \(fetus\) and \(C.\) fetus subsp. \(venerealis\). Such controls should be set up for each isolation attempt.
Chapter 2.4.5. – Bovine genital campylobacteriosis

**e) Identification of *Campylobacter* species**

i) Colony morphology

Colonies of *C. fetus* usually appear on culture media after 2–5 days. To prevent overgrowth of specific colonies by contaminants, it is recommended that the media be evaluated daily and suspicious colonies be subcultured. After 3–5 days of incubation, colonies measure 1–3 mm in diameter. They are slightly grey-pink, round, convex, smooth and shiny, with a regular edge.

ii) Macroscopic morphology

*Campylobacter* is motile, a property that may disappear during sub-culturing. *Campylobacter* often takes the form of a thin, curved bacillus, 0.3–0.4 µm wide and 0.5–8.0 µm long. Short forms (comma-shaped), medium forms (S-shaped), and long forms (helical with several spirals) may be observed simultaneously in the living state. Old cultures may contain cocccoid bacteria.

iii) Biochemical tests: see Table 1.

iv) Atmosphere: *Campylobacter* does not grow under aerobic conditions.

**f) Immunological identification of *Campylobacter fetus***

The IFAT can be applied to identify the organism directly from samples or to confirm the identification of a strain after isolation. It can not differentiate between different subspecies.

i) Preparation of immune sera

*Campylobacter* strains, preferably standard strains from recognised culture collections (*C. fetus* subsp. *venerealis* or *C. fetus* subsp. *fetus*), are grown on blood-based medium at 37°C under microaerobic conditions for 3 days. The organisms are harvested into PBS, and washed twice by centrifugation. Rabbits aged 3 months are inoculated intramuscularly with 2 ml of 10¹¹ organisms/ml of a *C. fetus* subspecies resuspended in PBS and Freund’s incomplete adjuvant. Inocula are administered at four sites, 0.5 ml at each site. The animals are bled before inoculation and at weekly intervals thereafter. When the serum titres reach high levels, as estimated by the immunofluorescence test or agglutination test, 0.1–1.0 ml of 10¹⁰ viable organisms/ml are injected intravenously. The rabbits are bled for serum 7 days later. Heterologous sera are pooled. In a recent study, a conjugate prepared from chicken IgY was described as an alternative to rabbit antibodies. Monoclonal antibodies that can be used for immunodiagnostic detection of *C. fetus* have been described (2).

ii) Preparation of conjugates

Conjugates are prepared as described by Harlow *et al.* (9). The working dilution of the conjugate is determined by checkerboard titration against smears of a *C. fetus* culture using positive and negative control dilutions, and selecting twice the lowest concentration that produces brilliant fluorescence with *C. fetus* bacteria.

iii) Sample preparation

The genital fluid (fetal abomasal content, preputial smegma or CVM) samples are rinsed into approximately 5 ml PBS 1% formalin. Two centrifugation steps are carried out. First, samples are centrifuged at 600 g for 10 minutes at 4°C to remove debris. Subsequently, the supernatant is centrifuged at 8000 g for 30 minutes at 4°C. The pellet is dissolved in ~100 µl remaining supernatant.

iv) Immunofluorescence test (14)

The sample (20 µl) is applied in duplicate to microscopic slides. The material is air-dried and fixed in acetone at −20°C for 30 minutes or ethanol at 18 - 25°C for 30 minutes. Glass slides will be air-dried and the fluorescein isothiocyanate isomer (FITC)-conjugated antiserum is added at the appropriate dilution. Staining is carried out in a humid chamber at 37°C for 30 minutes in dark condition. Subsequently, the slides are washed three times for 10 minutes in PBS. The slides are mounted in buffered glycerol (90% glycerol: 10% PBS). The cover-slips are sealed to prevent drying, and the slides are examined under ultraviolet light in an epifluorescent microscope. Positive and negative control slides will be used each time the test is done. *Campylobacter fetus* subsp. *venerealis* and *C. fetus* subsp. *fetus* reference strains are used as positive controls, and another *Campylobacter* species are used as negative control. Samples that show fluorescent bacteria presenting the typical morphology of *C. fetus* is considered positive.
g) Biochemical identification of *Campylobacter fetus* subspecies

Tests described in Table 1 must be done on pure cultures.

**Table 1. Differential characteristics of Campylobacter species potentially isolated from the bovine genital tract and aborted fetuses (according to Bergey's Manual 2nd edition, 2005)**

<table>
<thead>
<tr>
<th></th>
<th>25°C</th>
<th>42°C</th>
<th>Oxidase</th>
<th>Catalase</th>
<th>NaCl 3.5%</th>
<th>Glycine 1%</th>
<th>H₂S(b)</th>
<th>Nalidixic acid</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>C. fetus</em> subsp. <em>venerealis</em></td>
<td>V</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>V</td>
</tr>
<tr>
<td><em>C. fetus</em> subsp. <em>fetus</em></td>
<td>+</td>
<td>V^(d)</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td><em>C. jejuni</em></td>
<td>–</td>
<td>V^(d)</td>
<td>+</td>
<td>V</td>
<td>–</td>
<td>V</td>
<td>–</td>
<td>S(b)</td>
</tr>
<tr>
<td><em>C. hyointestinalis</em></td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>V</td>
<td>V</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td><em>C. sputorum</em></td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>V</td>
<td>+</td>
<td>+</td>
<td>V</td>
<td>–</td>
</tr>
</tbody>
</table>

(a) = Although *C. fetus* does not belong to the thermophilic *Campylobacter*, a considerable number of strains of this species grows at 42°C; (b) = On triple sugar iron agar medium; (c) *C. jejuni* subsp. *jejuni* is positive, *C. jejuni* subsp. *doylei* is negative; (d) *C. jejuni* subsp. *jejuni* is positive, *C. jejuni* subsp. *doylei* is variable; (e) according to Bergey’s Manual strains are sensitive, however resistant strains have frequently been reported; (+) = positive reaction or growth and (–) = negative reaction or absence of growth of the strain on an appropriate medium under specified conditions (see Section B.1.d ii); V = variable results; S = sensitive; R = resistant.

i) **Growth at 25°C and 42°C**

A cell-suspension (~McFarland no. 1) is inoculated onto two blood-based medium-plates. Each plate is incubated under the specified atmospheric conditions (see Section B.1.d.ii) at 25°C and 42°C. Control strains are tested in parallel.

ii) **Oxidase and catalase**

Tests are performed according to a standard bacteriological protocol. Control strains are tested in parallel.

iii) **Growth in the presence of sodium chloride**

A cell-suspension (~McFarland no. 1) is inoculated onto blood medium containing 3.5% NaCl (15 ml of blood medium + 2.04 ml of 5 M sodium chloride solution), and on to plain blood medium. Incubation is performed under the specified atmospheric conditions (see Section B.1.d.ii). Control strains are tested in parallel.

iv) **Growth in the presence of 1% glycine**

A cell-suspension (~McFarland no. 1) is inoculated onto a glycine medium (15 ml of blood-based medium + 1.65 ml of 10% aqueous solution of filter sterilised glycine), and onto the same medium without glycine. Incubation is performed under the specified atmospheric conditions (see Section B.1.d.ii). Two control strains (of subspecies *venerealis* and *fetus*) are tested in parallel. As all strains are fastidious, small changes in media can be important, and lack of growth in the presence of glycine should be considered to be a presumptive test for *C. fetus* subsp. *venerealis*. The reproducibility of the assay is poor and intermediate strains have been described (18).

v) **Hydrogen sulphide (H₂S) production in TSI medium**

This hydrogen sulphide (H₂S) test is done on triple sugar iron agar (TSI) under the specified growth conditions (see Section B.1.d.ii). The medium contains peptone (20 g/litre), meat extract (2.5 g/litre), yeast extract (3 g/litre), sodium chloride (5 g/litre), ferric citrate (0.5 g/litre), sodium thiosulphate (Na₂S₂O₃) (0.5 g/litre), lactose (10 g/litre), sucrose (10 g/litre), glucose (1 g/litre), phenol red (0.024 g/litre), agar (11 g/litre), and distilled water (to 1 litre). The medium is sterilised after distribution into tubes by autoclaving at 115°C for 15 minutes and are solidified to obtain a slope. A cell-suspension (~McFarland no. 1) is inoculated onto the slope and into the medium by a loop. A colour change from red to black indicates H₂S production. Control strains are tested in parallel.

vi) **Hydrogen sulphide production (H₂S) in cysteine medium (not listed in the Table 1)**

The H₂S test is done in a Brucella broth medium containing 0.02% cysteine. H₂S production is detected by a lead-acetate strip that is attached inside the top of the tube. A cell suspension (~McFarland no. 1) is inoculated into the medium. Blackening of the lead acetate strip is considered as a positive reaction. Control strains are tested in parallel.
vii) Sensitivity to cephalothin and nalidixic acid

Sensitivity to cephalothin (CN) and nalidixic acid (NA) is tested by the disks containing CN (30 µg) or NA (30 µg).

For the test, 72-hour cultures are suspended in PBS at a concentration of 10^9 bacteria/ml. The culture medium is dried before the culture is deposited on the surface. Using the suspension, 100 µl are spread onto the basic blood medium. The sensitivity disks are then placed on top. These plates are incubated at 37°C in the specified atmosphere (see Section B.1.d.ii), and examined after 48 hours and 72 hours. A zone of inhibition of at least 3 mm around a disk indicates that the strain is sensitive to this antibiotic. All C. fetus subsp. fetus strains and most of the C. fetus subsp. venerealis strains are resistant to NA (16). All C. fetus are sensitive to CN (16).

h) Molecular identification of Campylobacter fetus subspecies

Several molecular methods for the identification of C. fetus subspecies have been described, including 16S sequencing (8, 17), PFGE (17), AFLP (29), and MLST (23). However, most of these methods are time consuming and/or require expensive apparatus and knowledge. Routine diagnostic laboratories would be served best by a simple PCR. Several PCRs have been claimed to be subspecies specific including those developed by Hum et al. (12), Wang et al. (30), and more recently by Tu et al. (22) and Van Bergen et al. (25).

The multiplex PCR described by Hum et al. (12) is currently the most cited PCR. It enables the amplification of a C. fetus-specific DNA fragment (approximately 200 bp smaller than the 960 bp described in the original publication), as well as a C. fetus subsp. venerealis-specific fragment. Thus, performance of this multiplex PCR allows differentiation of the two subspecies (C. fetus = one amplification product vs C. fetus subsp. venerealis = two amplification products). Campylobacter fetus subsp. venerealis biovar intermedius strains have not been evaluated in Hum’s study, but isolates identified as belonging to biovar intermedius with AFLP, classify in the PCR of Hum as either C. fetus subsp. fetus or C. fetus subsp. venerealis (23). Comparison of this PCR against AFLP and MLST (23) and against the glycine test (31) confirms that PCR can give false positive and negative reactions.

The PCR described by Wang et al. (30) reveals only a C. fetus subsp. fetus-specific product. These results were obtained only for a very limited number of strains. Recent evaluations of its value for subspecies differentiation using larger sets of strains yielded both false positive and negative reactions (25).

The random amplification of polymorphic DNA (RAPD)-PCRs described by Tu et al. (22) are published only recently, and are apparently evaluated with a very limited number of C. fetus subsp. venerealis strains. Their value should be evaluated more extensively with a larger group of strains.

The recently described PCR by Van Bergen et al. (25) showed full consistency with the C. fetus subsp. venerealis as defined by AFLP and is therefore considered as the best PCR for detection method of C. fetus subsp. venerealis currently available. However, C. fetus subsp. venerealis biovar intermedius as defined by AFLP is not identified by this PCR.

2. Serological tests/antibody detection

An ELISA is available to detect antigen-specific secretory IgA antibodies in the vaginal mucus following abortion due to C. fetus subsp. venerealis. These antibodies are long lasting, and their concentration remains constant in the vaginal mucus for several months (13).

Initial sampling can be done after the early involution period (usually 1 week after abortion) when mucus becomes clear.

An ELISA for the detection of the serum humoral IgG response after vaccination is described.

a) Antigen preparation and coating

Cultures are transferred to PBS with 0.5% formalin for 1 hour, centrifuged at 17,000 g, washed twice with PBS, and then resuspended in 0.05 M carbonate buffer, pH 9.6. The final absorbance is adjusted to OD_<610 nm> = 0.2. Flat-bottomed polystyrene microtitre plates coated with 10 µl of antigen are left overnight at 4°C, and then stored at ~20°C. Before use, the plates are rinsed twice with distilled water and then tapped gently to remove moisture.

- Test procedure

i) Diluted vaginal mucus (100 µl) is added to each well, and the plate is incubated at 37°C for 2 hours. The plates are then washed as before, and 100 µl of rabbit anti-bovine IgA is added. After 2 hours
incubation at 37°C, the plates are washed and 100 μl of goat anti-rabbit IgG conjugated to horseradish peroxidase is added to each well. After a further 2 hours incubation at 37°C, the plates are washed, and 100 μl of substrate is added (0.8 mg/μl 5 amino-salicylic acid; pH 6.0), immediately activated by the addition of 2% 1 M hydrogen peroxide. The plates are left at room temperature for 30 minutes and the reaction is stopped by the addition of 50 μl of 3 M sodium hydroxide. The absorbance is measured on an ELISA reader at 450 nm. Each sample is tested in duplicate, and positive and negative controls are included in each plate. The absorbance measurements yielded by the test sample are corrected for the absorbance measurement of positive and negative controls according to the formula:

\[
\text{Result} = \frac{\text{Absorbance}_{\text{sample}} - \text{Absorbance}_{\text{negative control}}}{\text{Absorbance}_{\text{positive control}} - \text{Absorbance}_{\text{negative control}}} \times 100
\]

The test is considered to be positive if the result is above 40. Vaccinated animals will not react to IgA ELISA as their vaginal mucus contains only IgG isotype antibodies.

### C. REQUIREMENTS FOR VACCINES AND DIAGNOSTIC BIOLOGICALS

Two groupings of antigens of *C. fetus* are recognised: the thermolabile ‘H’ flagellar antigens and the thermostable ‘O’ somatic antigens. In addition, a capsular ‘K’ antigen should be present. The K antigen is easily destroyed under *in vitro* conditions. The vaccine must incorporate these different antigens. Other vaccine preparations have also been described (5). Experimental *C. fetus* subsp. *fetus* vaccine confers immunity against *C. fetus* subsp. *venerealis* because both strains share common antigens (1), however, the addition of a second strain of *C. fetus* subsp. *venerealis* to the biological product is widely practised and strongly suggested. The presence of four to five heat-labile glycoprotein immunogens, shared by many *C. fetus* subsp. *venerealis* and *C. fetus* subsp. *fetus* strains, is critical. The presence of such immunogens should be confirmed. The vaccine concentration (dry weight) should be around 40 mg protein per dose in order to have a good protection level.

In infected herds, all breeding animals (bulls, cows and heifers) will be vaccinated twice prior to the breeding season. In most of the cases, the vaccine reduces the length of the infection and carrier-cows can keep the infection from one season to the next. Bulls require two vaccine doses annually, because the vaccine may not always be effective in terminating established infections. The next year’s bulls and replacement heifers are vaccinated, and from the third year, bulls are vaccinated annually.

In non-infected herds, only the bulls are vaccinated annually, and this will be done twice a year (two doses with 21 days interval; 2 weeks before the start of the breeding season).

#### 1. Seed management

**a) Characteristics of the seed**

The seed consists of a large, homogeneous batch of a culture of *C. fetus* subsp. *fetus* or *C. fetus* subsp. *venerealis* that has been thoroughly characterised as to identity and purity, preserved in small aliquots.

**b) Method of culture**

The initial growth of the seed is accomplished in semisolid medium. This consists of basal medium with the addition of 0.16% agar. Basal medium is composed of 2.8% *Brucella* broth, 0.5% yeast extract, 1.2% sodium succinate, and 0.001% calcium chloride. The initial culture is maintained for 3 days at 37°C under specified conditions (see Section B.1.d.ii). The growth is transferred to additional tubes with semisolid medium and incubated for 48 hours. The resulting growth is used for vaccine production.

This culture should be stored at 4°C.

**c) Validation as a vaccine**

The seed must be free from contaminating organisms. The purity of the seed must be checked by a suitable culture method.

It is not practicable to test efficacy under laboratory conditions. It is determined in the field on the basis of epidemiological observations.
2. **Method of manufacture**

The working seed material is seeded into broth medium consisting of basal medium with the addition of 0.025% sodium thioglycollate. These cultures are incubated at 37°C for 24 hours while being shaken at a rate of 80 rpm. The fluids are harvested, and formaldehyde is added to a final concentration of 0.2% (0.74 g/litre).

The vaccine is mixed with an oil-emulsion adjuvant.

3. **In-process control**

The identity of the organism should be checked by culture and identification, as well as the absence of contaminating organisms.

4. **Batch control**

a) **Sterility**

Tests for sterility and freedom from contamination of biological material may be found in Chapter 1.1.9.

b) **Safety**

The inactivation process must be complete and the method to insure inactivation should be validated before it can safely be used. Inactivation is checked by inoculating the equivalent of one dose on to the same medium under the same conditions as those used in the production process. This culture is incubated under the same conditions for 72 hours, after which there should be no evidence of bacterial growth. The final product must also be shown to be free from viable bacterial and fungal contaminants, using suitable culture methods.

Two guinea-pigs are inoculated with 2 ml of the product, either intramuscularly or subcutaneously. They must not have an adverse reaction attributable to the vaccine during a 7-day observation period following inoculation.

c) **Potency**

Potency of the vaccine may be measured by seroconversion in rabbits. Their serum titres are measured by immunofluorescence or by the tube agglutination test. Five rabbits, serologically negative at 1/100 serum dilution, are vaccinated twice subcutaneously with half the dose used in cattle, at an interval of 14 days. Serum from at least four of the five rabbits, collected 14 days after the second vaccination, must show at least a four-fold increase in titre.

5. **Tests on the final product**

a) **Safety**

See Section C.4.b.

b) **Potency**

See Section C.4.c.

• **Acknowledgement**

Parts of this chapter were taken from or based on the chapter on bovine genital campylobacteriosis in previous editions of the *Terrestrial Manual*. The authors are grateful to Dr C. Campero (Argentina) for fruitful discussions.

**REFERENCES**


31. **Willoughby** K., **Nettleton** P.F., **Quirie** M., **Maley** M.A., **Foster** G., **Toszeghy** M. & **Newell** D.G. (2005). A multiplex polymerase chain reaction to detect and differentiate *Campylobacter fetus* subspecies *fetus* and *Campylobacter fetus*-species *venerealis*: use on UK isolates of *C. fetus* and other *Campylobacter spp*. *J. Appl. Microbiol.*, 99, 758–766.

* * *

**NB:** There is an OIE Reference Laboratory for bovine genital campylobacteriosis (see Table in Part 3 of this *Terrestrial Manual* or consult the OIE Web site for the most up-to-date list: www.oie.int).
CHAPTER 2.4.6.

BOVINE SPONGIFORM ENCEPHALOPATHY

SUMMARY

Bovine spongiform encephalopathy (BSE) is a fatal neurological disease of adult cattle that was first recognised in Great Britain (GB) in 1986. It is a transmissible spongiform encephalopathy or prion disease. The archetype for this group of diseases is scrapie of sheep and goats (see Chapter 2.7.12 Scrapie).

The epizootic of BSE can be explained by oral exposure to a scrapie-like agent in the ruminant-derived protein of meat-and-bone meal included in proprietary concentrates or feed supplements. Initial cases of BSE in some countries were considered to be the result of exports from GB of infected cattle or contaminated meat-and-bone meal, although exports from other countries are now implicated. In others, initial cases are clearly indigenous, with no clear link with imported meat-and-bone meal, suggesting that earlier, undetected, cases may have occurred. As a result of control measures, the epizootics in many countries are in decline. Cases of BSE currently occur throughout most of Europe and have been detected in Asia and North America.

Experimental transmissibility of BSE to cattle has been demonstrated following parenteral and oral exposures to brain tissue from affected cattle. The BSE agent is also believed to be the common source, via dietary routes, of transmissible spongiform encephalopathies (TSEs) in some other ruminant species and in species of felidae. There is evidence of a causal link between the BSE agent and the variant form of the human TSE, Creutzfeldt-Jakob disease (vCJD). Recommendations for safety precautions for handling BSE-infected material now assume that BSE is a zoonosis and a containment category 3 (with derogation) has been ascribed.

Identification of the agent: In GB, BSE had a peak incidence in cattle aged between 4 and 5 years. The clinical course is variable but can extend to several months. Overt clinical signs are sufficiently distinctive to lead to suspicion of disease, particularly if differential diagnoses are eliminated. Early clinical signs may be subtle and mostly behavioural, and may lead to disposal of affected animals before suspicion of BSE is triggered. In countries with a statutory policy toward the disease, clinically suspect cases must be killed, the brain examined and the carcass destroyed. Now, in most countries, active surveillance identifies infected cattle before, or without, the recognition of clinical signs. No diagnostic test for the BSE agent in the live animal is presently available. The nature of the agents causing the TSE is unclear. A disease-specific partially protease-resistant, misfolded isoform of a membrane protein PrP\(^{\text{c}}\), originally designated PrP\(^{\text{Sc}}\), has a critical importance in the pathogenesis of these diseases and according to the prion hypothesis is the principal or sole component of the infectious agent. Confirmation of the diagnosis, formerly by histopathological examination of the brain, is now, therefore, by the application of immunohistochemical (IHC) and/or immunochemical methods to brain tissue for the detection of PrP\(^{\text{Sc}}\). PrP\(^{\text{Sc}}\) can be detected in specific neuroanatomical loci in the CNS of affected cattle by IHC methods in formalin-fixed material, or by immunoblotting and other enzyme immunoassay methods using unfixed brain extracts.

Transmission from infected brain tissue, usually to conventional or transgenic mice, is the only practical method currently available for detection of infectivity and has an important role in the confirmation or characterisation of agent strains. Variant or atypical forms of BSE have been detected across all continents that have experienced classical BSE. While in the majority of instances atypical phenotypes have been based on western immunoblot banding pattern, bioassay characterisation of some isolates provides emerging evidence of strain diversity in naturally occurring prion diseases of cattle.
**Serological tests:** Specific immune responses have not been detected in TSEs.

**Requirements for vaccines and diagnostic biologicals:** There are no biological products available currently. Commercial diagnostic kits for BSE are available and are used for diagnosis of BSE in many countries.

### A. INTRODUCTION

BSE is a fatal disease of domestic cattle, cases of which were first recognised in Great Britain (GB) in November 1986 (27, 37). It is a transmissible spongiform encephalopathy (TSE) or prion disease, originally typified in animal species by scrapie of sheep. Prion diseases are defined by the pathological accumulation, principally and consistently in the central nervous system (CNS) and more variably in the lymphoreticular system (LRS), of a misfolded, partially protease-resistant, isoform of a highly conserved, host-encoded membrane protein (PrP^C^), which was originally designated PrP^{Sc}. The function of PrP^C^ remains unclear. PrP^{Sc} is the only disease-specific macromolecule identified in the scrapie-like diseases. It is also variably referred to as PrP^{res}, to denote the protease resistant property of the pathological protein, PrP^d^ for disease-specific and PrP^{BSE} specifically in BSE. Here PrP^{Sc} is used generically to refer to the abnormal isoform of PrP^C^. The favoured scientific view is that the agent is composed entirely of the disease-specific isoform of PrP and that the altered form is capable of inducing conversion of the normal form: the protein only or ‘prion’ hypothesis. Data in support of alternative hypotheses, such as viral or bacterial origins or the involvement of cofactors such as mineral imbalances, remain elusive. The molecular basis for strain variation is still unclear, but according to the prion hypothesis strain characteristics are encoded in different conformations of the prion protein.

Initial characterisation of BSE isolates from GB by transmission to mice showed that over the main course of the epidemic the disease was caused by a single major strain of agent that differed from characterised strains of the scrapie agent in sheep (4). Uniformity of the pathology among most affected cattle has also supported the notion of a consistent disease phenotype for BSE (7, 30). The pattern of neuropathology in the host species is important in the phenotypic characterisation and consequent case definition of BSE used for confirmation of the disease. Reports since 2003 of variant features of pathology and/or molecular characteristics in several countries have raised issues of possible agent strain variations of prion disease in cattle (3, 8, 21, 44). Whether or not such findings represent true strain variation of the BSE agent, or different forms of prion infections of bovines, remains to be proven. Because of the detection of most of these cases by active surveillance, correlation with clinical histories is lacking, and most focus only on western immunoblotting data (3, 44). The most comprehensive description, providing immunohistochemical (IHC), histopathological and western immunoblotting characterisation relates to two aged cows in Italy (8). Transmissibility of certain isolates to mice, with features distinct from previous BSE transmissions has been confirmed (2, 5). Transmission studies of other isolates in cattle are in progress. An interesting common feature is that most of these isolates originate from older cattle.

The initial epidemiological studies of BSE in GB established that its occurrence was in the form of an extended common source epizootic, due to feed-borne infection with a scrapie-like agent in meat-and-bone meal used as a dietary protein supplement (1, 39). Although recorded initially in the United Kingdom (UK), BSE has now occurred, albeit at lower incidence, in many countries involving imported and/or indigenous cattle. Such cases are most likely to have resulted directly or indirectly from the export of infected cattle or infected meat-and-bone meal from countries with occurrences of BSE, including historically, the UK. It is clear that infection has subsequently been propagated within countries in which cases have occurred as highlighted by the evaluation of Geographical BSE Risk (GBR) in many countries by the Scientific Steering Committee of the European Union (13). Indeed, in some countries, the only cases detected reflect indigenous exposure rather than direct linkage with imported contaminated feed (41). Current statistics on BSE occurrence around the world are provided by the OIE (41).

There is no evidence of horizontal transmission of BSE between cattle and little data to support the existence of maternal transmission (27). Epidemiological and transmission studies have not revealed evidence of a risk from semen or milk or through embryos (27).

As a result of control measures, the epizootics in the UK and many other countries have declined, or show the effects of controls in the form of changes in age-specific incidence. In some countries the controls have not been in place long enough for the effects to be recognised. Interpretation of the status of epizootics has been enhanced by the introduction of active surveillance using rapid diagnostic tests, which have detected infected animals that have not been recognised as clinically suspect cases. While such active surveillance is capable of detecting a proportion of preclinical cases, retrospective investigation at farms of origin frequently confirms that some signs have been presented before slaughter, but had not triggered consideration of a clinical diagnosis of BSE.
The novel occurrence of TSEs in several species of captive exotic bovidae and felidae and in domestic cats during the course of the BSE epizootic is attributed to and, for several affected species, shown, to have been caused by the BSE agent (23). Exposure is presumed to have been dietary.

The emergence of a new form of the human prion disorder Creutzfeldt-Jakob disease (CJD), termed variant CJD (vCJD) in the UK (40) has also been shown by transmission and molecular studies (6, 10) to be causally linked to the BSE agent. Dietary exposure is considered the route of infection. In the past, no connection has been established between the exposure of humans to agents causing animal spongiform encephalopathies and the occurrence of the human TSE and thus BSE presents a precedent as a zoonotic TSE. It is therefore now recommended that safety precautions for handling the BSE agent be based on the assumption that BSE is transmissible to humans. The epizootic of vCJD in the UK in individuals homozygous for MM at codon 129 of the PrP gene, peaked in 2000; small numbers of cases have occurred in some other countries.

Consequent to the occurrence of vCJD, a risk-based approach should be adopted when determining the biocontainment level for conducting necropsies on BSE-suspect animals or handling tissues derived from such animals, but any procedure that creates aerosols must be conducted under containment level 3 (see Chapter 1.1.2 Biosafety and biosecurity in the veterinary microbiology laboratory and animal facilities), and the laboratory must comply with national biocontainment and biosafety regulations to protect staff from exposure to the pathogen. Recommended decontamination procedures may not be completely effective when dealing with high-titre material or when the agent is protected within dried organic matter. Recommended physical inactivation is by porous load autoclaving at 134°C –138°C for 18 minutes at 30 lb/in². However, temperatures at the higher end of the range may be less effective than those at the lower end and total inactivation may not be achieved under certain conditions, such as when the test material is in the form of a macerate. Disinfection is carried out using sodium hypochlorite containing 2% available chlorine, or 2 N sodium hydroxide, applied for more than 1 hour at 20°C for surfaces, or overnight for equipment (33).

B. DIAGNOSTIC TECHNIQUES

1. Identification of the agent

Clinical BSE, as it presented throughout the main epidemic, occurs in adult cattle, and most cases were observed in dairy cattle aged 4–5 years. With the decline of the epidemic, the impact of effective controls has been reflected in an increasing age at onset of clinical disease (27). Onset of clinical signs is not associated with season or stage of breeding cycle. BSE has an insidious onset and usually a slowly progressive course (24, 38). Occasionally, a case will present with acute signs and then deteriorate rapidly, although frequency of observation is a significant factor in determining early clinical signs. Presenting signs, though variable, usually include behavioural changes, apprehension, and hyper-reactivity. For example, affected cows may be reluctant to enter the milking parlour or may kick vigorously during milking. In dry cows especially, hind-limb incoordination and weakness can be the first clinical features to be noticed. Neurological signs predominate throughout the clinical course and may include many aspects of altered mental status, abnormalities of posture and movement, and aberrant sensation, but the most commonly reported nervous signs have been apprehension, pelvic limb ataxia, and hyperaesthesia to touch and sound. The intense pruritus characteristic of some sheep with scrapie is not prominent in cattle with BSE, though in a proportion of cases there is rubbing and scratching activity. Affected cows will sometimes stand with low head carriage, the neck extended and the ears directed caudally. Abnormalities of gait include swaying of the pelvic quarters and pelvic limb hypermetria; features that are most readily appreciated when cattle are observed at pasture. Gait ataxia may also involve the forelimbs and, with advancing severity of locomotor signs, generalised weakness, resulting in falling and recumbency, can dominate the clinical picture. Reports of reduced rumination, also bradycardia and altered heart rhythm, though not specific signs, suggest that autonomic disturbance is a feature of BSE. General clinical features of loss of bodily condition, decreasing live weight, and reduction in milk yield often accompany nervous signs as the disease progresses. There has been no change in the clinical picture of BSE over the course of the epizootic in the UK (24, 38). Clinical signs are essentially similar in other countries where BSE has occurred. The protracted clinical course, extending usually over a period of weeks or months, would eventually require slaughter on welfare considerations. However, a statutory policy to determine the BSE status of a country requires compulsory notification and diagnostic investigation of clinically suspect cases, their slaughter and post-mortem examination of the brain. Early in the disease course, the signs may be subtle, variable and nonspecific, and thus may prevent clinical diagnosis on an initial examination. Continued observation of such equivocal cases, together with appropriate clinical pathology procedures to eliminate differential diagnoses, especially metabolic disorders, will establish the essential progression of signs. Some early clinical signs of BSE may show similarities with features of nervous ketosis, hypomagnesaemia, encephalic listeriosis and other encephalitides. Subtle signs may sometimes be exacerbated following stress, such as that caused by transport. Video clips of cattle affected by BSE may be downloaded from the web site of the European Commission (EC) TSE Community Reference Laboratory/Veterinary Laboratories Agency (VLA) (15). DVD or videotape footage of the clinical signs is available from this and other sources (35).
The laboratory diagnosis of BSE has evolved in concert with increasing knowledge of the disease and technical advances (17). In the absence of in-vitro methods for isolation of the causative agent, the historical basis of confirmation of the diagnosis in this group of diseases was the demonstration of the morphological features of spongiform encephalopathy by histopathological examination. This remains necessarily, by definition, the only method by which this characteristic vascular pathology can be diagnosed. The original diagnosis of BSE was based on the histopathological features of a scrapie-like spongiform encephalopathy and the electron microscopic visualisation of fibrils, termed scrapie-associated fibrils (SAF), which are composed largely of PrPSc, in detergent extracts of affected brain. The material examined was invariably from suspect clinical cases. In GB, in the light of the rapidly increasing epizootic in the late 1980s, histopathological diagnosis based on examination of a single section of medulla oblongata taken at the level of the obex, was validated against more extensive examination of the brainstem (34). This simplified approach enabled modification of sampling of the fresh brain; instead of whole brain removal, the required section was taken from the brainstem removed via the foramen magnum, using customised instrumentation. With increasing recognition of the diagnostic specificity of PrPSc and, with availability of appropriate antibodies and increasing efficiency of detection methods, immunohistochemical methods of disease-specific PrP detection, including IHC techniques and Western blotting/SAF-immunoblotting, were used, in addition to histopathology, to confirm the diagnosis. The introduction of more rapidly performed in-vitro methods for the detection of PrPSc led to implementation of a variety of ‘rapid’, mostly enzyme-linked immunosorbent assay (ELISA)-based, tests, conducted on sub-samples of medulla oblongata, and these have become the principal approach for active surveillance diagnosis. Such tests provide a preliminary screening from which positive or inconclusive results are subject to examinations by IHC or Western blot confirmatory methods. Rapid test strategies are currently the main approach by which cases are detected and their wider use as part of the confirmatory process has been agreed in principle (15).

The use of a particular method will depend on the purpose to which the diagnosis is to be applied in the epidemiological context and its validation for that purpose. This range of purposes will extend from confirmation of the clinical diagnosis in the control of epizootic disease to the screening of healthy populations for evidence of covert or preclinical disease. The case definition adopted will also differ according to whether the diagnostic method is to be applied for confirmation of a clinical case or for screening of a population. Care should be taken in the interpretation of diagnostic data using methodologies that do not enable careful cross-referencing with the standards for confirmatory diagnosis that are defined here. Without appropriate comparison with previously published criteria defining the BSE phenotype, and in the absence of transmission studies, diagnostic results that claim the identification of a new strain may be premature. Quality control (QC) and quality assessment (QA) are essential parts of the testing procedures and advice can be supplied by the OIE Reference Laboratories (15, 42). Whether BSE-infected animals are to be identified by passive or active surveillance, it is a good practice to detect and confirm disease by a combination of at least two test methods. The primary test can be one of the confirmatory test methods described below or a rapid test, but it is important to apply a secondary test to confirm a positive or inconclusive primary test result. Where there is a conflict between primary and secondary test results, further tests using immunohistochemistry or scrapie-associated fibrils (SAF)-immunoblot (or approved alternative) should be applied or samples should be submitted to an OIE Reference Laboratory for resolution.

a) Sample preparation

The BSE status of a country, the relative implementation of passive and active surveillance programmes and the diagnostic methods applied, will all influence sampling strategy.

In all circumstances of passive surveillance of neurological disease in adult cattle where the occurrence of BSE within a country or state has not been established or is of low incidence, it is recommended that clinically suspect cases are subjected to a standard neuropathological approach in which representative areas of the whole brain are examined. Moreover, care must be taken to preserve suitable fixed and fresh brain samples for the immunohistochemical and immunoochemical detection of PrPSc. Departure from this approach may prevent appropriate characterisation of the case, to confirm whether or not it is typical of BSE. Cattle suspected of having the disease should be killed with an intravenous injection of a concentrated barbiturate solution preceded, if necessary, by sedation. The brain should be removed as soon as possible after death by standard methods.

Histopathology and IHC examinations are carried out Initially on a single block (0.5–1.0 cm in width) cut at the obex of the medulla oblongata (Fig. 1a and b, level A–A representing the centre of the block for examination), which should be selected for fixation for at least 5 days in 4% formaldehyde solution (i.e. 10% formal saline or 10% normal buffered formalin [NBF]) and subsequent histological processing by conventional paraffin wax embedding methods for neural tissue.

Fresh material for use in confirmatory immunoblotting to detect disease-specific PrP should be taken initially, as a complete coronal section (2–4 g) from the medulla, immediately rostral, or caudal, to the obex block taken for fixation. Alternatively, the medulla, at the level of the obex, could be hemi-sectioned, as described for active surveillance (see below). All other brain areas should be subdivided by a sagittal paramedian cut (0.5 cm off the median). The smaller portion is reserved for the PrPSc detection by immunoochemical methods (e.g. SAF-immunoblot) and is stored frozen prior to testing (if testing is not done during the confirmatory process has been agreed in principle (15).
immediately after sampling). After sampling of the obex region for fixation and sampling of fresh tissue, the larger portion of the brain tissue is placed, intact, in approximately 4–6 litres of 10% formalin fixative, which should be changed twice weekly. After fixation for 2 weeks, the brain is cut into coronal slices. The fixation time may be shortened by cutting the fresh brainstem (detached from the rest of the brain) into smaller coronal pieces, similarly to the initial removal of the obex region, but leaving intact the remaining diagnostically important cross-sectional areas at the levels of the cerebellar peduncles and the rostral colliculi (Figure 1a and b, levels B–B and C–C, respectively). Depending on some other factors (temperature, agitation, thickness of tissue block, use of microwave) the fixation time for these small pieces of brainstem may be reduced to 2–5 days. However, evaluation of the effects of this kind of processing on subsequent IHC protocols needs to satisfy proficiency testing standards. The other formol-fixed parts of the brain may be used for differential diagnosis after completing the standard two weeks’ fixation.

Fig 1. Brainstem after the removal of the cerebellum, from a) dorsal, and b) lateral aspects.

Recommended levels at which sections should be taken:
A–A = medulla, at the obex; B–B = medulla through caudal cerebellar peduncles; C–C = midbrain through rostral colliculi.

When the occurrence of BSE in a particular country has been established in the indigenous cattle population, and there is evidence that the distribution of lesions and other phenotypic determinants, are consistent with that seen in the brains of cattle from the UK epizootic, it is adequate, although not ideal, for monitoring purposes, to remove the brainstem alone.

This can be achieved via the foramen magnum without removal of the calvarium (Fig. 2). This will reduce the amount of fixative required and the time and equipment needed, thereby lowering costs and improving safety. The major target areas for histological examination can still be maintained. This method allows for collecting and examining a large number of samples for passive surveillance or for an active surveillance programme in abattoirs. The brainstem is dissected through the foramen magnum without opening the skull by means of a specially designed spoon-shaped instrument with sharp edges around the shallow bowl (Fig. 2). Such instruments are available commercially, made of plastic or metal. It is possible that variations in technique, including orientation, are required with different forms of the instrument, thus highlighting the need for training of operators once there is agreement on equipment to be used. Under abattoir conditions it has also been shown possible to obtain expulsion of intact brainstem via the foramen magnum, providing histologically good material, by application of fluid pressure (air or water) (20) through the entry wound in the skull when penetrative stunning has been used in slaughtering. Clearly the feasibility and efficacy of this method will be dependent on the slaughter method and before implementation for routine use requires to be subjected to risk assessment.

Where the index case is identified through active surveillance, the necessary brain areas for full phenotypic characterisation are unlikely to be available. In most countries, brainstem alone is collected, even before the first confirmation of BSE. Ideally, provision should be made for heads that have been sampled in the course of active surveillance to be retained until the outcome of initial testing is available. This would enable much more comprehensive sampling of the brain of positive animals and enable the recommended approach to the characterisation of cases. This is particularly important if unvalidated tests are used and where, in the absence of direct comparison with the methods described here, claims are
made that new phenotypes have been identified. Where rapid immunoassays are used as the primary surveillance tool, in the absence of a diagnosis of BSE having ever been made in a country, it is necessary to make provision for material to be available for further morphological and IHC examination that would allow identification of disease phenotype.

Fig. 2. After the head has been removed from the body by cutting between the atlas vertebra and the occipital condyles of the skull, it is placed on a support, ventral surface uppermost (A), with the caudal end of the brainstem (medulla oblongata) visible at the foramen magnum (see B, expanded drawing of cranium).

The instrument (C) is inserted through the foramen magnum between the dura mater and the ventral/dorsal aspect (depending upon the specific approach) of the medulla and advanced rostrally, keeping the convexity of the bowl of the instrument applied to the bone of the skull and moving with a side-to-side rotational action. This severs the cranial nerve roots without damaging the brain tissue. The instrument is passed rostrally for approximately 7 cm in this way and then angled sharply (i.e. toward the dorsal/ventral aspect of the brainstem, depending on the approach) to cut and separate the brainstem (with some fragments of cerebellum) from the rest of the brain. The instrument, kept in the angled position, is then withdrawn from the skull to eject the tissue through the foramen magnum.

- **Sampling of brainstem in active surveillance with use of rapid tests**

The sampling and processing of the brain tissue for use in the rapid test should be carried out precisely as specified by the supplier or manufacturer of the test method or kit. Details of this procedure vary from method to method and should not be changed without supportive validation data from the manufacturer for the variant methodology. The preferred sample for immunoassay should be at, or within 1.0 cm rostral, or caudal to, the obex, based on the caudo-rostral extent of the key target sites (Fig. 3) for demonstration of PrPSc accumulations and the evaluation of sampling for rapid tests. The choice of target site has to take into account the subsequent method of confirmation. At least a hemi-section of the medulla at the level of the obex should be fixed for immunohistochemistry/histology. Sampling the medulla rostral or caudal to the obex for rapid testing does not compromise examination by histological or IHC means. However, to obtain comparable samples for rapid and confirmatory testing, sampling by hemi-section of the medulla at the level of the obex is preferable. While there is resultant loss of the ability to assess the symmetry of vacuolar changes, this approach is less likely to compromise the more important IHC examination. If hemisectioning is adopted however, it becomes critical to ensure that the target sites are not compromised in either sample. For example, the nucleus of the solitary tract and the dorsal motor nucleus of the vagus nerve (target areas for lesions in cattle with BSE) are small, and lie relatively close to the midline (Fig. 3). If sampled tissue is autolysed to the point that anatomical orientation is not possible, an unidentified aliquot can still be taken and tested. A positive result in such cases is still a valid result, but a negative test result cannot be taken to indicate a negative animal, and it should be interpreted with caution and reported with appropriate qualification.
Inaccurate hemi-sectioning could easily result in the complete loss of a target area for confirmatory testing, and significantly reduce the effectiveness of the surveillance programme. Failure to accurately sample target areas may also arise through inappropriate placement of proprietary sampling tools. Such approaches therefore need to be implemented with a very clear policy and monitoring programme for training and quality assurance of sampling procedures. Because of the specifically targeted distribution of PrP<sup>Sc</sup>, sample size and location should be as described in the diagnostic kit or, if not specified, at least 0.5 g taken from the diagnostic target areas for all confirmatory tests as detailed in Fig 3. Performance characteristics of some of the tests may be compromised by autolysis, particularly due to loss of the ability to ensure inclusion of target areas in the sample taken from the diagnostic target areas detailed in Fig 3.

b) Diagnostic examination

i) Histological examination

Histopathology is no longer the diagnostic method of choice for investigation of suspect animals, or screening of healthy populations. However, an awareness of the histopathological changes is important, to facilitate detection of cases when conducting routine diagnostic histological examinations of cattle brains. For differential diagnosis, sections of medulla–obex are cut at 5 µm thickness and stained with haematoxylin and eosin (H&E). If tissue quality permits, the histopathological examination of H&E sections allows confirmation of the characteristic neuropathological changes of BSE (30, 36) by which the disease was first detected as a spongiform encephalopathy. These changes comprise mainly spongiform change and neuronal vacuolation and are closely similar to those of all other animal TSEs, but in BSE the high frequency of occurrence of neuroparenchymal vacuolation in certain anatomical nuclei of the medulla oblongata at the level of the obex, provides a satisfactory means of establishing a histopathological diagnosis on a single section of the medulla (34) in clinical suspect cases. As in other species, vacuolar changes in the brains of cattle, particularly vacuoles within neuronal perikarya of the red and oculomotor nuclei of the midbrain are an incidental finding (18). The histopathological diagnosis of BSE must therefore not rely on the presence of vacuolated neurons alone, particularly in these anatomical locations.

The diagnosis may be confirmed if completely typical morphological changes are present in the medulla at the level of the obex, but, irrespective of the histopathological diagnosis, immunohistochemistry is now routinely employed in addition, as unpublished evidence suggests that as many as 5% of clinical suspects (which are negative on H&E section examination for vacuolar changes at the obex) can be diagnosed by IHC examination. Clearly, this protocol, confined to examination of the medulla–obex, does not allow a full neuropathological examination for differential diagnoses to be established, nor does it allow a comprehensive phenotypic characterisation of any TSE. It is for this reason that it is recommended that whole brains are removed from all clinical suspects.

ii) Detection of disease-specific forms of PrP
The universal use of PrP detection methods now provide a disease specific means of diagnosis independent of the morphological changes defined by the histopathological approach. Many laboratories have therefore now supplemented or replaced histopathological examination by IHC and other PrP-detection methods. The detection of accumulations of PrPSc is the approach of choice for surveillance programmes and confirmatory diagnosis. It is possible (but not desirable) to undertake immunohistochemistry for PrP on material that has been frozen prior to fixation (12). Freezing prior to fixation will not compromise the immunoreactivity of a sample, but it may compromise the identification of target sites that need to be checked before a negative result can be recorded.

- **Immunohistochemical (IHC) methods**

During the examination to detect PrPSc accumulation is applied to sections cut from the same formalin-fixed paraffin-embedded material of medulla at the level of the obex as that used for the histopathological diagnosis (36). Several protocols have been applied successfully to the IHC detection of PrPSc for the diagnosis of BSE and although harmonisation toward a fully validated standardised routine diagnostic IHC method would seem desirable, experience has indicated that it is much more important to recognise robust methods that achieve a standardised output, as monitored by participation in proficiency testing exercises, and by comparison with the results of a standardised model method in a Reference Laboratory. The technique does not necessarily require lengthy tissue fixation, although for accuracy the guidelines established for histopathology still apply and, providing the tissue can be adequately processed histologically, it works well in autolysed tissues in which morphological evaluation is no longer possible (11, 25). However, it is still necessary to be able to recognise the anatomy of the sample to determine whether or not target areas are represented. This is essential for a negative diagnosis, and may also be pivotal in accurately interpreting equivocal immunolabelling. IHC detection of PrPSc accumulations approximates to the sensitivity of the Western blotting approach for detection of PrPSc (28). In combination with good histological preparations, immunohistochemistry allows detection of PrPSc accumulations and, as this, like the vacuolar pathology, exhibits a typical distribution pattern and appearance, it provides simultaneous evaluation or confirmation of this aspect of the disease phenotype. Current methods are available by reference to the OIE Reference Laboratories (15, 42).

In contrast to the diagnosis of scrapie of sheep, the limited detection of PrPSc in lymphoid tissues in BSE does not provide any scope for utilising such tissues for pre-clinical diagnosis by biopsy techniques.

- **Western blot methods**

Immunoblotting techniques, are carried out on fresh (unfixed) tissue, and can be applied successfully even when tissue is autolysed (19). The SAF-immunoblot (15) was the first such method for use in BSE diagnosis. It has similar diagnostic sensitivity to the IHC techniques, and remains the method of choice, along with immunohistochemistry, for the confirmation or dismissal of a BSE suspicion. In the last decade, alternative methods have been developed that are less time-consuming and less costly. Most of these techniques use a precipitation of PrPSc using phosphotungstic acid (PTA) or by other chemicals (31), and some are commercially available.

While Western blot methodology is now in general use around the world, analytical sensitivity when used to detect PrPSc varies significantly between methods and laboratories. Where in-house methods are preferred to published methods for confirmatory purposes, it is important that they are evaluated as being fit for purpose and validated in consultation with an OIE Reference Laboratory.

- **Rapid tests methods**

Automated rapid Western blot and ELISA techniques have been developed that allow screening of large numbers of brain samples and are commercially available. Such techniques can be performed in a few hours (see EC evaluations of rapid tests for the detection of BSE on known IHC positive and negative sample groups [14]).

While many countries, and an OIE ad hoc Group on BSE tests, accept EU approval as an indicator of test performance, others have established their own evaluation mechanisms, most notably the United States of America, Canada and Japan (14, 26). The OIE now also has an approval process and protocols for such evaluations are posted on the OIE web site (43), and the EU approval process has been accepted as the gold standard for future evaluations in terms of acceptable sensitivity and specificity.

The relative sensitivities of rapid tests, immunohistochemistry and other confirmatory methods remain to be determined. Acknowledgement of this is particularly important in evaluating rapid test performance when testing animals that are not presenting clinical signs of BSE. Evaluations completed in the EU were restricted to a comparison of the examination of a sample of brains of cattle identified as suspect clinical animals with histopathological changes characteristic of BSE and a sample of brains of cattle from New Zealand that were unexposed to BSE and histopathologically negative. Rapid tests provide a means of initial screening for animals in the last few months of the incubation period, for example in surveys of post-
mornet material collected from routinely slaughtered cattle. In countries conducting surveillance for the detection of the novel occurrence of BSE and in those countries in which a means, independent of the system of notification of suspect cases, of assessing the prevalence of BSE is considered necessary, these screening tests offer an efficient approach. Since their introduction for active screening in Europe from January 2001, such tests have been responsible for the identification of the majority of BSE-infected animals. In some countries, given the speed with which results can be obtained, the rapid tests are the preferred primary test, but confirmation of a diagnosis of BSE ideally requires either the examination of fixed brain by histopathology and/or immunohistochemistry or the application of an appropriate Western blot protocol. Nevertheless, in 2006, the OIE accepted that through their use in active surveillance programmes, commercial rapid tests have proved themselves to be very effective and consistent, provided they are performed by appropriately trained personnel. Indeed, at times they may out-perform the acknowledged standard of comparison if training and experience in the latter are deficient. Under such circumstances, it is now considered acceptable, even if not ideal, for rapid tests to be used in combination for both primary screening in active or passive surveillance programmes and subsequent confirmation. It will be essential however to ensure that the choice of primary and secondary test are compatible, and do not present a danger of generating false positive results through shared reagents. Consequently, an algorithm of preferred test combinations will be maintained on the VLA web site to assist those who wish to resort to this approach instead of histopathology and immunohistochemistry, or SAF immunoblot for confirmation (15). VLA will not change this web site without informing the OIE. The ideal combinations should include an ELISA and western blot method as they generate useful complementary data to assist in phenotypic characterisation of the sample in the absence of examination of fixed tissue.

Under certain circumstances, an EU or OIE approved rapid test could be used for the confirmation of BSE in bovines following an initial reactive result with an approved rapid test. Such approval is dependent on a review of reagents used in each rapid test to ensure that the pairs of tests used are compatible. On the basis of confidential data released by test manufacturers, a procedure is now available and is summarised below:

1. The confirmation must always be carried out in a National Reference Laboratory (NRL) for TSEs.
2. The second test must include a negative control and a bovine BSE sample as positive control.
3. The second test must be a different test (in other words, two positive results involving the same test is insufficient for confirmation).
4. If a rapid Western blot is used as the first test, this result must be documented and submitted to the NRL.
5. One of the two methods must be a Western blot.

The combination of the two rapid tests can only be used for the confirmation of a BSE case. A negative result by the secondary test is insufficient to define a case as negative following a primary positive result. BSE suspect cases with discordant rapid test results must therefore be investigated further using either the SAF-immunoblot (or approved alternative) or IHC for the demonstration of PrP\textsuperscript{Sc}, or if these methods are not available, by histopathology. If histopathology is unable to confirm the initial reactive result, samples should be submitted to an OIE Reference laboratory for further examination.

Although the test evaluation programmes conducted in Europe were in support of legislation on surveillance for BSE, the consequences are of relevance to other countries. The consequences of false-positive or false-negative results are so great that the introduction of new tests should be supported by thorough evaluation of test performance. Claims by test manufacturers should always be supported by data, ideally evaluated independently. It must be stressed that the process of full validation of all of these diagnostic methods for BSE has been restrained by the lack of a true gold standard and the consequent need to apply standards of comparison based on relatively small studies. There is therefore a continuing need for the publication of larger scale studies of assay performance, and none of the data published so far equate with recognised procedures for test validation for other diseases.

d) Other diagnostic tests

The demonstration of characteristic fibrils, the bovine counterpart of SAF (see Chapter 2.7.12 Scrapie), by negative-stain electron microscopy in detergent extracts of fresh or frozen brain or spinal cord tissue (32) has been used as an additional diagnostic method for BSE and has been particularly useful when histopathological approaches were precluded by the occurrence of post-mortem decomposition. With modification, the method may be applied successfully to formalin-fixed tissue (9). Detection of fibrils has been shown to correlate well with the histopathological diagnosis of BSE, but does not offer the sensitivity available from IHC or immunoblotting methods. BSE infectivity can be shown by intracerebral/intraperitoneal inoculation or by feeding of mice with brain tissue from terminally affected cattle, but bioassay is impractical for routine diagnosis because of the long incubation period. Transgenic mice, such as those over-expressing the bovine PrP gene, offer bioassays with reduced incubation periods for BSE, but none as yet represent practical diagnostic tools.
There remains the need for a test for BSE that can be applied to the live animal and has a sensitivity capable of detecting PrPSc at the low levels that may occur early in the incubation of the disease. As yet, the effectiveness of potential approaches has not been shown. The EC remains committed to the evaluation of in-vivo tests, and sets out protocols for the evaluation of such tests (16). The detection of certain protein markers of neurodegeneration, including apolipoprotein E (Apo E), the 14-3-3 protein and S-100 proteins in cerebrospinal fluid have not proved useful for diagnosis of preclinical cases of BSE. The diagnostic potential of the observation of IgG light chains as a surrogate marker for prion infection in the urine of scrapie infected hamsters (22, 29), has not been investigated for the diagnosis of BSE.

2. Serological tests

The infectious agents of prion diseases cannot easily be grown in vitro and do not induce a significant immune response in the host.

C. REQUIREMENTS FOR VACCINES AND DIAGNOSTIC BIOLOGICALS

There are no biological products available currently. As discussed previously, diagnostic kits have been licensed for use in many countries.

REFERENCES


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NB: There are OIE Reference Laboratories for Bovine spongiform encephalopathy (see Table in Part 3 of this Terrestrial Manual or consult the OIE Web site for the most up-to-date list: www.oie.int).
CHAPTER 2.4.7.

BOVINE TUBERCULOSIS

SUMMARY

Bovine tuberculosis is a chronic bacterial disease of animals and humans caused by Mycobacterium bovis. In a large number of countries bovine tuberculosis is a major infectious disease among cattle, other domesticated animals, and certain wildlife populations. Transmission to humans constitutes a public health problem.

Aerosol exposure to M. bovis is considered to be the most frequent route of infection of cattle, but infection by ingestion of contaminated material also occurs. After infection, nonvascular nodular granulomas known as tubercles may develop. Characteristic tuberculous lesions occur most frequently in the lungs and the retropharyngeal, bronchial and mediastinal lymph nodes. Lesions can also be found in the mesenteric lymph nodes, liver, spleen, on serous membranes, and in other organs.

Bovine tuberculosis infection in cattle is usually diagnosed in the live animal on the basis of delayed hypersensitivity reactions. Infection is often subclinical; when present, clinical signs are not specifically distinctive of this disease and might include weakness, anorexia, emaciation, dyspnoea, enlargement of lymph nodes, and cough, particularly with advanced tuberculosis. After death, infection is diagnosed by necropsy and histopathological and bacteriological techniques. Rapid nucleic acid methodologies such as the polymerase chain reaction (PCR) may also be used. These are demanding techniques and only validated procedures should be used. Traditional mycobacterial culture remains the routine method for confirmation of infection.

Identification of the agent: Bacteriological examinations may comprise the demonstration of acid-fast bacilli by microscopic examination (provides presumptive confirmation), the isolation of mycobacteria on selective culture media and their subsequent identification by cultural and biochemical tests or DNA techniques. Animal inoculation, which has been used in the past for confirming infection with M. bovis, is now rarely used because of animal welfare considerations.

Delayed hypersensitivity test: This test is the standard method for detection of bovine tuberculosis. It involves measuring skin thickness, injecting bovine tuberculin intradermally into the measured area and measuring any subsequent swelling at the site of injection 3 days later.

The comparative intradermal tuberculin test with bovine and avian tuberculin is used mainly to differentiate between animals infected with M. bovis and those sensitised to tuberculin due to exposure to other mycobacteria or related genera.

The test used generally depends on the prevalence of tuberculosis infection and on the level of environmental exposure to the other sensitising organisms.

Due to their higher specificity and easier standardisation, purified protein derivative (PPD) products have replaced heat-concentrated synthetic medium tuberculins. The recommended dose of bovine PPD in cattle is at least 2000 International Units (IU) and in the comparative tuberculin test, the doses should be no lower than 2000 IU each. The reactions are interpreted on the basis of appropriate schemes.

Blood-based laboratory tests: Diagnostic blood tests are now available, e.g. the gamma-interferon assay, the lymphocyte proliferation assay, and the enzyme-linked immunosorbent assay. The logistics and laboratory execution of some of these tests may be a limiting factor. The use of blood-based assays can be advantageous, especially with intractable cattle, zoo animals and wildlife, although interpretation of the test may be hampered by lack of data for some species.
Information on the use of various diagnostic tests in animal species other than bovine is provided in a recent review by Cousins & Florisson (9).

Requirements for vaccines and diagnostic biologicals: Vaccines are being developed and evaluated for use in bovine and wildlife species, but at this time are not routinely administered as they may compromise the use of the tuberculin skin test and other immunological tests to detect infected animals. There are standard methods for the production of bovine PPD tuberculins. PPD used for performing the tests specified should be prepared in accordance with the World Health Organization requirements and should conform to these requirements with respect to source materials, production methods and precautions, added substances, freedom from contamination, identity, safety, potency, specificity and freedom from sensitising effect. The bioassays for biological activity are of particular importance, and the potency should be expressed in IUs.

A. INTRODUCTION

*Mycobacterium bovis* is a zoonotic organism and, during diagnostic examination, should be treated as a risk/hazard group III organism with appropriate precautions to prevent human infection occurring.

Bovine tuberculosis is an infectious disease caused by *M. bovis*, and is usually characterised by formation of nodular granulomas known as tubercles. Although commonly defined as a chronic debilitating disease, bovine tuberculosis can occasionally assume an acute, rapidly progressive course. Any body tissue can be affected, but lesions are most frequently observed in the lymph nodes (particularly of the head and thorax), lungs, intestines, liver, spleen, pleura, and peritoneum.

It should be noted that other members of the *M. tuberculosis* complex, previously considered to be *M. bovis*, have been accepted as new species. These include *M. caprae* (3) (in some countries considered to be a primary pathogen of goats) and *M. pinnipedii* (8), a pathogen of fur seals and sea lions. In central Europe, *M. caprae* has been identified as a common cause of bovine tuberculosis (36). These two new species are zoonotic. Disease caused by *M. caprae* is not considered to be substantially different from that caused by *M. bovis* and the same diagnostic tests can be applied in its diagnosis.

In countries with tuberculosis eradication programmes, clinical evidence of tuberculosis in cattle is seldom encountered because the intradermal tuberculin test enables presumptive diagnosis and elimination of infected animals before signs appear. Prior to the national tuberculosis eradication campaigns, however, the signs associated with tuberculosis were commonly observed (7).

These signs vary with the distribution of tubercles in the body but, with few exceptions, the course of the disease is chronic. In many instances, characteristic signs are lacking, even in advanced stages of the disease when many organs may be involved. Lung involvement may be manifested by a cough, which can be induced by changes in temperature or manual pressure on the trachea.

Dyspnoea and other signs of low-grade pneumonia are also evidence of lung involvement. In advanced cases, lymph nodes are often greatly enlarged and may obstruct air passages, the alimentary tract, or blood vessels. Lymph nodes of the head and neck may become visibly affected and sometimes rupture and drain. Involvement of the digestive tract is manifested by intermittent diarrhoea and constipation in some instances. Extreme emaciation and acute respiratory distress may occur during the terminal stages of tuberculosis. Lesions involving the female genitalia may occur. Male genitalia are seldom involved.

Tubercles of cattle are most frequently seen at necropsy in bronchial, mediastinal, retropharyngeal and portal lymph nodes and may be the only tissue affected. In addition, the lung, liver, spleen and the surfaces of body cavities are commonly affected. Other anatomical sites must be considered as potential to become infected.

At necropsy, a tuberculous granuloma usually has a yellowish appearance and is caseous, caseo-calcareous, or calcified in consistency. Occasionally, its appearance may be purulent. Some nontuberculous granulomas occur in which purulent content with a greenish lustre is replaced by granulation tissue, which may have a resemblance to tuberculous granulomas. The caseous centre is usually dry, firm, and covered with a fibrous connective capsule of varying thickness. Fixed tissues in a tubercle are not easily removed intact, as is the case with some nontuberculous granulomas. Lesion size ranges from small enough to be missed by the unaided eye, to involvement of the greater part of an organ. Serial sectioning of organs and tissues is vital to detect lesions contained within the tissue. Lesions caused by *M. bovis* are often paucibacillary (having few organisms) and the absence of acid-fast organisms does not exclude tuberculosis in lymphadenitis of unknown aetiology. In cervidae and some exotic species, tuberculosis should be considered when thin-walled purulent abscesses are observed in the absence of specific aetiology.
Mycobacterium bovis has been identified in humans in most countries where isolates of mycobacteria from human patients have been fully typed. The incidence of pulmonary tuberculosis caused by M. bovis is higher in farm and slaughterhouse workers than in urban inhabitants. The transmission of M. bovis to humans via milk and its products is eliminated by the pasteurisation of milk. One of the results of bovine tuberculosis eradication programmes has been a reduction in disease and death caused by bovine tuberculosis in the human population.

Although cattle are considered to be the true hosts of M. bovis, the disease has been reported in many domesticated and nondomesticated animals. Isolations have been made from buffaloes, bison, sheep, goats, equines, camels, pigs, wild boars, deer, antelopes, dogs, cats, foxes, mink, badgers, ferrets, rats, primates, llamas, kudus, elands, tapirs, elks, elephants, sitatungas, oryxes, addaxes, rhinoceroses, possums, ground squirrels, otters, seals, hares, moles, raccoons, coyotes and several predatory felines including lions, tigers, leopards and lynx (12, 34).

Bovine tuberculosis in wildlife was first reported in 1929 in greater kudu (Tragelaphus strepsiceros) and common duiker (Sylvicapra grimmii) in South Africa. During the 1940s, greater kudu in the same region were endemic infected. In 1982 in Uganda, a prevalence of 10% in African buffalo and 9% in warthog (Phacochoerus aethiopicus) was found. In Zambia, M. bovis infection has been reported in Kafue lechwe (Kobus leche kafuensis) and in a single eland (Traurtragus oryx). An outbreak of tuberculosis in wild olive baboons (Papio cynocephalus anubis) was reported in Kenya. Mycobacterium bovis infection has also been diagnosed in African buffalo in the Kruger National Park in South Africa (4), and more recently spill over to other species such as chacma baboon (Papio ursinus), lion (Panthera leo) and cheetah (Acynonyx jubatus) as well as greater kudu has occurred.

The rigorous application of tuberculin testing and culling of reactor cattle has eliminated M. bovis infection from farmed bovine populations in some countries, but this strategy has not been universally successful. Extensive investigations of sporadic M. bovis reoccurrence have shown that wildlife reservoirs exist in some countries and can act as a source of infection for cattle, deer and other livestock. The detection of infection in a wildlife population requires bacteriological investigation or the use of a valid testing method for the species involved (the tuberculin test is not effective in all species) together with epidemiological analysis of information. The badger (Meles meles) in the United Kingdom (41) and the Republic of Ireland (34), the brush-tail possum (Trichosurus vulpecula) in New Zealand (2), and several wild living species in Africa have been shown to be capable of harbouring M. bovis infection. Control of transmission from the wildlife population to farmed species is complex and, to date has relied on the reduction or eradication of the infected wildlife population. The use of vaccination to control the disease in some species continues to be investigated.

Mycobacterium bovis has been isolated from farmed and free-living cervidae. The disease may be subacute or chronic, with a variable rate of progression. A small number of animals may become severely affected within a few months of infection, while others may take several years to develop clinical signs, which are related to lesions in the animal. The lesions produced may resemble those found in cattle (proliferative granuloma, caseation, granulation and calcification with ageing). The lesions may take the form of thin-walled abscesses with little calcification and containing purulent material. Thin-walled abscesses have also been observed in llamas. In cervids, tuberculosis should be considered when abscess-like lesions of no known aetiology are observed. The lymph nodes affected are usually those of the head and thorax. The mesenteric lymph nodes may be affected—large abscesses may be found at this site. The distribution of lesions will depend on the infecting dose, route of infection and the incubation period before examination.

The tuberculin test can be used in farmed deer. The test must be carried out on the side of the neck, with hair clipping at the site of testing, accurate intradermal injection, and careful pre- and post-inoculation skin thickness measurement using callipers to obtain results that are valid (6).

Mycobacterium bovis can cause severe economic losses due to its effects on domesticated livestock and zoonotic infections. In addition, the presence of infection in wildlife populations poses a threat to the survival of endangered wildlife species.

B. DIAGNOSTIC TECHNIQUES

When diagnostic techniques are used within official TB control or eradication programmes, it is recommended the Veterinary Administration authorises:

- The diagnostic test(s);
- Laboratories performing the tests; and
- Those persons applying diagnostic techniques to animals, i.e. skin tests.
1. Identification of the agent

In cattle, clinical evidence of tuberculosis is usually lacking until very extensive lesions have developed. For this reason, its diagnosis in individual animals and an eradication programme were not possible prior to the development of tuberculin by Koch in 1890. Tuberculin, a concentrated sterile culture filtrate of tubercle bacilli grown on glycerinated beef broth and, more recently, on synthetic media, provides a means of detecting the disease.

Immunological responses to *M. bovis* infections in cattle are being studied in an attempt to develop improved or alternative diagnostic methods, as skin testing sometimes has practical drawbacks. The gamma interferon test shows promise as a diagnostic blood test for tuberculosis in cattle and for other animals (e.g. deer, buffalo) and is available commercially. The lymphocyte proliferation test and the IgG1 enzyme-linked immunosorbent assay (ELISA) have proven to be useful as ancillary serial (to enhance specificity) and parallel (to enhance sensitivity) tests in farmed red deer.

The presence of *M. bovis* in clinical and post-mortem specimens may be demonstrated by examination of stained smears or tissue sections and confirmed by cultivation of the organism on primary isolation medium. Collection containers should be clean and preferably sterile (use of sample containers that are contaminated by environmental mycobacteria may result in the failure to identify *M. bovis* infection due to the rapid growth of the environmental mycobacteria); where feasible, one-use plastic, disposable containers, 50 ml in capacity, may be used for a variety of specimen types. Specimens that are to be sent to the laboratory must be cushioned and sealed to prevent leakage, and properly packaged to withstand breakage or crushing in transit. The International Air Transport Association (IATA), Dangerous Goods Regulations (DGR) for shipping specimens from a suspected zoonotic disease must be followed. The requirements are summarised in Chapter 1.1.1 Collection and shipment of diagnostic specimens. Prompt delivery of specimens to the laboratory greatly enhances the chances of cultural recovery of *M. bovis*. If delays in delivery are anticipated, specimens should be refrigerated or frozen to retard the growth of contaminants and to preserve the mycobacteria. In warm ambient conditions, when refrigeration is not possible, boric acid may be added (0.5% [w/v] final concentration) as a bacteriostatic agent, but only for limited periods, no longer than 1 week.

Precautions should be taken to prevent infection of laboratory personnel (see Chapter 1.1.2 Biosafety and biosecurity in the veterinary microbiology laboratory and animal facilities). All procedures involving culture should be performed in a biological safety cabinet.

a) Microscopic examination

*Mycobacterium bovis* can be demonstrated microscopically on direct smears from clinical samples, and on prepared tissue materials. The acid fastness of *M. bovis* is normally demonstrated with the classic Ziehl–Neelsen stain, but a fluorescent acid-fast stain may also be used. Immunoperoxidase techniques may also give satisfactory results. The presumptive diagnosis of mycobacteriosis can be made if the tissue has characteristic histological lesions (caseous necrosis, mineralisation, epithelioid cells, multinucleated giant cells and macrophages). As lesions are often paucibacillary, the presence of acid-fast organisms in histological sections may not be detected although *M. bovis* can be isolated in culture.

b) Culture of *Mycobacterium bovis*

In order to process specimens for culture, the tissue is first homogenised using a pestle and mortar, stomacher or blender followed by decontamination with either detergent, acid or an alkali, such as 0.375–0.75% hexadecylpyridiumchloride (HPC), 5% oxalic acid or 2–4% sodium hydroxide. The mixture is shaken for 10 minutes at room temperature and then neutralised. When using HPC, neutralisation is not required. The suspension is centrifuged, the supernatant is discarded, and the sediment is used for culture and microscopic examination.

For primary isolation, the sediment is usually inoculated on to a set of solid egg-based media such as Lowenstein–Jensen, Coletsos base or Stonebrinks; these media should contain either pyruvate or pyruvate and glycerol. An agar-based medium such as Middlebrook 7H10 or 7H11 or blood based agar medium (10) should also be used.

Cultures are incubated for a minimum of 8 weeks (and preferably for 10–12 weeks) at 37°C with or without CO₂. The media should be in tightly closed tubes to avoid desiccation. Slopes are examined for macroscopic growth at intervals during the incubation period. When growth is visible, smears are prepared and stained by the Ziehl–Neelsen technique. Growth of *M. bovis* generally occurs within 3–6 weeks of incubation depending on the media used. *Mycobacterium bovis* will grow on Lowenstein–Jensen medium without pyruvate, but will grow less well when glycerol is added.
If gross contamination of culture media occurs, or a specimen shows a negative culture result and a positive macroscopic and histopathology result, the culture process should be repeated using the retained inocula with an alternative decontamination method. The limiting factor in isolation is often the poor quality of the samples submitted and every effort should be made to ensure that the laboratory receives good quality samples.

Growth considered to be mycobacterial is subcultured on to egg-based and/or agar-based media or into Tween albumin broth, and incubated until visible growth appears. In some laboratories, sterile ox bile is used before inoculation to facilitate the dispersion of the bacterial mass into small viable units.

Characteristic growth patterns and colonial morphology can provide a presumptive diagnosis of \textit{M. bovis}; however every isolate needs to be confirmed. It is necessary to distinguish \textit{M. bovis} from the other members of the ‘tuberculosis complex’, i.e. \textit{M. tuberculosis} (the primary cause of tuberculosis in humans), \textit{M. africanum} (occupies an intermediate phenotypic position between \textit{M. tuberculosis} and \textit{M. bovis}), and \textit{M. microti} (the ‘vole bacillus’, a rarely encountered organism).

Identification of isolates is usually carried out by determining cultural and biochemical properties. On a suitable pyruvate-based solid medium, colonies of \textit{M. bovis} are smooth and off-white (buff) in colour. The organism grows slowly at 37°C, but does not grow at 22°C or 45°C. \textit{Mycobacterium bovis} is sensitive to thiophen-2-carboxylic acid hydrazide (TCH) and to isonicotinic acid hydrazide (INH). This can be tested for by growth on 7H10/7H11 Middlebrook agar medium or on egg-containing media. The egg medium should be prepared without pyruvate because it inhibits INH and could have a similar effect on TCH (which is an analogue of INH) and thus give false-positive (resistant) results. \textit{Mycobacterium bovis} strains are also sensitive to para-aminosalicylic acid and streptomycin. Effective drug concentrations are different for egg-based and agar-based media. Results for niacin production and nitrate reduction are negative in \textit{M. bovis}. In the amidase test, \textit{M. bovis} is positive for urease and negative for nicotinamidase and pyrazinamidase. It is a microaerophilic and nonchromogenic bacterium.

Sometimes \textit{M. avium} or other environmental mycobacteria may be isolated from tuberculosis-like lesions in cattle. In such cases, a careful identification is needed, and a mixed infection with \textit{M. bovis} should be excluded. \textit{Mycobacterium tuberculosis} may sensitise cattle to bovine tuberculin without causing distinct tuberculosis lesions.

Liquid culture systems are used routinely in some hospital and veterinary laboratories. Growth is assessed by radiometric or fluorometric means.

c) Nucleic acid recognition methods

Rapid identification of isolates to the level of \textit{M. tuberculosis} complex can be made by Gen Probe TB complex DNA probe or polymerase chain reaction (PCR) targeting 16S–23S rRNA, the insertion sequences IS6110 and IS1081, and genes coding for \textit{M.-tuberculosis}-complex-specific proteins, such as MPB70 and the 38 kDa antigen b have been used. Specific identification of an isolate as \textit{M. bovis} can be made using PCR targeting a mutation at nucleotide position 285 in the \textit{oxyR} gene (15, 26, 32, 35). Alternatively molecular typing techniques such as spoligotyping will identify \textit{M. bovis} isolates and provide some molecular-typing information on the isolate that is of epidemiological value.

PCR has been widely evaluated for the detection of \textit{M. tuberculosis} complex in clinical samples (mainly sputum) in human patients and has recently been used for the diagnosis of tuberculosis in animals. A number of commercially available kits and various ‘in-house’ methods have been evaluated for the detection of the \textit{M. tuberculosis} complex in fresh and fixed tissues. Various primers have been used, as described above. Amplification products have been analysed by hybridisation with probes or by gel electrophoresis. Commercial kits and the in-house methods, in fresh, frozen or boric acid-preserved tissues, have shown variable and less than satisfactory results in interlaboratory comparisons (31). False-positive and false-negative results, particularly in specimens containing low numbers of bacilli, have reduced the reliability of this test. Variability in results has been attributed to the low copy number of the target sequence per bacillus combined with a low number of bacilli. Variability has also been attributed to decontamination methods, DNA extraction procedures, techniques for the elimination of polymerase enzyme inhibitors, internal and external controls and procedures for the prevention of cross-contamination. Improvement in the reliability of PCR as a practical test for the detection of \textit{M. tuberculosis} complex in fresh clinical specimens will require the development of standardised and robust procedures. Cross contamination is the greatest problem with this type of application and this is why proper controls have to be set up with each amplification. However, PCR is now being used on a routine basis in some laboratories to detect the \textit{M. tuberculosis} group in paraffin-embedded tissues (29, 30). Optimal results are obtained when both direct PCR and isolation methods are used.
Genetic fingerprinting allows laboratories to distinguish between different strains of *M. bovis* and will enable patterns of origin, transmission and spread of *M. bovis* to be described. The most widely used method is spoligotyping (from ‘spacer oligotyping’), which allows the differentiation of strains inside each species belonging to the *M. tuberculosis* complex, including *M. bovis*, and can also distinguish *M. bovis* from *M. tuberculosis* (25). Other techniques that may be more definitive include restriction endonuclease analysis (REA), restriction fragment length polymorphism (RFLP) using IS6110 probe (especially where there are >3–4 copies of IS6110 in the isolate), the direct repeat (DR) region probe and the PGRS (polymorphic GC repeat sequence) probe (40). RFLP using a combination of the DR and pUCD probes (33) and characterisation of the VNTR profile (variable number tandem repeat) (13, 14, 18, 27) have recently been evaluated. Often a combination of techniques may be used to gain the maximum discrimination between strains (11).

The genome of *M. bovis* has been sequenced and this information has contributed to improved methods of genetic fingerprinting.

2. Delayed hypersensitivity test

- **The tuberculin test (the prescribed test for international trade)**

  In the past, heat-concentrated synthetic medium (HCSM) tuberculin was used, but, in most countries, HCSM tuberculin has been replaced by purified protein derivative (PPD) tuberculin. The HCSM tuberculins can have a good potency if correctly standardised for biological activity, but their specificity is inferior to PPD tuberculins. Moreover, it has been shown that bovine PPDs prepared with the *M. bovis* production strain AN5 are more specific for detecting bovine tuberculosis than human PPDs prepared with *M. tuberculosis*.

  The standard method for detection of bovine tuberculosis is the tuberculin test, which involves the intradermal injection of bovine tuberculin PPD and the subsequent detection of swelling (delayed hypersensitivity) at the site of injection 3 days later. This may be performed using bovine tuberculin alone or as a comparative test using avian and bovine tuberculins. The tuberculin test is usually performed on the mid-neck, but the test can also be performed in the caudal fold of the tail. The skin of the neck is more sensitive to tuberculin than the skin of the caudal fold. To compensate for this difference, higher doses of tuberculin may be used in the caudal fold.

  Delayed hypersensitivity may not develop for a period of 3–6 weeks following infection. Thus, if a herd/animal is suspected to have been in contact very recently with infected animals, delaying testing should be considered in order to reduce the probability of false-negatives. As the sensitivity of the test is less than 100%, it is unlikely that eradication of tuberculosis from a herd will be achieved with only a single tuberculin test. It should be recognised that when used in chronically infected animals with severe pathology, the tuberculin test may be unresponsive.

  The comparative intradermal tuberculin test is used to differentiate between animals infected with *M. bovis* and those sensitised to bovine tuberculin as a result of exposure to other mycobacteria. This sensitisation can be attributed to the antigenic cross-reactivity among mycobacterial species and related genera. The test involves the intradermal injection of bovine tuberculin and avian tuberculin into different sites, usually on the same side of the neck, and measuring the response 3 days later.

  The potency of tuberculins must be estimated by biological methods, based on comparison with standard tuberculins, and potency is expressed in International Units (IU). In several countries, bovine tuberculin is considered to be of acceptable potency if its estimated potency guarantees per bovine dose at least 2000 IU (±25%) in cattle. In cattle with diminished allergic sensitivity, a higher dose of bovine tuberculin is needed, and in national eradication campaigns doses of up to 5000 IU are recommended. The volume of each injection dose must not exceed 0.2 ml.

- **Test procedure**

  i) A correct injection technique is important. The injection sites must be clipped and cleansed. A fold of skin within each clipped area is measured with callipers and the site marked prior to injection. A short needle, bevel edge outwards and graduated syringe charged with tuberculin attached, is inserted obliquely into the deeper layers of the skin. The dose of tuberculin is then injected. The dose of tuberculin injected must be no lower than 2000 International Units (IU) of bovine or avian tuberculin. A correct injection is confirmed by palpating a small pea-like swelling at each site of injection. The distance between the two injections should be approximately 12–15 cm. In young animals in which
There is no room to separate the sites sufficiently on one side of the neck, one injection must be made on each side of the neck at identical sites in the centre of the middle third of the neck. The skin-fold thickness of each injection site is re-measured 72 hours after injection. The same person should measure the skin before the injection and when the test is read.

ii) A number of alternative methods of interpreting the skin test responses have been adopted, recognising that false-positive reactions may be caused by sensitisation by other mycobacteria and by local inflammation. It is important to recognise that there is a balance between sensitivity and specificity and achieving high concurrent values may not be possible. Appropriate policies need to be in place depending on disease prevalence and according to risk (e.g. where a wildlife reservoir is present). The interpretation is based on observation and the recorded increases in skin-fold thickness. In the single intradermal test (which requires a single injection of bovine tuberculin), the reaction is commonly considered to be negative if only limited swelling is observed, with an increase of no more than 2 mm and without clinical signs, such as diffuse or extensive oedema, exudation, necrosis, pain or inflammation of the lymphatic ducts in that region or of the lymph nodes. The reaction is considered to be inconclusive if none of these clinical signs is observed and if the increase in skin-fold thickness is more than 2 mm and less than 4 mm. The reaction is considered to be positive if clinical signs, as mentioned above, are observed or if there is an increase of 4 mm or more in skin-fold thickness. Moreover, in M.-bovis-infected herds, any palpable or visible swelling should be considered to be positive. Sometimes a more stringent interpretation is used, particularly in a high risk population or in contact animals. Animals that are inconclusive by the single intradermal test should be subjected to another test after an interval of 42 days to allow desensitisation to wane (in some areas 60 days for cattle and 120 days for deer are used). Animals that are not negative to this second test should be deemed to be positive. Animals that are positive to the single intradermal test may be subjected to a comparative intradermal test or blood test. Any retest should be performed in accordance with the local or national control programmes standard.

iii) In the interpretation of the intradermal comparative test, a reaction is usually considered to be positive if the increase in skin thickness at the bovine site of injection is more than 4 mm greater than the reaction shown at the site of the avian injection. The reaction is considered to be inconclusive if the increase in skin thickness at the bovine site of injection is from 1 to 4 mm greater than the avian reaction. The reaction is considered to be negative if the increase in skin thickness at the bovine site of injection is less than or equal to the increase in the skin reaction at the avian site of injection. This interpretation scheme is used in European Union (EU) countries and is recommended in Council Directive 64/432/EEC (16). Sometimes a more stringent interpretation is used.

iv) In the caudal fold test, a short needle, bevel edge outwards, is inserted obliquely into the deeper layers of the skin on the lateral aspect of the caudal fold, midway along the fold and midway between the hairline and the ventral aspect of the fold. The standard interpretation is that any palpable or visible change is deemed to be a reaction. A modified interpretation is also in use: a positive test is any palpable or visible swelling at the site of the injection that has a caudal fold thickness difference of 4 mm when compared with the thickness of the opposite caudal fold. If an animal has only one caudal fold, it is considered to be test positive if the caudal fold thickness is 8 mm or more.

3. Blood-based laboratory tests

Besides the classical intradermal tubercul in test, a number of blood tests have been used (22). Due to the cost and the more complex nature of laboratory-based assays, they are usually used as ancillary tests to confirm or negate the results of an intra-dermal skin test. There is also evidence that when an infected animal is skin tested, an enhanced blood test occurs. This allows for better separation of in-vitro blood test responses leading to greater test accuracy. The gamma-interferon assay and the lymphocyte proliferation assay measure cellular immunity, while the ELISA measures humoral immunity.

a) Gamma-interferon assay

In this test, the release of a lymphokine gamma interferon (IFN-γ) is measured in a whole-blood culture system. The assay is based on the release of IFN-γ from sensitised lymphocytes during a 16–24-hour incubation period with specific antigen (PPD-tuberculin). The test makes use of the comparison of IFN-γ production following stimulation with avian and bovine PPD. The quantitative detection of bovine IFN-γ is carried out with a sandwich ELISA that uses two monoclonal antibodies to bovine gamma-interferon. It is recommended that the blood samples be transported to the laboratory and the assay set up as soon as possible, and within 8–12 hours of collection. In some areas, especially where ‘nonspecificity’ is prevalent, some concerns about the accuracy have been expressed. However, because of the IFN-γ test capability of detecting early infections, the use of both tests in parallel allows detection of a greater number of infected animals before they become a source of infection for other animals as well as a source of contamination of the environment (19). The use of defined mycobacterial antigens such as ESAT 6 and CFP-10 promise to improve specificity (5). The use of such antigens may also offer the ability to differentiate vaccinated from unvaccinated animals. In animals that are difficult or dangerous to handle, such as excitable cattle or other
bovidae, the advantage of the IFN-γ test over the skin test is that the animals need be captured only once. The IFN-γ test has been approved for use in a number of national programmes including in the USA, New Zealand and Australia. In New Zealand, the IFN-γ test is used for serial (to enhance specificity) and parallel testing (to enhance sensitivity). Where the IFN-γ is used as a serial test, blood samples can be submitted to the laboratory up to 28 hours after collection (37).

b) Lymphocyte proliferation assay

This type of in-vitro assay compares the reactivity of peripheral blood lymphocytes to tuberculin PPD-B and a PPD from Mycobacterium avium (PPD-A). The assay can be performed on whole blood (5) or purified lymphocytes from peripheral blood samples (20). These tests endeavour to increase the specificity of the assay by removing the response of lymphocytes to ‘nonspecific’ or cross-reactive antigens associated with non-pathogenic species of mycobacteria to which the animal may have been exposed. Results are usually analysed as the value obtained in response to PPD-B minus the value obtained in response to PPD-A. The B–A value must then be above a cut-off point that can be altered in order to maximise either specificity or sensitivity of the diagnosis. The assay has scientific value, but is not used for routine diagnosis because the test is time-consuming and the logistics and laboratory execution are complicated (it requires long incubation times and the use of radio-active nucleotides). As with the IFN-γ test, the lymphocyte proliferation assay should be performed shortly after blood is collected. The test may be useful in wildlife and zoo animals. A blood test comprising lymphocyte transformation assays and ELISA has been reported to have a high sensitivity and specificity in diagnosis of M. bovis infection in deer (20). The test is relatively expensive and has not yet been subject to inter-laboratory comparisons.

c) Enzyme-linked immunosorbent assay

There have been numerous unsuccessful attempts to develop clinically useful serodiagnostic tests for tuberculosis. The ELISA appears to be the most suitable of the antibody detection tests and can be a complement, rather than an alternative, to tests based on cellular immunity. It may be helpful in anergic cattle and deer. An advantage of the ELISA is its simplicity, but both specificity and sensitivity are limited in cattle, mostly due to the late and irregular development of the humoral immune response in cattle during the course of the disease. The antibody response in deer however seems to develop earlier and more predictably and the sensitivity of a comparative ELISA has been reported to be as high as 85% in this species (21). Improvement may be possible by using different antigens, including proteins (e.g. MPB 70, which is very specific but lacks sensitivity). Moreover, in M.-bovis-infected animals, an anamnestic rise has been described, resulting in better ELISA results 2–8 weeks after a routine tuberculin skin test. A comparison of antibody levels to PPD-B and PPD-A has also been shown to be useful in increasing specificity in the ELISA (21). The ELISA may also be useful for detecting M. bovis infections in wildlife. In New Zealand, the ELISA is approved as an ancillary parallel test for farmed deer, carried out 13–33 days after the mid-cervical skin test (20).

C. REQUIREMENTS FOR VACCINES AND DIAGNOSTIC BIOLOGICALS

At present the only available vaccine against M. bovis infections is bacille-Calmette-Guerin (BCG), which is a live attenuated strain of M. bovis. This has shown variable efficacy in cattle trials, which may be attributable to various factors including vaccine formulation, route of vaccination, and the degree of exposure to environmental mycobacteria (39). Trials have been conducted on a number of other vaccines, but none has been shown to induce a superior protection to BCG. The efficacy of BCG has been shown to vary in a similar manner to that reported for humans. A number of new candidate vaccines are currently being tested. The DNA of the tuberculosis organism is now being studied in detail and the entire genome sequence has been published. This may be particularly useful in identifying genes associated with virulence and in advancing towards a DNA vaccine. In infected countries where there is no test and slaughter control scheme, BCG vaccination may be used to reduce the spread of infection in cattle. Before embarking on a vaccination programme, the vaccination schedule must be optimised for local conditions. Typical dosage would be from 10^4 to 10^6 colony-forming units given subcutaneously. Vaccine should be based on the standard reference strain, BCG Pasteur or Danish (43). It is important to recognise that use of vaccine will compromise tuberculin skin tests or other immunological tests. Cattle vaccination should not therefore be used in countries where control or trade measures based on such testing are in operation. BCG vaccines may also be used to reduce spread of M. bovis in wildlife reservoirs of infection. Prior to using the vaccine, it is essential to validate the delivery system for the particular wildlife species. The environmental impact of the vaccine on other wildlife species must also be considered.

Guidelines for the production of veterinary vaccines are given in Chapter 1.1.8 Principles of veterinary vaccine production. The guidelines given here and in Chapter 1.1.8 are intended to be general in nature and may be supplemented by national and regional requirements.
Tuberculins were preparations made from the heat-treated products of growth and lysis of *M. tuberculosis* or *M. bovis* (known as human and bovine tuberculins, respectively). At the beginning the culture medium used for their production was glycerol broth. In the 1940s, the ‘heat-concentrated synthetic medium tuberculins’ or HCSM tuberculins, prepared from cultures in a synthetic liquid medium, replaced the ‘old’ tuberculins. Currently, both the old and HSCM tuberculins have been replaced, almost world-wide, with the purified protein derivatives or PPD.

- **Production of tuberculin**
  
  1. **Seed management**
     
    a) **Characteristics of the seed**
    
    Strains of *M. bovis* used to prepare seed cultures must be identified as to species by appropriate tests. A record must be kept of their origins and subsequent history. Seed cultures must not be passaged more than five times. The production strains *M. bovis* AN5 or Vallee are the most commonly used.

    b) **Method of culture**
    
    If the source culture was grown on solid medium, it is necessary to adapt the organism to grow as a floating culture (e.g. by incorporating a sterile piece of potato in the culture flasks of liquid media, such as Watson Reid’s medium). When the culture has been adapted to liquid medium, it may be used to produce the master seed lot, which is preserved in freeze-dried form. This is used to inoculate media for the production of the secondary seed lots, which must not be more than four culture passages from the master seed. The secondary seed is used to inoculate production cultures (1, 23).

    The production culture substrate must be shown to be capable of producing a product that conforms to recognised international standards (World Health Organization [WHO], European Pharmacopoeia or other recognised control authorities). It must be free from ingredients known to cause toxic or allergic reactions.

    c) **Validation**
    
    The strains of *M. bovis* used as seed cultures must be shown to be free from contaminating organisms.

    Seed lots must be shown to be efficacious in producing tuberculin with sufficient potency. The necessary tests are described in Section C.4 below.

  2. **Method of manufacture**

    The organism is cultured in a synthetic medium, the protein in the filtrate is precipitated chemically (ammonium sulphate or trichloroacetic acid [TCA] are used), then washed and resuspended. PPD tuberculin is recommended as it can be standardised more precisely.

    An antimicrobial preservative that does not give rise to false-positive reactions, such as phenol (not more than 0.5% [w/v]), may be added. Glycerol (not more than 10% [w/v]) or glucose (2.2% [w/v]) may be added as a stabiliser. Mercurial derivatives should not be used. The product is also dispensed aseptically into sterile, neutral glass containers, which are sealed so as to preclude contamination. The product may be freeze-dried.

  3. **In-process control**

    The production flasks, inoculated from suitable seed cultures, are incubated for the appropriate time period. Any flasks showing contamination or grossly abnormal growth should be discarded after autoclaving.

    As incubation proceeds, the surface growth of many cultures becomes moist and may sink into the medium or to the bottom of the flask.

    In PPD tuberculins, the pH of the dissolved precipitate (the so-called concentrated tuberculin) should be 6.6–6.7.

    The protein level of the PPD concentrate is determined by the Kjeldahl or other suitable method. Total nitrogen and TCA precipitable nitrogen are usually compared.

    The final product should be bioassayed in guinea-pigs. Potency and specificity assays are carried out in comparison with a reference tuberculin (PPD). Further dilutions are made with a buffer according to the protein content and the required final concentration, usually 1.0 mg/ml (1, 23).
4. Batch control

Samples should comply with the officially recognised standards for the production of tuberculin as set out in the European Pharmacopoeia or equivalent regulatory standards.

a) Sterility

Sterility testing is generally performed according to international guidelines (see also Chapter 1.1.9 Tests for sterility and freedom from contamination of biological materials).

b) Safety

Two guinea-pigs, each weighing not less than 250 g and that have not been treated previously with any material that will interfere with the test, are injected subcutaneously with 0.5 ml of the tuberculin under test. No abnormal effects should occur within 7 days.

Tests on tuberculin for living mycobacteria may be performed either on the tuberculin immediately before it is dispensed into final containers or on samples taken from the final containers themselves. A sample of at least 10 ml must be taken and this must be injected intraperitoneally into at least two guinea-pigs, dividing the dose between them. It is desirable to take a larger sample, such as 50 ml, and to concentrate any residual mycobacteria by centrifugation or membrane filtration. The guinea-pigs are observed for at least 42 days and are then examined macroscopically at post-mortem. Any lesions found are examined microscopically and by culture.

c) Sensitising effect

To test the sensitising effect, three guinea-pigs that have not been treated previously with any material that could interfere with the test are injected intradermally on each of three occasions with the equivalent of 500 IU of the preparation under test in a 0.1 ml volume. Each guinea-pig, together with each of three control guinea-pigs that have not been injected previously, is injected intradermally 15–21 days after the third injection with the same dose of the same tuberculin. The reactions of the two groups of guinea-pigs should not be significantly different when measured 24–28 hours later.

d) Potency

Potency is determined by comparison with a reference preparation of bovine tuberculin in guinea-pigs sensitised with M. bovis.

In the 1970s, countries in the then European Economic Community (EEC, now the EU) recognised a standard for bovine HCSM tuberculins. This EEC standard for bovine HCSM has a potency of 65,000 provisional Community tuberculin units per ml.

As early as the 1960s, the EEC recognised an EEC standard for bovine PPD, which was given a potency of 50,000 provisional Community tuberculin units per mg of PPD, and was dispensed in the lyophilised state. Unfortunately, the number of freeze-dried ampoules was not sufficient for the WHO's requirements and therefore it was decided to produce a new bovine PPD preparation that could be designated by the WHO as the new international standard for bovine PPD tuberculins.

This new bovine PPD standard had to be calibrated against the existing EEC standard. Based on international collaborative assays, both in guinea-pigs and cattle, it was found that the new bovine standard had a relative potency of 65% against the EEC standard. Therefore, in 1986, the WHO officially gave the international standard for bovine PPD tuberculins a unitage of 32,500 IU/mg. This means that the provisional Community tuberculin units are equipotent with the IUs. The European Pharmacopoeia has also recognised the WHO international standard for bovine PPD.

In order to save the stock of the actual international standard, it is desirable that the countries where bovine PPD tuberculin is produced, establish their own national reference preparations for bovine PPD as working standards. These national reference preparations must have been calibrated against the official international standard for bovine PPD, both in guinea-pigs and cattle (28, 38, 42).

• Standardisation in guinea-pigs

The guinea-pigs are sensitised with a low dose (e.g. 0.001 or 0.0001 mg wet weight) of live bacilli of a virulent strain of M. bovis 5–7 weeks prior to the assay. The bacilli are suspended in physiological saline, and a deep intramuscular injection of 1 ml is made on the medial side of the thigh. At the time of the assay,
the guinea-pigs infected with the low dose of *M. bovis* should still be in good health and the results of numerous post-mortem examinations carried out shortly after the standardisation assays should show that the guinea-pigs do not suffer from open tuberculosis and thus are not excreting tubercle bacilli.

An alternative, but less reliable, potency test can be used that does not use live pathogenic mycobacteria and is more suitable for laboratories that do not have isolation areas for safe housing of infected guinea-pigs. This tuberculin potency test is performed as follows: the PPD tuberculin is bioassayed in homologously sensitised guinea-pigs against the standard for bovine PPD tuberculin by an eight-point assay comprising four dilutions corresponding to about 20, 10, 5 or 2.5 IU. The injection volume is 0.1 ml. In this assay, two test tuberculins are compared with standard tuberculin in eight guinea-pigs, applying eight intradermal injections per animal and employing a Latin square design. The guinea-pigs are sensitised with inactivated bacilli of *M. bovis*, 5–7 weeks before the assay. The bacilli are suspended in buffer and made into an emulsion with Freund’s incomplete adjuvant. A deep intramuscular injection is made on the medial side of the thigh, using a dose of 0.5 ml.

A suitable assay for potency is as follows: The produced PPD tuberculins are bioassayed in homologously sensitised guinea-pigs against the standard for bovine PPD tuberculin by a six-point assay comprising three dilutions at five-fold intervals of each tuberculin. The dilutions of the tuberculin preparations are made in isotonic buffer solution containing 0.0005% (w/v) polysorbate 80 (Tween 80). Volumes of 0.001, 0.0002 and 0.00004 mg tuberculoprotein corresponding to the international standard for PPD of 32, 6.4 and 1.28 IU, respectively, are chosen because these amounts give good readable skin reactions with acceptable limits. The injection volume is 0.2 ml. In one assay, two test tuberculins are compared with the standard tuberculin in nine guinea-pigs, applying eight intradermal injections per animal and employing a balanced incomplete Latin square design (17).

Normally, the reading of the assays is done 24 hours after the injection of the tuberculins, but a second additional reading can be performed after 48 hours. The different diameters of erythema are measured with callipers in millimetres and recorded on assay sheets. The results are statistically evaluated using standard statistical methods for parallel-line assays according to Finney (17). The relative potencies of the two test tuberculins are calculated with their 95% confidence limits, the slopes of the log dose–response curves for each preparation (increase in mean reaction per unit increase in log dose) and the F ratios for deviations from parallelism.

According to the European Pharmacopoeia, the estimated potency for bovine tuberculins must be not less than 66% and not more than 150% of the potency stated on the label.

- **Standardisation of bovine tuberculin in cattle**

According to WHO Technical Report Series No. 384, potency testing should be performed in the animal species and under the conditions in which the tuberculins will be used in practice (42). This means that bovine tuberculins should be assayed in naturally infected tuberculous cattle. As this requirement is difficult to accomplish, routine potency testing is conducted in guinea-pigs. However, periodic testing in tuberculous cattle is necessary and standard preparations always require calibration in cattle. The frequency of testing in cattle can be reduced if it is certain that the standard preparations are representative of the routine issue tuberculins and that the production procedures guarantee consistency.

A suitable potency assay for bovine tuberculins in cattle is as follows: The test tuberculins are assayed against a standard for bovine PPD tuberculin by a four-point assay using two dilutions at five-fold intervals of each tuberculin. For the standard, 0.1 and 0.02 mg of tuberculoprotein are injected as these volumes correspond with about 3250 and 650 IU if the international standard for bovine PPD tuberculin is used. The test tuberculins are diluted in such a way that the same weights of protein are applied. The injection volume is 0.1 ml, and the distance between the middle cervical area injection sites is 15–20 cm. In one assay, three test tuberculins are compared with the standard tuberculin in eight tuberculous cattle, applying eight intradermal injections per animal in both sides of the neck, and employing a balanced complete Latin square design. The thickness of the skin at the site of each injection is measured with callipers in tenths of a millimetre, as accurately as possible before and 72 hours after injection (24).

The results are statistically evaluated using the same standard methods for parallel-line assays as employed in the potency tests in guinea-pigs.

e) **Specificity**

A suitable assay for specificity is as follows: three bovine test tuberculins are assayed against the standard for avian PPD tuberculin (or three avian test tuberculins against the standard for bovine PPD tuberculin) by a four-point assay in heterologously sensitised guinea-pigs, comprising two dilutions at 25-fold intervals of each tuberculin. Quantities of 0.03 mg and 0.0012 mg of test tuberculoprotein, corresponding to approximately 1500 and 60 IU, are chosen because these doses give good readable skin reactions. The injection doses of the standard are lower, namely 0.001 mg and 0.0004 mg. In one assay, three test
tuberculins are compared with the standard tuberculin in eight guinea-pigs by applying eight intradermal injections per animal and employing a balanced complete Latin square design. The reading of the results and the statistical evaluation are identical with the potency test.

f) Stability
Provided the tuberculins comply with the legislative standards required for production and are stored at a temperature of between 2°C and 8°C and protected from light, they may be used up to the expiry date as specified in the licence for production of tuberculin. For long-term storage, it is recommended to keep the PPD in a concentrated form rather than the diluted form and the concentrate should also be stored in the dark.

g) pH control
The pH should be between pH 6.5 and 7.5.

h) Protein content
The protein content is determined as indicated in Section C.3 In-process control.

i) Storage
During storage, liquid bovine tuberculin should be protected from light and held at a temperature of 5±3°C. Freezing of the liquid product may compromise the quality. However, freeze-dried preparations can be prepared and they may be stored at higher temperatures (but not exceeding 25°C) and protected from light. Periods of exposure to higher temperatures or to direct sunlight should be kept to a minimum.

j) Preservatives
Antimicrobial preservatives or other substances that may be added to a tuberculin must have been shown not to impair the safety and effectiveness of the product.

The maximum permitted concentration for phenol is 0.5% (w/v), and for glycerol it is 10% (v/v).

k) Precautions (hazards)
Experience both in humans and animals led to the observation that appropriately diluted tuberculin, injected intradermally, results in a localised reaction at the injection site without generalised manifestations. Even in very sensitive individuals, severe, generalised reactions are extremely rare and limited. But experience has shown that a hypersensitive operator can acquire severe generalised signs after accidental intradermal contact (needle stab-wound) with bovine tuberculin. These individuals should be advised not to carry out the tuberculin skin test with the high dose of 2000–5000 IU tuberculin, which is about 1000 times the normal human dose of 5 IU.

5. Tests on the final product

a) Safety
A test for the absence of toxic or irritant properties must be carried out (see Section C.4.b).

b) Potency
The potency of tuberculins must be estimated by biological methods. These methods must be used for HCSM and PPD tuberculins; they are based on comparison of the tuberculins to be tested with a standard reference preparation of tuberculin of the same type (see also Section C.4.d).

REFERENCES


* * *

**NB:** There are OIE Reference Laboratories for Bovine tuberculosis (see Table in Part 3 of this *Terrestrial Manual* or consult the OIE Web site for the most up-to-date list: www.oie.int).
CHAPTER 2.4.8

BOVINE VIRAL DIARRHOEA

SUMMARY

Cattle of all ages are susceptible to infection with bovine viral diarrhoea virus (BVDV) (see also Chapter 4.3 in the Terrestrial Animal Health Code). Distribution of the virus is world-wide. The clinical signs range from subclinical to the fulminating fatal condition called mucosal disease. Acute infections may result in transient diarrhoea or pneumonia, usually in the form of group outbreaks. Acute forms of the disease associated with high mortality have also been described, often, but not always, associated with a haemorrhagic syndrome. However, most infections in the young calf are mild and go unrecognised clinically. The virus spreads mainly by contact between cattle. Vertical transmission plays an important role in its epidemiology and pathogenesis.

Infections of the bovine fetus may result in abortions, stillbirths, teratogenic effects or persistent infection in the neonatal calf. Persistently viraemic animals may be born as weak, unthrifty calves or may appear as normal healthy calves and be unrecognised clinically. Some of these animals may later develop mucosal disease with anorexia, gastrointestinal erosions, and profuse diarrhoea, leading invariably to death. Mucosal disease can arise only in persistently infected animals.

It is important to avoid the trade of viraemic animals. It is generally considered that serologically positive, nonviraemic cattle are ‘safe’, providing that they are not pregnant. Antibody-positive pregnant cattle carrying persistently infected fetuses are important transmitters of the virus between herds. About 15% of persistently viraemic animals have antibody to the NS/2 protein and a lower percentage to the E2 glycoprotein. Therefore, seropositivity cannot be equated with ‘safety’. Latent infections are not generally thought to occur following recovery from acute infection, though semen from acutely infected animals and, very rarely, recovered animals may be suspect.

Identification of the agent: BVDV is a pestivirus in the Flaviviridae and is closely related to classical swine fever and ovine Border disease viruses. BVDV occurs in two forms: noncytopathogenic and cytopathogenic. There are two antigenically distinct genotypes (types 1 and 2), and virus isolates within these groups exhibit considerable biological and antigenic diversity.

Persistently viraemic healthy animals resulting from congenital infection can be readily identified by isolation of noncytopathogenic virus in cell cultures from blood or serum. It is necessary to use an immune-labelling method to detect the growth of virus in the cultures. Alternative methods based on direct detection of viral antigen or viral RNA in leukocytes are also available. Persistence of virus should be confirmed by resampling after an interval of at least 3 weeks. These animals will usually have no or low levels of antibodies to BVDV.

Viraemia in acute cases is transient and can be difficult to detect. In fatal cases of haemorrhagic disease, virus can be isolated from tissues post-mortem. Confirmation of mucosal disease can be made by isolation of the cytopathogenic biotype of BVDV, particularly from intestinal tissues. Noncytopathogenic virus may also be detected, especially in blood.

Serological tests: Acute infection with BVDV is best confirmed by demonstrating seroconversion using sequential paired samples from several animals in the group. The testing of paired (acute and convalescent samples) should be done a minimum of 21 days apart and samples should be tested side by side. The enzyme-linked immunosorbent assay for antibody and the virus neutralisation test are the most widely used.

Requirements for vaccines and diagnostic biologicals: There is no standard vaccine for BVD, but a number of commercial preparations are available. Modified live virus vaccine should not be
Chapter 2.4.8. – Bovine viral diarrhoea

administered to pregnant cattle (or to their sucking calves) due to the risk of transplacental infection. There is also a risk of inducing mucosal disease in persistently infected animals. Killed virus vaccines generally require booster vaccinations. An ideal vaccine should be able to prevent transplacental infection in pregnant cows.

BVDV is a particularly important hazard to embryo transfer and the manufacture of biological products for other diseases due to the high frequency of contamination of batches of fetal calf serum used as a culture medium supplement dams subject to embryo transfer, which makes use of this material, may be at risk of infection.

A. INTRODUCTION

Bovine viral diarrhoea virus (BVDV) is a pestivirus in the family Flaviviridae and is closely related to classical swine fever and ovine Border disease viruses (23). Two antigenically distinct genotypes of BVDV exist, types 1 and 2, with further subdivisions discernable by genetic analysis (74). The two genotypes may be differentiated from each other, and from other pestiviruses, by monoclonal antibodies (MAbs) directed against the E2 and ENS major glycoproteins, or by genetic analysis (65, 68). Multiplex polymerase chain reaction (PCR) enables virus typing direct from blood samples (33). Type 1 virus is generally more common although the prevalence of type 2 is reported to be almost as high as type 1 in North America. BVDV of both genotypes may occur in noncytopathogenic and cytopathogenic forms (biotypes), classified according to whether or not it produces visible change in cell cultures. Usually, it is the noncytopathogenic biotype that circulates in cattle populations. Each biotype has a specific role in a variety of clinical syndromes – acute, congenital and chronic infections (5, 11). Type 2 viruses are usually noncytopathogenic and have been associated with outbreaks of severe acute infection and a haemorrhagic syndrome (16). However recent type 2 viruses isolated in the United Kingdom have been associated with a disease indistinguishable from that seen with the more frequently isolated type 1 viruses. Some type 1 isolates have been associated with particularly severe and fatal disease outbreaks in adult cattle (20) clinically mild and inapparent infections are common with both genotypes.

Although ubiquitous, control of BVDV can be achieved at the herd level, and even at the national level, as evidenced by the progress towards eradication made in many European countries (56).

B. DIAGNOSTIC TECHNIQUES

a) Acute infections

Acute infections of cattle occur particularly in young animals, and may be clinically inapparent or associated with diarrhea (1). Affected animals may be predisposed to secondary infections, for example those leading to shipping disease, perhaps due to an immunosuppressive effect of the virus. Bulls may suffer a temporary depression of fertility and can show transient shedding of virus in the semen (62). Cows may also suffer from infertility, likely associated with changes in ovarian function (35) and secretions of gonadotrophin and progesterone (30). During acute infections, a brief viraemia may be detectable and nasal shedding of virus may occur. There may also be a transient leukopenia, thrombocytopenia or temperature response, but these can vary greatly among animals. A serological response is the most certain means of diagnosing a previous infection. The clinical picture is generally one of high morbidity and low mortality, though more severe disease is sometimes seen (12). In particular, outbreaks of a severe form of acute disease with haemorrhagic lesions, thrombocytopenia and high mortality have been reported sporadically from some countries (1, 6) and infection with Type 2 viruses in particular has been demonstrated to cause altered platelet function (76). Other acute outbreaks may show fever, pneumonia, diarrhea and sudden death in any age group, with haemorrhagic signs (16).

b) Congenital infection

If noncytopathogenic virus infects the bovine fetus, this may result in abortion, stillbirth, teratogenic effects or a congenital infection that persists in the neonatal calf (1, 11, 26, 55). Confirmation that an abortion is caused by BVDV is often difficult to establish (69), but virus may be isolated from fetal tissue in some cases, or viral antigen or genome may be demonstrated. An attempt should also be made to detect specific antibody in samples of fetal fluids or serum, or in the supernatant fluid from a tissue suspension. Stillbirths or teratogenic effects may be associated with an active fetal immune response to the virus during mid-to-late gestation. The dam will often have high antibody titres (>1/2000) to BVDV, which is suggestive of fetal infection and is probably due to the fetus providing the dam an extended challenge of virus (47).

Although congenital infection with BVDV often leads to abortion, it is not always recognised in the field. Infection during the first third of the gestation period can result in the abortion of a conceptus that is small
and goes unnoticed by the farmer. The cow would return to service and the failure to maintain pregnancy would be classified as an example of early embryonic death. Another possible outcome of infection is the death and subsequent resorption of fluids from the fetus that results in mummification. It is frequently observed that aborted fetuses have subcutaneous oedema and copious pleural and peritoneal effusions. There may also be congenital abnormalities that result in growth retardation and in selective central nervous system (CNS) defects, such as cerebellar hypoplasia and dysmyelination (70), and eye defects, such as cataracts and retinal atrophy. Sometimes there are skeletal defects, the most advanced of which is arthrogryposis.

Stillborn calves has been reported to be sequel to congenital infection before 150 days of gestation and the calves usually appear to be fully developed at parturition, but fail to survive. However, it has been reported, that in many cases, BVD virus cannot be isolated from these animals and they are PCR negative. If infection occurs after day 150 of pregnancy, the immune system of the fetus will be developed and infection of the fetus will usually result in an antibody response and the birth of a normal calf.

c) Persistent infection

When infections of the fetus occur before approximately 110 days of gestation and before immunocompetence, the calf may be born with a persistent infection. Identification of these animals is readily made by detection of noncytopathogenic BVDV in blood. The virus can also be identified in the skin by immunohistochemistry. Furthermore, animals with a persistent infection will also lack specific antibody, but diagnosis in the young calf, up to approximately 3 months of age, may be confused by the presence of maternal antibody to BVDV. Maternal antibody may also interfere with virus isolation. In older animals with persistent viraemia infection, low levels of antibody may be present due to their ability to seroconvert to strains of BVDV (including vaccines) ‘heterologous’ (antigenically different) from the persisting virus (12). To confirm a diagnosis of persistent infection, animals should be retested after an interval of at least 3 weeks.

There are no pathognomonic lesions in the viraemic calf. Depending on the gestational age at infection, lesions may be mediated entirely by the effects of the virus on the differentiating cells of the fetus, they may be mediated by the maturing immune system of the developing fetus, or both. The clinical signs vary from the apparently normal healthy animal to the weak, unthrifty calf that has difficulty in standing and sucking. These latter calves can show CNS defects, such as muscular tremors, incoordination and blindness. They often die within days of birth, thus contributing to the ‘weak calf syndrome’.

Approximately 1–2% of cattle within a population are persistently infected, with many viraemic animals surviving to sexual maturity and retained for breeding. Calves born to these infected dams are always persistently viraemic, and are often weak at birth and fail to thrive. Persistently viraemic animals are a continual source of infective virus to other cattle, and thus their rapid identification and removal from the herd are required. Animals being traded should first be screened for the absence of persistent BVD viraemia.

Bulls that are persistently infected usually have poor quality, highly infective semen and, as a result, reduced fertility (45, 67). All bulls used for natural or artificial insemination should be screened for persistent BVD infection. A rare event, possibly brought about by acute infection during pubescence, can result in persistent infection of the testes and thus strongly seropositive bulls (59, 75). This phenomenon has also been observed following vaccination with an attenuated virus (34). Female cattle used as embryo recipients should always test negative for BVD viraemia before first use. Donor cows that are persistently infected with BVDV also represent a potential source of infection, as oocysts without an intact zona pellucida are shown to be susceptible to infection in vitro (73). However, a limited study of two persistently infected animals revealed that the majority of oocysts were BVDV-negative (71). Embryos may also become contaminated following acute infection of the donor (3). Biological materials used for in-vitro fertilisation techniques (bovine serum, bovine cell cultures) have a high risk of contamination and should be screened for BVDV (9). Recent incidents of apparent introduction of virus via such techniques (24, 48) have highlightened this risk. It is considered essential that serum supplements used in media should be sterilised as detailed in chapter 4.4. Article 4.4.5 of the OIE Terrestrial Animal Health Code (Terrestrial Code) and outlined in Section B.1.a of this chapter. Importing countries may consider requesting additional tests to confirm sterilisation, detailed in Article 4.4.6 of the Terrestrial Code.

d) Mucosal disease

It is well established that persistently viraemic animals may later succumb to mucosal disease (11); however, cases are rare. This syndrome has been shown to be associated with the presence of the noncytopathogenic biotype, which can arise either through superinfection (5, 14), recombination between noncytopathogenic biotypes, or mutation of the persistent biotype (50). Consequently, confirmatory diagnosis of mucosal disease should include the isolation of cytopathogenic virus from affected cattle. This biotype may sometimes be isolated from blood, but it can be recovered more consistently from a variety of other tissues, in particular intestinal and Peyer’s patch tissue (17). Virus isolation is also readily
accomplished from spleen. This is easy to collect and is seldom toxic for cell culture after preparation for viral isolation. Isolation from gut samples may be difficult if autolysis has occurred; in this case suspensions from lymph nodes or tonsil should then be tested. Noncytopathogenic virus can also be detected, particularly from blood or blood-associated organs. Cryostat tissue sections from mucosal disease cases can be stained for viral antigen by immunofluorescence or immunoperoxidase labelling.

Mucosal disease is invariably fatal. Its onset may be so rapid that the first signs seen are dead or moribund animals. However, it is more common for animals to become anorexic over a period of several days, to be disinclined to move and to show signs of abdominal pain. They can develop a profuse diarrhoea and rapidly lose bodily condition. Erosions can often be seen in the mouth, particularly along the gingival margin. Lacrimation and excessive salivation occur. Generally, cases of mucosal disease are sporadic and rare.

Post-mortem examination reveals erosions in the mucosa at various sites along the gastrointestinal tract. The most noticeable are those overlying the lymphoid Peyer’s patches in the small intestine and in the ileocaecal lymph nodes. On histological examination, there is a clear demonstration of destruction of the lymphoid tissue within the gut-associated lymphoid tissue. Most of the Peyer’s patch lymphoid cells have been lysed and replaced by inflammatory cells, debris and cells from the overlying collapsed epithelium.

Severe acute BVD infection can be clinically similar to mucosal disease and confusion can arise, particularly when a number of animals are so affected. Mucosal disease can occur among cohorts of persistently infected animals when oestrus synchronisation has been carried out. Differentiation requires a careful examination of case histories and testing for antibody as well as antigen or virus among infected and any recovered animals. Seroconversion among recovered animals is indicative of acute infection, whereas two antigen or virus positive results on samples from an affected animal, taken 3 weeks apart, is diagnostic of mucosal disease. Generally, animals with mucosal disease are antibody negative, though low levels of antibody can sometimes be detected.

1. Identification of the agent (the prescribed test for international trade)

All test methods must be validated by testing on known noninfected and infected populations of cattle, including animals with low- and high-titre viraemias. Methods based on MAb-binding assays or on nucleic acid recognition must be shown to detect the full range of antigenic and genetic diversity found among BVD viruses. There are two designated OIE Reference Laboratories for BVD (see Table given in Part 3 of this Terrestrial Manual; the reference laboratories for classical swine fever could also be approached to offer advice.

a) Virus isolation

The virus may be isolated in a number of bovine monolayer cell cultures (e.g. kidney, lung, testis or turbinate). Growth of both biotypes is usually satisfactory. Noncytopathogenic BVDV is a common contaminant of fresh bovine tissue, and cell cultures must be checked for freedom from adventitious virus by regular testing (8, 28). Primary or secondary cultures can be frozen as cell suspensions in liquid nitrogen. These can then be tested over a series of passages, or seeded to other susceptible cells and checked before routine use. Such problems may be overcome by the use of continuous cell lines, which can be obtained BVD-free (8).

The fetal bovine serum that is selected for use in cell culture must also be free not only from virus, but also and of equal importance, from BVDV neutralising antibody (28). Heat treatment (56°C for 30–45 minutes) is inadequate for the destruction of BVDV in contaminated serum; irradiation at 25 kiloGrays (2.5 Mrad) is more certain. Commercial batches of fetal bovine serum mostly test positive by PCR even after the virus has been inactivated by irradiation. Where appropriate, horse serum can be substituted for bovine fetal serum, although it is often found to have poorer cell-growth-promoting characteristics.

Buffy coat cells, whole blood, washed leukocytes or serum are suitable for isolation of the virus from live animals. Maternal antibody may interfere with isolation from serum in young calves. Tissue suspensions from post-mortem cases should be prepared by standard methods. Semen can also be examined, but a blood sample from the donor bull is preferable if it can be obtained. There is a report of an atypical persistent shedding of BVDV in semen from a bull that was not viraemic (75). Raw semen is cytotoxic and must be diluted in culture medium. Extended semen can usually be inoculated directly on to cell monolayers, but may occasionally cause cytotoxicity. For these reasons, it is important to monitor the health of the cells by microscopic examination at intervals during the incubation.

There are many variations of procedure in use for virus isolation. All should be optimised to give maximum sensitivity of detection of a standard virus preparation. This may include one or more in-vitro passage(s). Conventional methods for virus isolation are used, with the addition of a final immune-labelling step (fluorescence or enzymatic) to detect growth of noncytopathogenic virus. Thus tube cultures should include
flying cover-slips, while plate cultures can be fixed and labelled directly in the plate. Examples are given below.

- **Microplate immunoperoxidase method for mass screening for virus detection in serum samples** (54)
  i) 10 µl of the serum sample is placed into each of four wells of a 96-well tissue-culture grade microplate. This is repeated for each sample. Known positive and negative controls are included.
  ii) 100 µl of a cell suspension of 150,000 cells/ml in medium without fetal calf serum (FCS) is added to all wells. *NB* the sample itself acts as the cell-growth supplement. If testing samples other than serum, use medium with 10% FCS that is free of antibodies to ruminant pestiviruses.
  iii) The plate is incubated at 37°C for 4 days, either in a 5% CO₂ atmosphere or with the plate sealed.
  iv) Each well is examined microscopically for evidence of cytopathic effect (CPE), or signs of cytotoxicity.
  v) The plate is emptied by gentle inversion and rinsed in phosphate buffered saline (PBS).
  vi) The plate is fixed as follows: the plate is dipped into a bath of 20% acetone in PBS, emptied immediately, and then transferred to a fresh bath of 20% acetone in PBS for 10 minutes. The plate is drained thoroughly and as much fluid as possible is removed by tapping and blotting. The plate is dried thoroughly for at least 3 hours at a temperature of 25–30°C (e.g. using radiant heat from a bench lamp). *NB*: the drying is part of the fixation process.
  Alternative fixation methods include paraformaldehyde or heat (see Chapter 2.8.3 Classical swine fever, Section B.2.b.viii).
  vii) The fixed cells are rinsed by adding PBS to all wells.
  viii) The wells are drained and the BVD antibody (50 µl) is added to all wells at a predetermined dilution in PBS containing 1% Tween 80 (PBST) and 5% horse serum. (Horse serum may be added to reduce nonspecific staining.) The plate is incubated at 37°C for 15 minutes.
  ix) The plate is emptied and washed three times in PBST.
  x) The plate is then drained and appropriate antispecies serum conjugated to peroxidase is added at a predetermined dilution in PBST (50 µl per well) for 15 minutes at 37°C.
  xi) The plate is emptied and washed three times in PBST.
  xii) The plate is rinsed in distilled water. All fluid is tapped out from the plate.
  xiii) Freshly prepared hydrogen peroxide substrate with a suitable chromogen, e.g. 3-amino-9-ethyl carbazole (AEC) is added. The stock solution is: AEC (0.1 g) dissolved in dimethyl formamide (15 ml). For use, the stock (0.3 ml) is added to 0.05 M acetate buffer (5 ml, pH 5.0), and then 30% H₂O₂ (5 µl is added). An alternative substrate can be made, consisting of 9 mg diaminobenzidine tetrahydrochloride and 6 mg sodium perborate tetrahydrate dissolved in 15 ml of PBS. Though the staining is not quite so intense, these chemicals have the advantage that they can be shipped by air.
  xiv) The plate is examined microscopically. Virus-positive cells show red-brown cytoplasmic staining.

- **Tube method for tissue or buffy coat suspensions, or semen samples**
  *NB*: this method can also be conveniently adapted to 24-well plastic dishes.
  i) Tissue samples are ground up and a 10% suspension in culture medium is made. This is then centrifuged to remove the debris. Raw semen is diluted 1/10 in culture medium.
  ii) Test tube cultures (with cover-slips) with newly confluent or subconfluent monolayers of susceptible bovine cells are inoculated with 0.1 ml of the sample. The culture is left to adsorb for 1 hour at 37°C.
  iii) The culture is washed with 1 ml of medium; this is then discarded and 1 ml of culture maintenance medium is added.
  iv) The culture is incubated for 4–5 days at 37°C, and examined microscopically for evidence of CPE or signs of cytotoxicity.
  v) Culture may then either be frozen and thawed for passage to fresh cultures, or the cover-slip may be removed, fixed in acetone and stained with direct immunofluorescent conjugate to BVDV. In this case, examine under a fluorescent microscope for diffuse, cytoplasmic fluorescence characteristic of pestiviruses.

Alternatively, cultures may be freeze/thaw harvested and passaged on to microtitre plates for culture and staining by the immunoperoxidase method (see section on microplate immunoperoxidase method for mass screening of serum samples above) or by the immunofluorescent method described here.
b) Enzyme-linked immunosorbent assay for antigen detection

Several methods for the enzyme-linked immunosorbent assay (ELISA) for antigen detection have been published (e.g. ref. 29) and a number of commercial kits are available. Most are based on the sandwich ELISA principle, with a capture antibody bound to the solid phase, and a detector antibody conjugated to a signal system, such as peroxidase. Both monoclonal- and polyclonal-based systems are described. The test is suitable for detection of persistently infected animals, and usually measures BVD antigen (NS2-3 or ERNS) in lysates of peripheral blood leukocytes; the new generation of antigen-capture ELISAs (ERNS capture ELISAs) is able to detect BVD antigen in blood as well as in plasma or serum samples. The best of the methods gives a sensitivity similar to virus isolation, and may be preferred in those rare cases where persistent infection is combined with seropositivity. Due to transient viraemia, the antigen ELISA appears to be less useful for virus detection in acute BVD infections.

The NS2-3 ELISA may be less effective in young calves that have had colostrum due to the presence of BVDV maternal antibodies. The reverse transcription PCR (RT-PCR) is probably the most sensitive detection method for this circumstance, but the ERNS ELISA has also been shown to be a sensitive and reliable test, particularly when used with ear-notch samples (18).

c) Immunohistochemistry

Enzyme-labelled methods are useful to detect BVDV antigen in tissue sections (77), particularly where suitable MAbs are available. It is important that the reagents and procedures used be fully validated, and that nonspecific reactivity be eliminated. For persistently infected cattle almost any tissue can be used, but particularly good success has been found with lymph nodes, thyroid gland, skin, brain, abomasum and placenta. Skin biopsies, such as ear-notch samples, have shown to be useful for in-vivo diagnosis of persistent BDV infection (17).

d) Nucleic acid detection

The RT-PCR method can be adapted to the detection of BVD viral RNA for diagnostic purposes (10, 36, 44, 46). This may have a special value where low-level virus contamination is suspected, for example in screening batches of FCS, or biological products such as vaccines (38). Caution is needed in the interpretation of results, as the detection of viral RNA does not imply per se that infective virus is present. A multiplex PCR can be used to amplify and type virus from cell culture, or direct from blood samples, by producing different sized PCR products (33). Newer methodologies incorporate the use of DNA fluorescently labelled probes, which confirm the identity of the PCR product, provide automated reading and can also differentiate pestiviruses in real time (53). Testing for virus after inoculation of cell cultures using PCR should be avoided as it may give false positive results if commercial bovine fetal serum contaminated with ruminant pestiviruses has been used in the growth medium. Primers should be selected in conserved regions of the genome, ideally the 5'-noncoding region, or the NS3 (p80 gene). Molecular tests can be prone to contamination in unskilled hands. Stringent precautions should therefore be taken to avoid DNA contamination in the test system, and rigorous controls must be mounted (see Chapter 1.1.5 Validation and quality control of polymerase chain reaction methods used for the diagnosis of infectious diseases).

The RT-PCR technique is also sensitive enough to enable the detection of persistently infected lactating cows in a herd of up to 100 animals or more, by testing the somatic cells within bulk milk (25, 66). A positive result indicates that at least one such animal is present in the milking herd. Follow-up virus isolation or antigen detection tests are required to identify the individual(s).

Viral nucleic acid in tissues can be detected by in-situ hybridisation with enzyme-linked riboprobes (22). This is a sensitive technique that can be applied to formalin-fixed paraffin-embedded tissue, thereby allowing a retrospective analysis. Extraction of nucleic acid and RT-PCR from such samples has been described in this context, also allowing phylogenetic analysis (2).

2. Serological tests

Antibody to BVDV can be detected in cattle sera by a standard virus neutralisation (VN) test or by ELISA, using one of several published methods (27, 40, 43, 63). Control positive and negative standard sera must be included in every test. These should give results within predetermined limits for the test to be considered valid. ELISA for antibody in bulk milk samples can give a useful indication of the BVD status of a herd (58). A high ELISA value of 1.0 or more absorbance units indicates a high probability of the herd having been exposed to BVDV in the recent past, most likely through one or more persistently viraemic animals being present. In contrast, a very low or negative value (≤0.2) indicates that it is unlikely that persistently viraemic animals are present. Further categorisation has been suggested for intermediate values, but this is dependent on the husbandry system in use. ELISA values have been shown to be an unreliable indicator of the presence of persistently infected animals on farms, due to differing husbandry (78), and also due to the presence of viral antigen in bulk milk, which may interfere with the antibody assay itself (60). Determination of the antibody status of a small number of young stock (9-18 months) has also been suggested as an indicator of recent exposure to BVDV (39), but these are
likewise dependent on the degree of contact between different groups of animals in the herd. Rapid ‘spot tests’ can be used for initial screening as part of BVD control and eradication schemes (49).

a) Virus neutralisation test

Because it makes the test easier to read, most laboratories use highly cytopathogenic, laboratory-adapted strains of BVDV for VN tests, although immune-labelling techniques are now available that allow simple detection of the growth or neutralisation of noncytopathogenic strains where this is considered desirable. No single strain is likely to be ideal for all circumstances, but in practice one should be selected that detects the highest proportion of serological reactions in the local cattle population. Two widely used cytopathogenic strains are ‘Oregon C24V’ and ‘NADL’. Low levels of antibody to BVD type 2 virus may not be detectable by a neutralisation test that uses type 1 strain of the virus, and vice versa (32). It is important that BVD type 1 and BVD type 2 be used in the test and not just the one that the diagnostician thinks is present, as this can lead to under reporting.

An outline protocol for a microtitre VN test is given below (27):

i) The test sera are heat-inactivated for 30 minutes at 56°C.

ii) From a starting dilution of 1/5, serial twofold dilutions of the test sera are made in a cell-culture grade flat-bottomed 96-well microtitre plate, using cell culture medium as diluent. For each sample, two or four wells are used at each dilution depending on the degree of precision required. Control positive and negative sera should also be tested.

iii) An equal volume (e.g. 50 µl) of a stock of cytopathogenic strain of BVDV containing 100 TCID₅₀ (50%) tissue culture infective dose is added to each well. A back titration of virus stock is also done in some spare wells to check the potency of the virus (acceptance limits 30–421 TCID₅₀).

iv) The plate is incubated for 1 hour at 37°C.

v) A flask of suitable cells (e.g. bovine turbinate, bovine testis) is trypsinised and the cell concentration is adjusted to 3 × 10⁵/ml. 50 µl of the cell suspension is added to each well of the microtitre plate.

vi) The plate is incubated at 37°C for 4–5 days, either in a 5% CO₂ atmosphere or with the plate sealed.

vii) The wells are examined microscopically for CPE. The VN titre for each serum is the dilution at which the virus is neutralised in 50% of the wells. This can be calculated by the Spearman–Kärber method. A seronegative animal will show no neutralisation at the lowest dilution (1/5), equivalent to a final dilution of 1/10.

b) Enzyme-linked immunosorbent assay

Both indirect and blocking types of test can be used (40, 43, 63). A number of commercial kits are available. The chief difficulty in setting up the test lies in the preparation of a viral antigen of sufficient potency. The virus must be grown under optimal culture conditions using a highly permissive cell type. Any serum used in the medium must not inhibit growth of BVDV. The optimal time for harvest should be determined experimentally for the individual culture system. The virus can be concentrated and purified by density gradient centrifugation. Alternatively, a potent antigen can be prepared by treatment of infected cell cultures with detergents, such as Nonidet P40, N-decanoyl-N-methylglucamine (Mega 10), Triton X-100 or 1-octyl-beta-D-glucopyranoside (OGP). Some workers have used fixed, infected whole cells as antigen. In future, increasing use may be made of artificial antigens manufactured by expressing specific viral genes in bacterial or eukaryotic systems (72). Such systems should be validated by testing sera specific to a wide range of different virus strains. In the future, this technology should enable the production of serological tests complementary to subunit or marker vaccines, thus enabling differentiation between vaccinated and naturally infected cattle.

An example outline protocol for an indirect ELISA is given below (27).

i) Roller cultures of secondary calf testis cells with a high multiplicity of infection (about one), are inoculated with BVDV strain Oregon C24V, overlaid with serum-free medium and incubated for 24 hours at 37°C.

ii) The cells are scraped off and pelleted. The supernatant medium is discarded. The pellet is treated with two volumes of 2% OGP in PBS for 15 minutes at 4°C, and centrifuged to remove the cell debris. The supernatant antigen is stored in small aliquots at −70°C, or freeze-dried. Non-infected cells are processed in parallel to make a control antigen.

iii) The antigen is diluted to a predetermined dilution in 0.05 M boric acid buffer, pH 9.6. Alternate rows of an ELISA-grade microtitre plate are coated with virus and control antigens overnight at 4°C. The plates are then washed in PBS with 0.05% Tween 20 or Tween 80 (PBST) before use in the test.
iv) Test sera are diluted 1/50 in serum diluent (0.5 M NaCl; 0.01 M phosphate buffer; 0.05% Tween 20; 0.001 M ethylene diamine tetra-acetic acid; 1% polyvinyl pyrrolidone, pH 7.2) and added to virus- and control-coated wells for 1 hour at 37°C. The plates are then washed five times in PBST.

v) Rabbit anti-bovine IgG peroxidase conjugate is added at a predetermined dilution (in serum diluent) for 1 hour at 37°C, then the plates are again washed five times in PBST.

vi) A suitable enzyme substrate is added, such as hydrogen peroxide/tetramethyl benzidine. After colour development, the reaction is stopped with sulphuric acid and the absorbance is read on an ELISA plate reader. The value obtained with control antigen is subtracted from the test reaction to give a net absorbance value for each serum.

vii) It is recommended to convert net absorbance values to sample:positive ratio (or percentage positivity) by dividing net absorbance by the net absorbance on that test of a standard positive serum that has a net absorbance of about 1.0. This normalisation procedure leads to more consistent and reproducible results.

C. REQUIREMENTS FOR VACCINES AND DIAGNOSTIC BIOLOGICALS

Infection via the oropharynx and respiratory tract is probably the most important route of transmission of BVDV on farms. Protection against spread in this way would have a beneficial effect on controlling disease due to the virus, particularly in the young animal. The formulation of a vaccine that will provide protection to the fetus is also required in order to prevent the wide range of syndromes that result from in utero infection (13).

A standard vaccine for protection against infection has not yet been developed, but a number of commercial preparations are available in, for example, Europe and North America. Traditionally, BVD vaccines have been based on a cytopathogenic strain of the virus and fall into two classes: modified live virus or inactivated vaccines.

Although live virus vaccines are available in some countries, they should be used under careful veterinary control because a cytopathogenic strain may precipitate mucosal disease by superinfection of persistently viraemic animals, while in pregnant cattle, a noncytopathogenic component of the vaccine may cross the placenta and infect the fetus as described in Section B.b. Live virus vaccine may also be immunosuppressive and precipitate other infections. On the other hand, modified live virus vaccines may only require a single dose. Properly constituted vaccines containing killed virus are safe to use but, to obtain satisfactory levels of immunity, they usually require booster vaccinations, which may be inconvenient. A combined vaccination protocol using inactivated followed by live vaccine may reduce the risk of adverse reaction to the live strain (31).

Experimental inactivated vaccines based on baculovirus-expressed BVD viral glycoprotein E2 have been described. They offer a future prospect of ‘marker vaccines’ when used in connection with a complementary serological test (15). However, it should be noted that such vaccines for the closely related classical swine fever virus have not proven so effective, probably because of their inability to induce a strong cell-mediated immune response.

BVDV is particularly important as a hazard in the manufacture of biological products for other diseases because of the high frequency of contamination of batches of FCS used as a culture medium supplement (38). Particular attention should be paid to sera designed for administration to animals, or used as a growth supplement in embryo transfer or in-vitro fertilisation procedures. Serum used for such purposes should be treated so as to assure sterility. It is recommended that post-treatment tests, such as are detailed in Chapter 1.1.9 Tests for sterility and freedom from contamination of biological materials, be used to ensure that serum is free of BVDV.

Guidelines for the production of veterinary vaccines are given in Chapter 1.1.8 Principles of veterinary vaccine production. The guidelines given here and in Chapter 1.1.8 are intended to be general in nature and may be supplemented by national and regional requirements.

1. Seed management

a) Characteristics of the seed

An ideal vaccine should contain a strain (or strains) of virus that has been shown to give protection against the wide diversity of antigenicity that has been demonstrated by BVDV. A good appreciation of the antigenic characteristics of individual strains can be obtained by screening with panels of MAb (64). The identity of the seed virus should be confirmed by sequencing (68).

The emergence of genotype 2 BVD has raised questions regarding the degree of protection conferred by type 1 vaccines against genotype 2. An in-vitro study of the neutralising ability of sera induced by one
vaccine revealed broad reactivity with diverse strains from Europe and the USA, including type 2 strains (37). Other work has shown that vaccine derived from one genotype can afford a degree of protection from the other (19, 21, 52). However, the efficacy of vaccination of whatever genotype, particularly with a killed vaccine, in preventing transplacental transmission is less predictable, as viraemia is rarely completely prevented.

Isolates of cytopathic virus are often mixed with the noncytopathic biotype. The separation and purification of the two biotypes from an initial mixed culture depends on either three cycles of a limiting dilution technique for the noncytopathogenic virus, or three cycles of plaque selection for the cytopathogenic virus. Purity of the cytopathogenic virus should be confirmed by at least one additional passage at limiting dilution. When isolates have been cloned, their identity should be confirmed by direct or indirect staining with specific antibody linked to fluorescein or enzyme.

b) Method of culture

Both biotypes will grow in a variety of cell cultures of bovine origin. Standard procedures may be used, with the expectation for harvesting noncytopathogenic virus on days 5–7 and cytopathogenic virus on days 2–4. The details for optimal yield depend on several factors, including the cell culture and isolate used and the initial seeding rate of virus (42).

c) Validation as a vaccine

All vaccines should pass standard tests for safety and efficacy. It is crucial to ensure that the cell cultures and fetal bovine serum included in culture medium be free from adventitious BVDV and antibody (described in Section B), and other microorganisms. Live vaccines must either be demonstrated to be safe in pregnant cattle (i.e. no transmission to the fetus), or should be licensed with a warning not to use them in pregnant animals. Live vaccines containing cytopathogenic strains should have an appropriate warning of the risk of inducing mucosal disease in persistently infected cattle.

Efficacy tests of BVD vaccines in non-pregnant cattle are limited by the difficulty of establishing a satisfactory challenge model. Tests should include as a minimum the demonstration of seroconversion following vaccination, a reduction in virus shedding after challenge in vaccinated cattle, and a diminution in measurable clinical parameters, such as rectal temperature response and leukopenia (4, 13, 42). Vaccines intended for use in adult breeding cattle should be evaluated for their efficiency in reducing transplacental transmission, ideally achieving complete prevention. In this case, a suitable challenge system can be established by intranasal inoculation of noncytopathogenic virus into pregnant cows at under 90 days gestation (13). Usually this system will reliably produce persistently viraemic offspring in non-immune cows.

2. Method of manufacture

There is no standard method for the manufacture of a BVD vaccine, but conventional laboratory techniques with stationary, rolled or suspension (micro-carriers) cell cultures may be used. Inactivated vaccines can be prepared by conventional methods, such as binary ethylenimine or beta-propiolactone inactivation (42, 61). A variety of adjuvants may be used (42, 57).

3. In-process control

Cultures should be inspected regularly to ensure that they are free from contamination, and to monitor the health of the cells and the development or absence of CPE, as appropriate.

4. Batch control

a) Sterility

Tests for sterility and freedom from contamination of biological materials may be found in Chapter 1.1.9.

b) Safety

It is essential that all the infectivity be removed during preparation of an inactivated vaccine, and samples should be subjected to several passages in cell culture to ensure the absence of live BVDV. It may also be necessary to ensure the absence of various proscribed agents (prior to inactivation) before use of the vaccine is permitted.

c) Potency

Ideally, the potency of the vaccine should be determined by inoculation into seronegative and virus negative calves, followed by monitoring of the antibody response; however, this is prohibitively expensive for batch control. Antigen content can be assayed by ELISA and adjusted as required to a standard level for the
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particular vaccine (4, 51). Standardised assay protocols applicable to all vaccines do not exist. Live vaccine batches may be assayed by infectivity titration.

d) Duration of immunity

There are few published data on the duration of antibody following vaccination with a commercial product. Protocols for their use usually recommend a primary course of two inoculations and boosters at yearly intervals. Only limited data are available on the antibody levels that correlate with protection against respiratory infections (7, 41) or in utero infection (13).

e) Stability

There are no accepted guidelines for the stability of BVD vaccines, but it can be assumed that attenuated virus vaccine (freeze-dried) should remain potent for at least 1 year if kept at 4°C. Inactivated virus vaccine could have a longer shelf life at 4°C. Lower temperatures could prolong shelf life for either type, but adjuvants in killed vaccine may preclude this.

f) Precautions

BVDV is not considered to be a human health hazard. Standard good microbiological practice should be adequate for handling the virus in the laboratory.

5. Tests on the final product

a) Safety tests

The safety of the final product formulation of both live and inactivated vaccines should be assessed in susceptible calves for any local reactions following administration, and in pregnant cattle for their effects on the unborn calf. Tests for individual batches are described in Section C.4.b.

b) Potency tests for antigenicity

BVD vaccines must be demonstrated to produce adequate immune responses, as outlined in Section C.4.c above, when used in their final formulation according to the manufacturer’s published instructions. In-vitro assays (Section C.4.c) may be used to monitor individual batches.

REFERENCE


Chapter 2.4.8. — Bovine viral diarrhoea


Chapter 2.4.8. – Bovine viral diarrhoea


**NB:** There are OIE Reference Laboratories for Bovine viral diarrhoea (see Table in Part 3 of this *Terrestrial Manual* or consult the OIE Web site for the most up-to-date list: www.oie.int).
SUMMARY

Contagious bovine pleuropneumonia (CBPP) is a disease of cattle caused by Mycoplasma mycoides subsp. mycoides SC (MmmSC; SC = small colonies). It is manifested by anorexia, fever and respiratory signs such as dyspnoea, polypnoea, cough and nasal discharges. Diagnosis depends on the isolation of the aetiological agent. The main problems for control or eradication are the frequent occurrence of subacute or subclinical infections and the persistence of chronic carriers after the clinical phase.

Identification of the agent: Samples to be taken from live animals are nasal swabs and/or broncho-alveolar washings or pleural fluid obtained by puncture. Samples to be taken at necropsy are lung lesions, lymph nodes, pleural fluid and synovial fluid from those animals with arthritis. Direct examination of the exudate or smears is possible, but requires great skill.

For cultivation of the pathogen, the tissues are ground in medium containing antibiotics and inoculated into media that contain inhibitors to prevent the growth of contaminating bacteria. The growth of MmmSC takes several days.

In broth, growth is visible within 3–10 days as a homogeneous cloudiness with whirls when shaken; on agar, small colonies develop, 1 mm in diameter, with the classical ‘fried-egg’ appearance. The biochemical characteristics of MmmSC are the following: sensitivity to digitonin, reduction of tetrazolium salts, fermentation of glucose, absence of arginine hydrolysis, and the absence of phosphatase and proteolytic activities. Special media have been described that are recommended for these tests. Diagnosis is confirmed by immunological tests, such as the growth inhibition and immunofluorescence tests (both use hyperimmune sera). The polymerase chain reaction is now used as a rapid, specific, sensitive and easy to use test.

Serological tests: For diagnosis, the modified Campbell & Turner complement fixation test remains the prescribed test for international trade. However, it has significant limitations regarding sensitivity and specificity. The competitive enzyme-linked immunosorbent assay was designated as an OIE prescribed test for international trade by the OIE International Committee in May 2004. An immunoblotting test has undergone evaluation and is highly specific and sensitive.

Requirements for vaccines: Attenuated strains now recommended for vaccine production: the T1/44 and T1sr. The minimal recommended titre is $10^7$ mycoplasmas per vaccinal dose, but higher titres of at least $10^8$ are recommended.

A. INTRODUCTION

Contagious bovine pleuropneumonia (CBPP) is a contagious disease of cattle caused by Mycoplasma mycoides subsp. mycoides SC (MmmSC; SC = small colonies). CBPP has been known to occur in Europe since the 16th century but it gained a world-wide distribution only during the second half of the 19th century because of increased international trade in live cattle. It was eradicated from many countries by the beginning of the 20th century through stamping-out policies. However the disease persists in many parts of Africa. The situation in Asia is unclear. There have been no reported outbreaks in Europe since 1999. In natural conditions, MmmSC affects only the ruminants of the Bos genus, i.e. mainly bovine and zebu cattle. MmmSC (bovine biotype) has been isolated from buffaloes in Italy (Bubalus bubalis) (36), and from sheep and goats in Africa and more recently in Portugal and in India (37). Among wild animals, one single case has been reported in American buffaloes (Bison bison) and none in African buffaloes (Syncerus caffer) or other wild ruminants. Wild animals do not play a role in the epidemiology of the disease. CBPP is manifested by anorexia, fever and respiratory signs, such as dyspnoea, poly pneumia, cough and nasal discharges. In the case of acute outbreaks under experimental conditions, the mortality rate may be as high as 50% in the absence of antibiotic treatment. When an outbreak
First occurs in an area, the mortality will be high but is often lower in the field following the primary outbreak. Clinical signs are not always evident; subacute or asymptomatic forms occur frequently as the clinical signs in affected animals subside with partial recovery. In this case their lungs show typical encapsulated lesions called ‘sequestra’. These animals may be responsible for unnoticed persistence of the infection in a herd or a region and play an important role in the epidemiology of the disease. Transmission of the disease occurs through direct contact of an infected animal with a naïve one. There is no evidence of transmission through fomites as MmmSC does not persist in the environment. In most continents, control strategies are based on the early detection of outbreaks, control of animal movements and a stamping-out policy. In Africa control of the disease is based on vaccination campaigns using attenuated MmmSC strains such as T1/44 or T1sr. Although the use of antibiotics is theoretically prohibited, they are widely applied in the field. The consequences of these antibiotic treatments in terms of clinical efficacy, emergence of resistant strains, and persistence of chronic carriers have not been evaluated yet. However, recent work has shown that antibiotic treatment of cattle may greatly reduce the transmission to healthy contacts but this requires treatment of all affected cattle in a group (20). The M. mycoides cluster consists of six mycoplasma species or groups of strains, originating from bovines and goats (11, 32, 39). This cluster can be subdivided in two groups, capricolum and mycoides, comprising very closely related species. These six mycoplasmas share serological and genetic characteristics, and this causes taxonomic and diagnostic problems (11) with standard techniques. Specific identification of MmmSC can now be achieved by polymerase chain reaction (PCR) or the use of specific monoclonal antibodies (MAbs). Although MmmSC has been considered to be a very homogeneous biotype, recent molecular techniques, such as enzymatic digestion of whole DNA or southern blotting using an insertion element as a probe, were able to identify differences among strains. A recently described technique that provides an easier way to perform molecular epidemiology of CBPP is a multi-locus sequence analysis (or typing). This technique allows the three main lineages that correlate with the geographical origins (Europe, Southern Africa, rest of Africa) to be distinguished (24). Quite interestingly, the strains of European origin can be clearly differentiated from African ones (10, 16, 42). Recent European strains form a particular cluster and differ from all other strains by no duplication of a long 17 kb DNA fragment (15) and deletion of a 8.4 kb fragment. They are not able to oxidise glycerol, which may account for an apparent lower pathogenicity (19, 43). However, the oldest European strain kept in collection (1967) appears as an unique strain without the deletion and duplication. African strains seem to be more diverse. The sequence of the complete genome of the reference strain PG1 has been published recently (45). There is no doubt that further technical development will allow for a finer characterisation of strains.

B. DIAGNOSTIC TECHNIQUES

1. Identification of the agent

The causal organism can be isolated from samples taken either from live animals or at necropsy. Samples taken from live animals are nasal swabs or nasal discharges, broncho-alveolar lavage or transtracheal washing and pleural fluid collected aseptically by puncture made in the lower part of the thoracic cavity between the seventh and eighth ribs. Blood may also be cultured (21). Samples taken at necropsy are lungs with lesions, pleural fluid ('lymph'), lymph nodes of the broncho-pulmonary tract, and synovial fluid from those animals with arthritis. The samples should be collected from lesions at the interface between diseased and normal tissue.

The agent can be detected by culture, nucleic acid methods and immunological tests described below. Bacteriological identification of the agent is more complex and can be done by biochemical tests, nucleic acid recognition methods and immunological methods. These methods are described here in general terms; however, it is recommended that the definitive identification be done by an OIE Reference Laboratory.

The presence of pathogens varies greatly with the stage of development of the lesions, and a negative result is not conclusive, particularly after treatment with an antibiotic.

When dispatching samples to the laboratory, it is advisable to use a transport medium that will protect the mycoplasmas and prevent proliferation of other bacteria (heart-infusion broth without peptone and glucose, 10% yeast extract, 20% serum, 0.3% agar, 500 International Units [IU]/ml penicillin, thallium acetate 0.2 g/litre).

The samples must be kept cool at 4°C if stored for a few days or frozen at or below −20°C for a longer period. For laboratory-to-laboratory transfer, lung fragments or pleural fluid can also be freeze-dried.

a) Culture

MmmSC needs appropriate media to grow (35). In attempting isolation, 2–3 blind passages may be required. Many attempts to isolate fail because the organism is labile, is often present in small quantities, and is demanding in its growth requirements. The media should contain a basic medium (such as heart-infusion or peptone), yeast extract (preferably fresh), and horse serum (10%). Several other components can be added, such as glucose, glycerol, DNA, and fatty acids, but the effects vary with the strains. To avoid growth of other bacteria, inhibitors, such as penicillin, colistin or thallium acetate, are necessary. The media can be used as broth or solid medium with 1.0–1.2% agar. All culture media prepared should be subjected to quality control and must support growth of Mycoplasma spp. from small inocula. The reference
strain should be cultured in parallel with the suspicious samples to ensure that the tests are working correctly.

After grinding in broth containing antibiotics, the lung samples are diluted tenfold to minimise contaminating bacteria and are inoculated into five tubes of broth and on to solid medium. The pleural fluid can be inoculated directly without previous dilution. Hermetic sealing of the Petri dishes or the use of incubators with controlled humidity are recommended in order to avoid desiccation. To ensure the best conditions for mycoplasma growth, a CO₂ incubator or candle jar should be used. The tubes and Petri dishes are inspected at day 5 and at day 10. In fluid medium, a homogeneous cloudiness usually appears within 2–4 days, frequently with a silky, fragile filament called a ‘comet’, which is characteristic of MmmSC (or M. capricolum subsp. capripneumoniae, the cause of contagious caprine pleuropneumonia). During the following days a uniform opacity develops with whirls when shaken. On agar media, the colonies are small (1 mm in diameter) and have the classical appearance of ‘fried eggs’ with a dense centre. At this stage, the indirect fluorescent antibody (IFA) test or PCR can be performed.

b) Biochemical tests

For routine field use, the immunological tests and PCR are sufficient, but where these give dubious results, biochemical tests may be used. These biochemical tests should be carried out by a reference laboratory. For this purpose, after two or three subcultures, antibiotics should be omitted from the medium to check if the isolate is a mycoplasma or an L-form of a bacterium that will regain its original form in the medium without inhibitors. Once this test is done and after cloning (at least three colonies should be selected), the organism can be identified using biochemical tests (2, 14).

MmmSC is sensitive to digitonin (like all members of the order Mycoplasmatales), does not produce ‘film and spots’, ferments glucose, reduces tetrazolium salts (aerobically or anaerobically), does not hydrolyse arginine, has no phosphatase activity, and has no or weak proteolytic properties.

For these tests, special media have been developed that include the same basic ingredients (heart-infusion broth or Bacto PPLO [pleuropneumonia-like organisms] broth, horse serum, 25% yeast extract solution, 0.2% DNA solution), to which is added 1% of a 50% glucose solution for glucose hydrolysis, 4% of a 38% arginine HCl solution for arginine hydrolysis, and 1% of a 2% triphenyl tetrazolium chloride solution for tetrazolium reduction, plus a pH indicator (e.g. phenol red). (Note: a pH indicator should not be added to a medium containing triphenyl tetrazolium chloride.) For demonstration of proteolysis, growth is carried out on casein agar and/or coagulated serum agar.

Once the biochemical characteristics have been checked, one of the following immunological tests can be performed to confirm the identification: disk growth inhibition test (DGIT), fluorescent antibody test (FAT), and the dot immunobinding on a membrane filter (MF-dot) test. The isolation and identification of the CBPP agent can be difficult and time consuming and depends on careful use of the appropriate procedures and media. When possible, classical bacteriology laboratories should set up a special section for work only with mycoplasmas.

c) Nucleic acid recognition methods

Radiolabelled or enzyme probes have been developed, but have been superseded by the more convenient and safe PCR technology.

The PCR is sensitive, highly specific, rapid and relatively easy to perform, Primers specific for the M. mycoides cluster (38) and for MmmSC (12, 29, 31) have been reported and PCR assays have been developed (5, 12, 29), including a new technique that permits the specific identification of the T1 vaccinal strains (25). Using samples such as lung exudate allows the PCR to be performed directly after differential centrifugations to remove inflammatory cells and pellet mycoplasmas. For lung fragments, the PCR is applied after DNA extraction. The PCR can also be performed on urine or blood. The main advantage of the PCR technique is that it can be applied to poorly preserved samples (contaminated or without any viable mycoplasmas as may occur following antibiotic treatment). If direct detection of DNA from the organ under test fails, specimens should be enriched by culturing them in an appropriate medium for 24–48 hours, followed by attempted detection of DNA from the culture. The PCR has become the primary tool for identification of MmmSC. If a sample is PCR positive in a CBPP-free zone, the test should be confirmed by a second and different PCR; infection can be confirmed by the use of only one immunological test.

One of the problems with PCR is the possible occurrence of contamination if the necessary precautions and quality management system are not implemented correctly in the diagnostic laboratory. Great care must be taken to respect the strict separation between those parts of the laboratory that may be contaminated with PCR products (such as the electrophoresis room) and those parts of the laboratory devoted to preparing the PCR reagents.
The onset of real-time PCR assays should solve this possible troubleshooting as fluorescence resulting from genomic amplification is measured directly without opening the tubes. This technique has already been applied to \textit{MmmSC} detection (17) and further developments are expected in the near future.

\textbf{d) Immunological tests}

The aetiological agent or its antigens can be demonstrated by immunochromic tests on infected tissues, tissue fluids and/or cultures of the organism. However, as some of these tests are dependent on a minimum number of organisms being present in the sample, only positive results are taken into account.

\textit{i) Indirect fluorescent antibody test}

The IFA test can be performed on smears from clinical material using hyperimmune rabbit serum against \textit{MmmSC} and labelled anti-bovine IgG. Hyperimmune bovine serum has been used, but may have cross-reactive antibodies. The test is satisfactory when applied to pleural fluid smears, but is less satisfactory with lung smears due to considerable nonspecific fluorescence. However, good results can be obtained using lung smears counterstained with Erichrome black.

\textit{Broth culture:} Place two drops on a microscope slide. Fix for 15 minutes with methyl alcohol, and leave in contact with the labelled hyperimmune serum for 30 minutes at 37°C in a humid chamber. Rinse three times with phosphate buffered saline (PBS, pH 7.2) and examine under an epifluorescence microscope (×80).

\textit{Colonies grown on solid medium:} Cut a block of agar supporting a number of young colonies and place on a slide with the colonies facing upwards. Place one or two drops of the labelled hyperimmune serum on the block and leave it in a humid chamber for 30 minutes. Place the block into a tube and wash twice for 10 minutes with PBS. Place the block on a slide with the colonies facing upwards and examine as before.

\textit{Petri dish culture:} The gel should not be too thick (no more than 3 mm) and should contain as little horse serum as possible. Rinse the gel three times with PBS, flood the surface with 1 ml of labelled serum and incubate for 30 minutes in a humid chamber. Rinse four times with PBS and examine directly under the microscope. The FAT in a Petri dish is used mainly just after isolation and before cloning, as it is very useful in the case of mixed infection with several mycoplasma species.

\textit{Interpretation of the FAT:} With broth culture, the mycoplasmas appear bright green on a dark background. However, experience is required for the FAT carried out with colonies on agar, because the background appears dark green.

\textit{ii) Fluorescent antibody test}

The FAT is commonly performed on broth and agar cultures. It is slightly less specific than the IFA test.

\textit{Broth culture:} Place two drops on a microscope slide. Fix for 15 minutes with methyl alcohol, and leave in contact with the labelled hyperimmune serum for 30 minutes at 37°C in a humid chamber. Rinse three times with phosphate buffered saline (PBS, pH 7.2) and examine under an epifluorescence microscope (×80).

\textit{Colonies grown on solid medium:} Cut a block of agar supporting a number of young colonies and place on a slide with the colonies facing upwards. Place one or two drops of the labelled hyperimmune serum on the block and leave it in a humid chamber for 30 minutes. Place the block into a tube and wash twice for 10 minutes with PBS. Place the block on a slide with the colonies facing upwards and examine as before.

\textit{Petri dish culture:} The gel should not be too thick (no more than 3 mm) and should contain as little horse serum as possible. Rinse the gel three times with PBS, flood the surface with 1 ml of labelled serum and incubate for 30 minutes in a humid chamber. Rinse four times with PBS and examine directly under the microscope. The FAT in a Petri dish is used mainly just after isolation and before cloning, as it is very useful in the case of mixed infection with several mycoplasma species.

\textit{Interpretation of the FAT:} With broth culture, the mycoplasmas appear bright green on a dark background. However, experience is required for the FAT carried out with colonies on agar, because the background appears dark green.

\textit{iii) Disk growth inhibition test}

The DGIT is based on the direct inhibition of the growth of the agent on a solid medium by a specific hyperimmune serum (14). However, cross-reactions within the mycoides cluster are common and great care should be taken to differentiate \textit{MmmSC} (bovine biotype) from \textit{MmmLC} (caprine biotype; LC: large colonies). It is a simple test to perform, but some results require experience to be interpreted: small inhibition zones (less than 2 mm wide), partial inhibition with ‘breakthrough colonies’, false-negative and false-positive reactions (very rare). The quality of the hyperimmune serum used in this test is critical for good results.

\textit{iv) Agar gel immunodiffusion test}

The agar gel immunodiffusion (AGID) test can detect the specific antigen present at the surface of \textit{MmmSC} and the circulating galactan invading the haemolymph system of sick animals (18). Pleural fluid, ground lung fragments or even sequestra can be tested against a hyperimmune serum in two wells cut 5 mm apart in the gel. The gel is composed of Noble agar (12 g) and thallium acetate (0.2 g/litre) in PBS, pH 7.2 (1000 ml). The test is considered to lack sensitivity and little is known about its specificity, but it has served as a screening test and only positive reactions should be taken into account. The results are better when the plate is incubated at 37°C and can be read within 24 hours. A simpler field test has been developed using impregnated paper discs instead of wells (34).

\textit{v) Dot immunobinding on membrane filtration}

The MF-dot test can be used for routine identification tests in the laboratory (33). Specific SC biotype specific MAbs have been developed to overcome cross-reactions within the mycoides cluster (8).
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vi) Immunohistochemistry

MnmSC immunoreactive sites can be detected in lung lesions using the peroxidase–antiperoxidase method on sections of paraffin-embedded material (13). Because the isolation of the agent is not always achieved from chronic cases and after treatment with antimicrobial drugs, this test is only supplementary to the diagnosis of CBPP (6); a negative result is not conclusive.

2. Serological tests

Serological tests for CBPP are valid at the herd level only. Tests on single animals can be misleading, either because the animal is in the early stage of disease, before specific antibodies are produced, or it may be in the chronic stage of the disease when very few animals are seropositive.

a) Complement fixation (a test suitable for determining freedom from disease and a prescribed test for international trade)

The Campbell & Turner complement fixation (CF) test remains the recommended procedure (although the current method is slightly different from the original one), and it is widely used in all countries where infection occurs (35). The CF test, as a micromethod, has been harmonised in the European Union (27). For antigen titration and harmonisation purposes, an international standard positive bovine serum is available from the OIE Reference Laboratory in Teramo, Italy. However, the CF test is still difficult to perform, requiring well-trained and experienced personnel.

- Reagents
  i) Veronal buffer (VB), pH 7.3. A concentrated stock solution is used diluted 1/5 in sterilised double-distilled water.
  ii) The serum samples, free from erythrocytes, must be inactivated at 56°C for 30 minutes and diluted 1/10 in VB.
  iii) The antigen is a suspension of MnmSC, previously checkerboard titrated and used at a dose of 2 complement fixing units (CF units). It must be kept at 4°C and not frozen. It is produced, tested and delivered by Internationally recognised laboratories.
  iv) The complement (C’) is obtained from normal guinea-pig serum. It is freeze-dried and reconstituted with double-distilled water. It must be kept at –20°C after reconstitution. It is titrated by making a close dilution series in VB containing an appropriate quantity of the antigen to be used in the test. After incubation at 37°C for 2 hours, an appropriate quantity of sensitised sheep red blood cells (SRBC) is added to each dilution. The titration is read after incubation for a further hour. The highest dilution giving complete haemolysis of the SRBC equals 1 C’ unit, from which can be calculated the dilution required for 2.5 units in 25 µl.
  v) The haemolysin is a hyperimmune rabbit serum to SRBC. The quantity used is 6 haemolytic doses read at 50% end-point (HD₅₀ [50% haemolysing dose]).
  vi) The SRBC are obtained by aseptic puncture of the jugular vein. They can be preserved in Alsever’s solution or with sodium citrate. They are used in a 6% suspension.
  vii) The haemolytic system (HS) is prepared by diluting haemolysin in VB to give a dose of 12 HD₅₀. An equal volume of 6% SRBC suspension is added, and the system is sensitised in a water bath at 37°C for 30 minutes with periodic shaking.
  viii) The positive bovine standard sera has been obtained from a naturally infected animals negative to antibodies against Brucella, bovine viral diarrhoea virus, respiratory syncytial virus, infectious bovine rhinotracheitis virus, adenovirus, bovine herpes virus 4, foot and mouth disease viruses, bovine leukosis virus, and parainfluenza 3 virus. The infected animals are also negative for adventitious viruses.
  ix) The negative control serum (NS) is a healthy bovine serum, negative to the above microorganisms.

- Test procedure (using microplates)
  i) Dispense 25 µl of the test serum samples (already diluted 1/10). Add 25 µl of antigen at a dose of 2 CF units.
  ii) Add 25 µl of C’ at a dose of 2.5 units. Shake vigorously and incubate at 37°C for 30 minutes with periodic shaking.
  iii) Add 25 µl of HS. Shake vigorously and incubate at 37°C for 30 minutes with periodic shaking.

It is necessary to set up the following controls:

Complement: 0.5 units, 1 unit and 2.5 units.
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### Haemolytic system
75 µl of VB + 25 µl of HS.

### Antigen
25 µl of 2 CF units of antigen + 25 µl of C' at 2.5 units + 25 µl of HS = 25 µl of veronal buffer.

Note: the microplates must be shaken vigorously twice during the incubation period. The above-mentioned controls, the PS and the NS are always used in each microplate or in a series of microplates where the same batches of reagents are used.

iv) **Reading and interpreting the results:** After centrifugation of the microplates at 125 g for 2 minutes, the reading is carried out based on the percentage of complement fixation observed.

Positive result: 100% inhibition of haemolysis at 1/10;

Doubtful results: 25, 50 or 75% inhibition of haemolysis at 1/10.

It is recommended that any fixation of complement, even partial (25, 50 or 75%), at a serum dilution of 1/10 should be followed by additional investigations.

The limitations of the CF test are well known. With a sensitivity of 70% and a specificity of 98% (7), the CF test can detect nearly all sick animals with acute lesions, but a rather smaller proportion of animals in the early stages of the disease or of animals with chronic lesions. In addition, therapeutic interventions and improperly conducted prophylactic operations (partial slaughter of the herd) may increase the number of false-negative reactions. However, for groups of animals (herd or epidemiological unit) the CF test is capable of detecting practically 100% of infected groups.

The nature of the pathogenesis of the disease is such that the incubation period, during which antibodies are undetectable by the CF test, may last for several months.

Despite the high specificity of the CF test, false-positive results can occur, of which an important cause is serological cross-reactions with other mycoplasmas, particularly other members of the *M. mycoides* cluster. The validity of the results has to be confirmed by post-mortem and bacteriological examination, and serological tests on blood taken at the time of slaughter.

b) **Competitive enzyme-linked immunosorbent assay (a prescribed test for international trade)**

A competitive enzyme-linked immunosorbent assay (C-ELISA) developed by the OIE Collaborating Centre for the diagnosis and control of animal diseases in tropical countries (see Table given in Part 3 of this *Terrestrial Manual*) (23), has undergone evaluation (3). An indirect ELISA based on the use of a lipoprotein antigen is currently being validated by the IAEA (1, 9). In May 2004, the C-ELISA was designated as an OIE prescribed test for international trade by the OIE International Committee. Compared with the CF test, the C-ELISA has equal sensitivity and greater specificity. Advice on the availability of reagents can be obtained from the OIE Reference Laboratories for CBPP, or the OIE Collaborating Centre for ELISA and Molecular Techniques in Animal Disease Diagnosis (see Table given in Part 3 of this *Terrestrial Manual*).

Validation tests (3, 23) that have been carried out in several African and European countries would indicate i) that the true specificity of the C-ELISA has been reported to be at least 99.9%; ii) that the sensitivity of the C-ELISA and the CF test are similar; and iii) antibodies are detected by the C-ELISA in an infected herd very soon after they can be detected by the CFT and C-ELISA antibody persists for a longer period of time (30).

This C-ELISA is now provided as a ready made kit that contains all the necessary reagents including precoated plates kept in sealed aluminium foil. The kit has been especially designed to be robust and offer a good repeatability. As a consequence, sera are analysed in single wells. The substrate has been modified and is now TMB (tetramethyl benzidine) in a liquid buffer and the reading is at 450 nm. The substrate colour turns from pale green to blue in the first place and becomes yellow once the stopping solution has been added. MAb controls exhibit a darker colour while strong positive serum controls are very pale. The cut-off point has been set at 50% and should be valid in every country.

**Reagents**

i) Stock antigen is prepared by washing a concentrated suspension of mycoplasma (2 mg/ml) and lysis with sodium dodecyl sulphate at 0.1%. The stock is kept at –20°C until use.

ii) MAbs are available from the OIE Collaborating Centre for the Diagnosis and Control of Animal Diseases in Tropical Regions (see Table given in Part 3 of this *Terrestrial Manual*).

iii) The conjugate DAKO P260 is diluted in PBS according to the manufacturer’s instructions, with the addition of 0.5% horse serum and 0.05% Tween 20.
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iv) Substrate is made of 1 mM ABTS (2,2’-azino-bis-[3-ethylbenzothiazoline-6-sulphonic acid]) and H₂O₂ in citrate buffer.

- Test procedure
  i) ELISA plates are coated with a lysed antigen solution in PBS, pH 7.4 (100 µl/well) and incubated overnight at 4°C.
  ii) The plates are washed once in PBS diluted 1/5 with 0.05% Tween 20.
  iii) Sera that have not been heat inactivated (diluted 1/10) and MAb diluted in PBS with 0.5% horse serum and 0.05% Tween 20 are left in contact with the antigen for 1 hour at 37°C under moderate agitation in a humid chamber. Heat-inactivated serum will not give satisfactory results.
  iv) The plates are washed twice and conjugate is added to all the wells (100 µl); the wells are then incubated for 1 hour at 37°C.
  v) The plates are washed three times and the substrate is added to all the wells (100 µl).
  vi) Reading is performed at 405 nm when the absorbance in the control MAb has reached 0.8–1.6.

c) Immunoblotting test

An immunoenzymatic test designated the immunoblotting test (IB test) has been developed and is of diagnostic value. A field evaluation indicated a higher sensitivity and specificity than the CF test. A core profile of antigenic bands, present both in experimentally and naturally infected cattle are immunodominant. The more accurate picture of the immune status of animals given by this test is due to the possibility of a more precise analysis of the host’s immune response in relation to the electrophoretic profile of MnmSC antigens; thus the test overcomes problems related to nonspecific binding. It should be used primarily as a confirmatory test, after other tests and should be used in all cases in which the CF test has given a suspected false result.

- Preparation of antigen strips
  i) The antigen is prepared by harvesting and washing a suspension of mycoplasma cells obtained from a 48-hour culture.
  ii) A 4% stacking/5–15% gradient-resolving SDS/PAGE (sodium dodecyl sulphate/polyacrylamide gel) is prepared and used to perform electrophoresis of the sample with appropriate molecular weight standards.
  iii) The separated proteins are transferred to a 14 × 14 cm 0.45 µm nitrocellulose membrane at 70 V constant voltage in transfer buffer (20% methanol in 193 mM glycine, 25 mM Tris/HCl, pH 8.3).
  iv) The membrane is dried and labelled on the side on which the proteins were electrophoresed. The nitrocellulose membrane is incubated in blocking buffer (PBS containing 5% skim milk, 1 M glycine and 1% egg albumin) for 2 hours at room temperature. After washing at room temperature for three 15-minute washes in 0.1% (v/v) Tween 20 in PBS, the nitrocellulose membrane is then washed again in PBS alone. The sheet is then dried and one strip cut and tested from the edge of the membrane. Specific bands are identified at 110, 98, 95, 62/60 and 48 kDa.
  v) The nitrocellulose membrane sheet is cut into strips, 0.4 cm wide and each strip is labelled. These strips are the antigen used for blotting.

- Test procedure
  NB: The strips must be kept with the antigen side up during the procedure.
  i) Serum samples for testing are diluted 1/3 and positive and negative control sera are prepared using dilution buffer (PBS containing 0.1% skim milk and 0.1% egg albumin).
  ii) An antigen strip is placed in each test sample (and controls) and incubated at 37°C for 2 hours with continuous agitation. Strips are then washed, as above.
  iii) Strips are incubated for 1 hour at room temperature in an appropriate dilution of peroxidase-conjugated anti-bovine IgG (H + L chains) in dilution buffer, with continuous agitation. Wash as above.
  iv) Substrate is made by adding 30 mg 4-chloro-1-naphthol dissolved in 10 ml methanol to 50 ml PBS and 30 µl H₂O₂. Substrate is added to the strips, which are then left in the dark with continuous agitation and examined periodically until the protein bands are suitably dark. The reaction is stopped with distilled water.
v) *Reading the results:* The strips are dried and examined for the presence of the core IgG immunoblot profile of five specific antigenic bands of 110, 98, 95, 62/60 and 48 kDa. Sera giving a similar immunological profile are considered to be positive.

d) *Other tests*

A rapid field slide agglutination test (SAT) with either whole blood or serum (41) has been developed to detect specific agglutinins: the antigen is a dense suspension of stained mycoplasmas that is mixed with a drop of blood or serum. Due to a lack of sensitivity, the test detects only animals in the early stages (i.e. acute phase) of the disease. It should be used only on a herd basis. A latex agglutination test has been developed that is easier to interpret than the SAT (4).

For CBPP, the CF test and ELISAs can be used in screening and eradication programmes, but the highly specific IB test should be used as a confirmatory test. However, the IB test is not fit for mass screening and may be difficult to standardise in countries with marginal laboratory facilities so IB testing should be performed in a reference laboratory.

C. REQUIREMENTS FOR VACCINES AND DIAGNOSTIC BIOLOGICALS

Since the beginning of the 20th century, many vaccines against CBPP have been described (e.g. killed vaccines, and heterologous vaccines), but none of them has proven to be really satisfactory. Today, the only vaccines commonly used are produced with attenuated *Mmm* SC strains.

1. Seed management

a) **Characteristics of the seed**

Two strains are used for preparing CBPP vaccines: strain T1/44, a naturally mild strain isolated in 1951 by Sheriff & Piercy in Tanzania, and strain T1sr (44, 46). The 44th egg-passage of strain T1, called T1/44, was sufficiently attenuated to protect cattle without post-vaccinal severe reactions, however such reactions may still occur in the field although rarely. Their frequency is unpredictable. Cattle breeds should be assessed for their sensitivity before mass vaccination. It should be noted that when given by intubation, the vaccine can produce CBPP lesions (28); however, as the vaccine is to be injected subcutaneously, this should not create a serious disease problem (22).

The identity of the strain can be verified with the insertion sequence profile or by the specific PCR assay (25).

The master seed strain is kept in freeze-dried form at −20°C. It is deposited at an international laboratory from African Unity, PANVAC.

b) **Method of culture**

For vaccine production, a system of freeze-dried seed lots originating from master seed cultures is used. These seed lots are kept at −20°C.

The media used for seed cultures are usually the same than for batch production. However there is no specific requirement, they should ensure a correct growth of the vaccine strain.

For vaccine bulk cultures, in order to avoid the risk of inadvertent cloning of vaccine seed, the whole content of a vaccine seed vial should be inoculated directly into a tube filled with production medium. A second tube may be seeded as a dilution from the first one.

2. Method of manufacture

The media used for vaccine production may differ slightly from media for isolation purpose. In the case of a vaccine production, what matters more is the final titre that can be obtained rather than the speed of growth. Furthermore the harvested mycoplasmas should withstand the freeze drying process without excessive titre loss.

Examples of vaccine culture medium are Gourlay’s medium or F66, however modifications of these media are licit and may include addition of buffers.

Vaccine bulk cultures must be obtained with a maximum of three successive passages of the seed. A passage is defined here by a 1/100 dilution of a culture in the exponential phase of growth.
For example, 0.5 ml of culture from the seed are transferred to 50 ml of fresh medium and, when turbidity is observed, these 50 ml are used to seed 5000 ml of medium, which represents the final product when the optimum titre has been reached. Each vaccine producer should then evaluate the speed of growth of the vaccine strain in the medium that is used to optimise the harvest time.

A stabiliser can be added to final cultures before freeze drying. The manufacturer should ensure an homogeneous distribution in the vials and use of a proper freeze dryer to have identical titres in all the vials when the freeze drying process is finished.

3. **In-process control**

Good Manufacturing Practice should be observed to avoid contaminations at each step of the production and to ensure purity of the final product.

As an example, phase contrast microscopic examination of cultures easily allows the detection of contaminations by bacteria or fungi.

4. **Batch control**

a) **Purity and identity**

Suitable media must be seeded with the final product to ensure purity of the final product and absence of contamination with classical bacteria and fungi. All media should remain sterile (35). Tests for sterility and freedom from contamination of biological materials may be found in Chapter 1.1.9.

Absence of contamination by other mycoplasmas must be checked. For example a growth inhibition test with the final product and a hyperimmune serum to MmmSC (preferably raised with T1/44 antigen) can be performed. The presence of mycoplasma colonies within the inhibition zone must be followed by identification of these colonies to rule out the presence of other mycoplasmas than the vaccine strain.

The identity of the vaccine strain present in the final product must be guaranteed by the producer.

For example a specific PCR can be used to identify T1 strains. In addition streptomycin resistance can be used to differentiate T1/44 from T1sr.

b) **Titration**

The minimum titre is \(10^7\) live mycoplasmas per vaccine dose, but higher titres are recommended because of the loss of titre between production plant and actual injection to animals. Titration is performed after reconstitution of the freeze-dried vaccine in the diluent recommended for vaccination and preferably with the diluent provided by the vaccine manufacturer. Titrations should be performed on at least three vials per batch. This titre must be evaluated with a titration technique that allows a precision of +/- 0.25 logs. A batch passes the test if three vials chosen randomly have titres above this limit. The manufacturer must ensure that the minimum titre is retained until the expiry date if the product is kept at the correct temperature.

c) **Safety**

After reconstitution, the vaccine is inoculated subcutaneously into two mice, intraperitoneally into two mice and intraperitoneally into two male guinea-pigs. None of the animals should die within the following month, and the guinea-pigs should not show signs of orchitis. Safety tests should be carried out on (at least two) cattle or zebu cattle. These are inoculated with ten vaccinal doses each, and observed for adverse effects for at least 4 weeks.

d) **Potency**

Potency tests are not performed routinely with production batches as there is no laboratory animal that would allow this test to be performed at low cost. Potency tests in cattle are also not performed because of the cost. Getting statistically significant protection rates would involve using at least 50 naïve animals.

Potency of the final product is ensured by using a master seed lot of well known origin for which the potency test has already been performed, by strictly following the production standard protocols (avoiding multiple passages) and by ensuring that the final titres are correct.

e) **Duration of immunity**

Strain T1/44 confers protection for approximately 1 year (21), but the protection conferred by the T1sr strain may only be 6 months long. Serological conversion (CF test) takes place in some animals. The antibodies disappear 3 months after vaccination.
f) **Stability**

Periodic titration of the stored vaccine allows the shelf life to be calculated. Freeze-dried vaccine must be stored at −20°C. At this temperature its storage life is at least 1 year (35), viability may even be conserved for many years without loss of titre allowing for the constitution of emergency stocks. The titres of these stocks naturally need to be controlled regularly.

g) **Preservatives**

For lyophilisation, stabilisers can be added. For example, dried skimmed milk can be added: 45 g/litre of culture medium. For reconstitution of a freeze-dried vaccine normal sterile saline solution (9 g/litre) is preferably used. Alternatively, a molar solution of magnesium sulphate (248 g per litre) is used at room temperature. This molar solution protects mycoplasmas against inactivation by heat (35). The purity of the salts used is important. When using magnesium sulphate as a diluent for vaccine reconstitution, it is also important to insure that the pH of the final product does not drop below 6.5 as this may induce a loss of titre (26).

h) **Precautions (hazards)**

Procedures for use in the field and reconstitution of freeze-dried vaccines have been described by Provost et al. (35).

Intense reactions may appear when infected animals are vaccinated, as occurred recently following emergency vaccination campaigns in East Africa. These reactions usually occur within 2–3 days. Local reactions may also appear at the site of injection after 2–3 weeks with strain T1/44. These reactions are known as a ‘Willems reaction’, and consist of an invading oedema that leads to death if antibiotic treatment, such as tetracyclin or tylosin, is not given. Strain T1sr is completely devoid of residual pathogenicity, which makes it an alternative choice to T1/44, although the duration of immunity is shorter. Concerns were raised about the ineffectiveness of T1sr to control outbreaks in southern Africa leading to its suspension (40). The general sensitivity of a given bovine population should be first tested by vaccinating sample groups (35).

5. **Tests on the final product**

These tests should be performed after reconstitution of a pool of at least five vials of the freeze-dried vaccine in the recommended diluent.

a) **Safety**

Safety tests should be carried out on cattle or zebu cattle, according to Section C.4.c.

b) **Potency**

The test is carried out according to the protocol described in Section C.4.d. Because CBPP cannot be easily reproduced experimentally, and due to its cost, only one potency test need be performed on each seed lot, providing the titre is satisfactory and that production parameters have not been changed.

**REFERENCES**


* * *

**NB**: There are OIE Reference Laboratories for Contagious bovine pleuropneumonia (see Table in Part 3 of this *Terrestrial Manual* or consult the OIE Web site for the most up-to-date list: www.oie.int).
CHAPTER 2.4.10.

DERMATOPHILOSIS

SUMMARY

Dermatophilosis (also known as streptothrichosis) is an exudative, pustular dermatitis that mainly affects cattle, sheep and horses, but also goats, dogs and cats, many wild mammals, reptiles and, occasionally, humans. The severe disease in ruminants is promoted by immunomodulatory effects induced by infestation with the tick, Amblyomma variegatum.

Laboratory diagnosis of dermatophilosis depends on the demonstration of the bacterium Dermatophilus congolensis in material from the skin or other organs. Sites other than the skin are rarely affected.

Identification of the agent: Dermatophilus congolensis normally affects the epidermis, causing the formation of scabs. It may be demonstrated in smears made from scabs emulsified or softened in water or in impression smears from the base of freshly removed adherent scabs. The organism is Gram positive, but its morphology is more readily appreciated in smears stained with Giemsa. In stained smears, the organism is seen as branching filaments containing multiple rows of cocci. This characteristic appearance is diagnostic. In wet or secondarily infected scabs, only free cocci may be present, so that staining by immunofluorescence is necessary. Dermatophilus congolensis is demonstrated in histopathological sections by Giemsa staining or by immunofluorescence. Dermatophilus cheloniae may be found in crocodiles, chelonids and cobras.

Isolation of D. congolensis from freshly removed scabs is straightforward, but the organism is readily overgrown by other bacteria. When cultured from contaminated sites, special techniques involving filtration, chemotaxis, or selective media are necessary.

Demonstration and identification of D. congolensis by immunofluorescence is a reliable and very sensitive method of diagnosis, but requires that laboratories make their own diagnostic antisera as these are not available commercially. Although antigenic cross-reaction with Nocardia spp. has been reported, this is likely to give only weak fluorescence. Ideally, a monoclonal antibody specific to D. congolensis should be used. Polymerase chain reaction (PCR)-based characterisation has also been developed.

Serological tests: A variety of serological tests has been used in studies of the epidemiology and pathogenesis of dermatophilosis. Antibody can be demonstrated in all but fetal blood in healthy ruminants, but the elevated levels associated with clinical infection can be used to identify animals that have been infected with the disease.

Requirements for vaccines and diagnostic biologicals: Despite the identification of several virulence factors, no vaccines are available currently.

A. INTRODUCTION

Dermatophilosis (also known as streptothrichosis, or in sheep as ‘lumpy wool disease’) is an exudative, pustular dermatitis that affects mainly cattle, sheep and horses, but also goats, dogs and cats, many wild mammals, reptiles and, occasionally, humans. Dermatophilosis is caused by the bacterium Dermatophilus congolensis, the type species of the genus Dermatophilus, which is a member of the order Actinomycetales. Dermatophilosis is the commonest skin disease of crocodiles in Australia and has an impact on farming of this species (2). It is provoked by Dermatophilus cheloniae, which has also been isolated from chelonids and cobra.

There is considerable variation in the clinical appearance of the disease and in the affected areas of the body. Typically, infection gives rise to the formation of dense scabs on the skin, but in certain areas, such as the
perineum in ruminants and the pastern in horses, moist lesions with thickened, folded skin may occur. In such lesions, relatively thin scabs are found. Where lesions are exposed to prolonged wetting, with or without secondary infection, exudative lesions may be present.

Scabs characteristically comprise alternating layers of parakeratotic keratinocytes invaded with branching bacterial filaments and infiltrates of neutrophils in serous exudate. This gives a palisaded appearance in stained sections. *D. congolensis* filaments remain confined to the epidermis and very rarely infect the dermis.

Extensive acute dermatophilosis cannot be reproduced easily in experimental conditions. *Dermatophilus congolensis* itself is not highly pathogenic, and a combination of factors is necessary for the development of clinical lesions. Malnutrition, intense rainfalls and mechanical traumas have been incriminated as favouring the disease. However, where dermatophilosis has an important economic impact in West and Central Africa as well as on some Caribbean islands, the major risk factor is the infestation by *Amblyomma variegatum* ticks. Severe disease may be promoted by immunomodulatory effects of saliva secreted during tick bite (1), but the fine underlying mechanisms are not understood. Susceptibility to dermatophilosis is also greatly influenced by the genetic background of ruminant breeds, animals from temperate regions and especially dairy cattle being extremely susceptible when introduced in regions at risk.

**B. DIAGNOSTIC TECHNIQUES**

1. **Identification of the agent**

   a) **Microscopic observation**

   Diagnosis can usually be made by demonstrating the causal organism in scabs from the lesions or in exudate beneath the scabs. The organism has a characteristic microscopic appearance – its septate, branching filaments become longitudinally, as well as transversely, divided to form ribbons of spherical or ovoid cocci, each about 0.5 µm in diameter, in multiple rows. This appearance is diagnostic, provided that cocci are found in transverse rows of four or more, and is readily seen in stained preparations. However, the distinctive formation can be disrupted during the preparation of smears for examination if the material is spread too vigorously over the slide.

   Impression smears may be made from the moist, concave undersurfaces of freshly removed scabs. Otherwise, thick smears are best prepared from scabs emulsified in sterile distilled water. Alternatively, scabs can be soaked overnight in sterile water or saline to sufficiently moisten them so that the undersurface of the scab can be used to make effective impression smears by firmly pressing this surface on to a microscope slide. Smears are then air-dried, fixed by heating or immersion in methanol for 5 minutes, and stained. The organism stains well in dilute carbol fuchsin or methylene blue stain, but Gram’s stain or, preferably, a 1 in 10 dilution of Giemsa stain for 30 minutes, gives better differentiation in thick smears, the darkly stained *D. congolensis* contrasting with the paler or pink counterstained background of keratinocytes and neutrophils. Gram staining does not give as good results as Giemsa because it may overstain the background and does not clearly show the characteristic laddering of the coccoid forms.

   Wet or secondarily infected scabs often contain few, if any, intact filaments, and the organism may not stain Gram positive. In such material, the cocci cannot be differentiated morphologically from other coccoid bacteria, so that staining by immunofluorescence is required. However, specific antisera for immunofluorescence are not commercially available. Thin, heat-fixed smears are used. In difficult cases and when infection of organs other than the skin is suspected, histopathological examination of biopsy or necropsy material is advisable. Giemsa stain or immunofluorescence is used.

   The characteristic appearance of the lesions and of the organism in smears from typical bovine dermatophilosis makes culture unnecessary in most cases. However, in the rare cases in which a Giemsa-stained smear does not give a definitive result, confirmation of the diagnosis may be made by isolating the bacterium. Cultures are made on blood agar and incubated at 37°C. Growth is accelerated under microaerophilic conditions; rough, usually haemolytic, greyish-yellow colonies, about 1 mm in diameter, are seen pitting the medium after 24 hours. Incubation in air produces similar pinpoint colonies at 24 hours that grow to about 1 mm at 48 hours. The rough colonies are formed by the branching filaments, but continued growth in air stimulates the production of the cocci, which are commonly yellow in colour. Colonies take on a smooth, often yellowish, appearance. The cocci are normally vigorously motile when taken from young cultures. The colonies must be differentiated from *Nocardia* spp. and *Streptothyces* spp., neither of which produces filaments that break up into multiple rows of motile cocci.
b) Culture

For isolation, material can be streaked out directly from the moist undersurfaces of freshly removed, uncontaminated scabs or from scab emulsions, but the relatively slow-growing \textit{D. congolensis} is readily overgrown by other bacteria. Special isolation techniques are thus required for contaminated specimens. In most specimens, free cocci, whether motile or not, will be present in emulsions of the material. Filtration of the emulsion through a 0.45 µm membrane filter is usually sufficient to reduce or eliminate contaminants and permits isolation from the filtrate, as described above. Alternatively, Haalstra’s method (4) may be used. Small pieces of scab are placed in a bijou bottle containing 1 ml of sterile distilled water and allowed to stand at room temperature for 3–4 hours. The open bottle is then placed for 15 minutes in a candle jar. Samples of the surface liquid are removed with a bacteriological loop and cultured. The method depends on the release from the scab of the motile cocci of \textit{D. congolensis} and their chemotropic attraction towards the carbon-dioxide-rich atmosphere of the candle jar. A selective medium consisting of 1000 units/ml of polymyxin B in blood agar can also be used, and is effective when the contaminants are sensitive to this antibiotic.

c) Immunological methods

Immunofluorescence staining of smears or tissues is the most reliable and sensitive immunological technique for the identification of \textit{D. congolensis} antigens and for the diagnosis of dermatophilosis. Polyclonal antibody obtained from animals inoculated with \textit{D. congolensis} can be easily prepared using standard methods, but there is a risk of possible cross-reaction with some strains of \textit{Nocardia} spp. Monoclonal antibody to species-specific antigen (5) is preferable. However, monoclonal antibodies have not been widely distributed and validated by interlaboratory tests. Thin, heat-fixed smears of scab emulsions, or impression smears, are stained. Known positive and negative control specimens should always be included.

d) Nucleic acid recognition methods

In absence of extensive genome sequence information, randomly amplified polymorphic DNA methods (RAPD) as well as pulsed-field gel electrophoresis (PFGE) have been used and proved to be useful for the molecular typing of \textit{D. congolensis} (7). An alkaline ceramidase gene was cloned from RAPD fragments, and a polymerase chain reaction (PCR) using primers designed from the nucleotide sequence from this gene gave an amplification product with \textit{D. congolensis} DNA. No amplification product was observed with \textit{M. bovis}, \textit{C. propinquum} and \textit{D. cheloniae}, suggesting a possible use in diagnosis or detection of \textit{D. congolensis} (3). Alternatively, 16S rDNA sequence obtained after amplification can be used to confirm the presence of \textit{D. congolensis}.

2. Serological tests

Clinical diagnosis is best performed using the methods described above rather than serological methods. Antibody can be demonstrated in all but fetal blood in healthy ruminants, but levels are raised following clinical infection. The enzyme-linked immunosorbent assay (ELISA) has proved to be a sensitive and convenient assay technique, and elevation of titres above baseline values can be used in epidemiological studies to identify animals that have had the disease (9). The test being based on a crude antigen, cross-reactivity with other bacteria can occur as in immunofluorescence. At present, the ELISA remains as a research and investigation method. Serology, either using ELISA or older methods such as haemagglutination and counter-immunoelectrophoresis, is not used for routine diagnosis of dermatophilosis where direct detection of the bacterium is easy.

C. REQUIREMENTS FOR VACCINES AND DIAGNOSTIC BIOLOGICALS

\textit{Dermatophilus congolensis} produces virulence factors such as haemolysin, phospholipases, ceramidases and proteolytic enzymes, which may be used to penetrate the epidermis barrier and interact with the inflammatory response of the host. These virulence factors are considered candidate antigens for vaccines. Research on vaccines for prevention of dermatophilosis has been conducted (6, 10); however, no vaccine is currently available. Research in this domain is hampered by the inability to reproduce the disease experimentally and the poor understanding of skin immunity. Much emphasis has therefore been put on tick control and identification of genetic markers of resistance or susceptibility with promising results in cattle (8).

REFERENCES

Chapter 2.4.10. – Dermatophilosis


* *
CHAPTER 2.4.11.
ENZOOTIC BOVINE LEUKOSIS

SUMMARY

Enzootic bovine leukosis (EBL) is a disease of adult cattle caused by the retrovirus, bovine leukaemia virus (BLV). Cattle may be infected at any age, including the embryonic stage. Most infections are subclinical, but a proportion of cattle (~30%) over 3 years old develop persistent lymphocytosis, and a smaller proportion develop lymphosarcomas (tumours) in various internal organs. Natural infection has also been recorded in buffaloes, sheep and capybaras. Clinical signs, if present, depend on the organs affected. Cattle with lymphosarcomas almost invariably die either suddenly, or weeks or months after the onset of clinical signs.

Identification of the agent: Virus can be isolated following in-vitro culture of peripheral blood lymphocytes from infected animals by electron microscopy or by BLV antigen detection in the culture supernatant. Proviral DNA can be detected in peripheral blood lymphocytes or tumours by the polymerase chain reaction.

Serological tests: The antibody detection methods widely used are the agar gel immunodiffusion (AGID) assay using serum and the enzyme-linked immunosorbent assay (ELISA) using serum or milk. These tests have formed the basis for successful eradication policies in many countries. Other tests, such as radio-immunoassay, can also be used. A number of AGID and ELISA kits are available commercially.

Requirements for vaccines and diagnostic biologicals: No vaccine against BLV is available.

A. INTRODUCTION

There may be several causes of lymphosarcomas in cattle, but the only definitely known cause is the retrovirus, bovine leukaemia virus (BLV), which causes enzootic bovine leukosis (EBL). The term sporadic bovine leukosis (SBL) is usually reserved for young animals (calves) as well as cutaneous and thymic types of lymphoma, which is defined by the age of the animal affected and the distribution of the tumours. The cause or causes of SBL are not known. There may also be lymphosarcomatous conditions that do not fall into either the SBL or EBL categories, i.e. adult multicentric lymphoma with sporadic occurrence of unknown aetiology. Only lymphomas caused by BLV infection should be termed leukosis or enzootic bovine leukosis (12).

Although animals can become infected with BLV at any age, tumours (lymphosarcomas) are seen typically in animals over 3 years of age. Infections are usually subclinical; only 30–70% of infected cattle develop persistent lymphocytosis, and 0.1–10% of the infected animals develop tumours. Signs will depend on the site of the tumours and may include digestive disturbances, inappetance, weight loss, weakness or general debility and sometimes neurological manifestations. Superficial lymph nodes may be obviously enlarged and may be palpable under the skin and by rectal examination. At necropsy, lymph nodes and a wide range of tissues are found to be infiltrated by neoplastic cells. Organs most frequently involved are the abomasum, right auricle of the heart, spleen, intestine, liver, kidney, omasum, lung, and uterus. The susceptibility of cattle to persistent lymphocytosis, and perhaps also to tumour development, is genetically determined. There is conflicting evidence of the role of the virus as a cause of immunological deficiency or increased cull rate. In one study it was demonstrated that BLV-infected herds have lower milk production (2.5–3% on a herd level), an increased cull rate, and are more susceptible to other diseases with infectious aetiology, e.g. mastitis, diarrhoea and pneumonia, but the effect on fertility is only minor (10).

B. DIAGNOSTIC TECHNIQUES

Virus can be detected by in-vitro cultivation of peripheral blood lymphocytes. The virus is present in peripheral blood lymphocytes and in tumour cells as provirus integrates into the DNA of infected cells. Virus is also found in
the cellular fraction of various body fluids (nasal and bronchial fluids, saliva, milk). Natural transmission depends on the transfer of infected cells, for example during parturition. Artificial transmission occurs, especially by blood-contaminated needles, surgical equipment, gloves used for rectal examinations etc. Lateral transmission in the absence of these contributory factors is usually slow (15). In regions where blood-sucking insects occur in large numbers, especially tabanids, these may transmit the virus mechanically.

Although several species can be infected by inoculation of the virus, natural infection occurs only in cattle (Bos taurus and Bos indicus), water buffaloes, and capybaras. Sheep are very susceptible to experimental inoculation and develop tumours more often and at a younger age than cattle. A persistent antibody response can also be detected after experimental infection in deer, rabbits, rats, guinea-pigs, cats, dogs, sheep, rhesus monkeys, chimpanzees, antelopes, pigs, goats and buffaloes.

BLV was probably present in Europe during the 19th century, from where it spread to the American continent in the first half of the 20th century. It may then have spread back into Europe and introduced into other countries for the first time by the import of cattle from North America (13). A number of countries are recognised as officially free from BLV infection.

Several studies have been carried out in an attempt to determine whether BLV causes disease in humans, especially through the consumption of milk from infected cows. There is, however, no conclusive evidence of transmission, and it is now generally thought that BLV is not a hazard to humans.

1. Identification of the agent

BLV is an exogenous retrovirus and belongs to the genus Delta-Retrovirus within the subfamily Orthoretrovirinae. It is structurally and functionally related to the human T-lymphotropic viruses 1 and 2 (HTLV-1 and HTLV-2). The major target cells of BLV are B lymphocytes (5, 12). The virus particle consists of single-stranded RNA, nucleoprotein p12, capsid (core) protein p24, transmembrane glycoprotein gp30, envelope glycoprotein gp51, and several enzymes, including the reverse transcriptase. Proviral DNA, which is generated after reverse transcription of the viral genome, integrates randomly into the DNA of the host cell where it persists without constant production of viral progeny. When infected cells are cultured in vitro, usually by co-cultivation of lymphocytes with indicator cells, infectious virus is produced, most readily through stimulation with mitogens.

a) Virus isolation

Mononuclear cells from 1.5 ml of peripheral blood in ethylene diamine tetra-acetic acid (EDTA) are separated on a ficoll/sodium metrizoate density gradient, cultured with 2 × 10^6 fetal bovine lung (FBL) cells, and subsequently grown for 3–4 days in 40 ml of minimal essential medium (MEM) containing 20% fetal calf serum. Virus causes syncytia to develop in the cell monolayer of the FBL cells. Short-term cultures can be prepared by culturing mononuclear cells in the absence of FBL cells in 24-well plastic trays for 3 days (14). The p24 and gp51 antigens can subsequently be detected in the supernatant of the cultures by radio-immunoassay (RIA), enzyme-linked immunosorbent assay (ELISA), immunoblot or agar gel immunodiffusion (AGID), and the presence of the BLV particles and of the BLV-provirus can be demonstrated by electron microscopy and by PCR.

b) Polymerase chain reaction

The use of the polymerase chain reaction (PCR) to detect BLV provirus has been described by various workers (3, 6, 17, 18, 21). Primers constructed to match the gag, pol and env regions of the genome have all been used with variable success. So far, nested PCR followed by gel electrophoresis is the most rapid and sensitive method. The method described is based on primer sequences from the env gene, coding for gp51. This gene is highly conserved, and the gene and the antigen are generally present in all infected animals throughout the course of infection. The technique is restricted to those laboratories that have the facilities for molecular virology, and the usual precautions and control procedures must be in place to ensure validity of the test results (see Chapter 1.1.5 Validation and quality control of polymerase chain reaction methods used for the diagnosis of infectious diseases, and Chapter 1.1.7 Biotechnology in the diagnosis of infectious diseases and vaccine development).

The nested PCR is applicable to the detection of BLV infection in individual animals in the following circumstances:

- Young calves with colostral antibodies,
- Tumour cases, for differentiation between sporadic and infectious lymphoma,
- Tumour tissue from suspected cases collected at slaughterhouses,
- New infections, before development of antibodies to BLV,
- Cases of weak positive or uncertain results in ELISA,
• The systematic screening of cattle in progeny-testing stations (before introduction into artificial insemination centres),
• Cattle used for production of vaccines, ensuring that they are BLV free.

PCR is not suitable for use as a herd test, but may be used as an adjunct to serology for confirmatory testing.

• Sensitivity and reliability of the method
  i) Analytical sensitivity
  Although the nested PCR assay has a theoretical sensitivity of one target molecule, in practice the analytical sensitivity is around five to ten target molecules of proviral DNA.
  ii) False-positive samples
  The high sensitivity of the nested PCR may cause problems of false-positive results due to contamination between samples (6). To minimise this, several special procedures are adopted during the analysis, such as the use of laminar air-flow hoods, separate rooms for different steps of the analysis, new gloves or the use of special tube openers for each individual assay, negative controls (water blanks), etc.
  iii) False-negative samples
  It should be noted that only a small proportion of the peripheral lymphocytes can be infected, thus limiting the sensitivity of the assay. The presence of inhibitory substances in some samples may cause false-negative results. To detect this, at least one positive control is used on every test run. In addition, internal controls (mimics) are added to each sample. The mimic is a modified target molecule that is amplified with the same primers as the real target, but that generates a PCR product with different size, which can be visualised by agarose gel electrophoresis. The mimic is added at a low concentration which favours the amplification of the real target (2). However, it is possible for the mimic to compete with the true target. It may therefore be necessary to analyse each sample with or without the mimic.

• Sample preparation
  Peripheral blood lymphocytes (PBL) are separated from EDTA blood samples by using the Ficoll-Paque separation method. Alternatively buffy coat may be used, or even whole blood, e.g. where samples have been frozen.
  Tumours or other tissues should be homogenised to a 10% suspension.

• DNA extraction
  Purification of total DNA is a prerequisite for achieving optimal sensitivity. Various purification methods are commercially available, e.g. NucleoSpin (Macherey-Nagel) or Chelating resin treatment (BioRad).
  Special precautions should be taken during all steps to minimise the risk of contamination (6).
  i) Approximately 100 µl chelating resin (Sigma C-7901 or Chelex from Bio-Rad) is added for each sample in a 1.5 ml eppendorf tube.
  ii) 100 µl of the samples and 10 µl of the mimic are added to the tubes with chelating resin. The samples are vortexed.
  iii) The eppendorf tubes are closed and incubated at 56–60°C for 20 minutes.
  iv) The tubes are vortexed for 10 seconds.
  v) The tubes are incubated at 98°C for 8–10 minutes.
  vi) The tubes are vortexed for 10 seconds and immediately put on ice.
  vii) Optional: all samples are equilibrated to a standard amount of DNA (500 ng/reaction) applying, for example, the Beta Globin-method (21).
  viii) The tubes are centrifuged at 15,000 g for 2 minutes.
  ix) 5 µl is used in the PCR assay.

• Nested PCR procedure
  i) Primer design and sequences
  Several PCR protocols for the detection of BLV provirus sequences have been published (3, 17, 18, 21). As an example, a PCR assay based on the one developed by Ballagi-Pordany et al. (6) is described in detail. The BLV region used as target is the gp51 (env) gene. The sequence used for designing the primers is available from GenBank, accession No. K02120. The sequences of the primers are:
Chapter 2.4.11. – Enzootic bovine leukosis

<table>
<thead>
<tr>
<th>Oligo</th>
<th>Sequence</th>
<th>Position in K02120</th>
</tr>
</thead>
<tbody>
<tr>
<td>OBLV1A</td>
<td>(5'-CTT-TGT-GTG-CCA-AGT-CTC-CCA-GAT-ACA-3')</td>
<td>5029</td>
</tr>
<tr>
<td>OBLV6A</td>
<td>(5'-CCA-ACA-TAT-AGC-ACA-GTC-TGG-GAA-GGC-3')</td>
<td>5442</td>
</tr>
<tr>
<td>OBLV3</td>
<td>(5'-CTG-TAA-ATG-GCT-ATC-CTA-AGA-TCT-ACT-GTC-3')</td>
<td>5065</td>
</tr>
<tr>
<td>OBLV5</td>
<td>(5'-GAC-AGA-GGG-AAC-CCA-GTC-ACT-GTT-CAA-CTG-3')</td>
<td>5376</td>
</tr>
</tbody>
</table>

PCR1-product size: 440 bp; PCR1I-product size: 341 bp; Mimic-product size: 761 bp.

ii) Reaction mixtures

Reaction mixtures are blended (except sample and mimic) before adding to the separate reaction tubes. One negative control (double distilled H₂O) per five samples, and one positive control should be added. Total volumes of mixtures are calculated by multiplying the indicated volumes by the total number of samples, including controls, plus one. Taq polymerase is used in a premade 1/10 dilution.

DNA samples and mimic¹ (2) should be added in separate rooms in the laboratory: laboratory room 1 for DNA preparations and mimics, and laboratory room 2 for PCR1I-products, to minimise contamination.

a) Reagents added in clean laboratory room

This mixture may be prepared in advance and stored at 4°C for up to 1 month.

<table>
<thead>
<tr>
<th>Reagents per reaction (conc.)</th>
<th>PCR1 reaction</th>
<th>PCR1I reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Double-distilled H₂O (standardised)</td>
<td>21 µl</td>
<td>21 µl</td>
</tr>
<tr>
<td>10 × PCR buffer (Perkin Elmer)</td>
<td>5 µl</td>
<td>5 µl</td>
</tr>
<tr>
<td>dNTP (10 mM)</td>
<td>4 × 1 µl</td>
<td>4 × 1 µl</td>
</tr>
<tr>
<td>Bovine serum albumin (1 mg/ml)</td>
<td>5 µl</td>
<td>5 µl</td>
</tr>
</tbody>
</table>

**Primers (10 pmol/µl):**

| OBLV1A | 1.5 µl | – |
| OBLV6A | 1.5 µl | – |
| OBLV3  | –      | 1.5 µl |
| OBLV5  | –      | 1.5 µl |

| In total: | 38 µl | 38 µl |

The following should be added just before starting the PCR

<table>
<thead>
<tr>
<th>Reagents per reaction (conc.)</th>
<th>PCR1 reaction</th>
<th>PCR1I reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>MgCl₂ (25 mM)</td>
<td>5 µl</td>
<td>5 µl</td>
</tr>
<tr>
<td>Taq polymerase (1 unit/reaction)</td>
<td>2 µl</td>
<td>2 µl</td>
</tr>
<tr>
<td>Mineral oil</td>
<td>2 drops</td>
<td>2 drops</td>
</tr>
</tbody>
</table>

| In total: | 45 µl | 45 µl |

b) Reagents added in laboratory room 1 (DNA) or 2 (PCR1I)

<table>
<thead>
<tr>
<th>Reagents per reaction (conc.)</th>
<th>PCR1 reaction</th>
<th>PCR1I reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA sample* (or water*)</td>
<td>5 µl</td>
<td>–</td>
</tr>
<tr>
<td>PCR1 product</td>
<td>–</td>
<td>5 µl</td>
</tr>
</tbody>
</table>

| In total: | 50 µl | 50 µl |

¹ Available from Dr S. Belák, Department of Virology, National Veterinary Institute, Box 585, Biomedical Centre, S-751 23, Uppsala, Sweden.
iii) PCR thermoprofiles

PCR\textsuperscript{I}-thermoprofile

\[\begin{align*}
5 \times & \quad 94^\circ C/45\text{ seconds}, 60^\circ C/60\text{ seconds}, 72^\circ C/90\text{ seconds} \\
30 \times & \quad 94^\circ C/45\text{ seconds}, 55^\circ C/60\text{ seconds}, 72^\circ C/90\text{ seconds} \\
1 \times & \quad 72^\circ C/420\text{ seconds} \geq 20^\circ C
\end{align*}\]

PCR\textsuperscript{II}-thermoprofile

\[\begin{align*}
5 \times & \quad 94^\circ C/45\text{ seconds}, 58^\circ C/60\text{ seconds}, 72^\circ C/90\text{ seconds} \\
30 \times & \quad 94^\circ C/45\text{ seconds}, 53^\circ C/60\text{ seconds}, 72^\circ C/90\text{ seconds} \\
1 \times & \quad 72^\circ C/420\text{ seconds} \geq 20^\circ C
\end{align*}\]

iv) Laboratory procedure

Mix PCR\textsuperscript{I}-reagents as described in step ii. Use separate gloves or tube openers for each individual tube when adding the DNA samples. Put the samples on ice. Heat the thermoblock to 80°C. Put samples in the thermoblock and start the PCR\textsuperscript{I}-programme (step iii).

Mix PCR\textsuperscript{II}-reagents as described in step ii. Use separate gloves or tube openers for each individual tube when adding the PCR\textsuperscript{I} product. Put the samples on ice. Heat the thermoblock to 80°C. Put samples in the thermoblock and start the PCR\textsuperscript{II}-programme (step iii).

• Agarose gel electrophoresis

Take the PCR\textsuperscript{II}-products to the electrophoresis laboratory. Load approximately 10–15 µl of the samples and 23 µl loading buffer on a 2% agarose gel containing ethidium bromide at 0.01%. Using 0.5 × Tris/borate/EDTA (TBE) buffer, electrophoresis is performed with 90 mA for 2 hours. To control the size of the amplification products, a 100 bp ladder is recommended. Analysis of PCR products is done by UV illumination.

• Interpretation of the results

i) Positive samples

Positive samples should have PCR products of the expected size (341 bp), similar to the positive control.

ii) Negative samples

Negative samples should have no PCR products of the expected size (341 bp), but mimic product (144 bp) should be present.

iii) Unclear results

The assay must be repeated if the positive controls (mimic or external positive control) are negative, or if the negative water controls are positive.

• Confirmatory testing

For confirmatory identification, the PCR products can be sequenced, hybridised to a probe, or analysed by restriction fragment length polymorphism (RFLP) analysis (11).

2. Serological tests

Infection with the virus in cattle is lifelong and gives rise to a persistent antibody response. Antibodies can first be detected 3–16 weeks after infection. Maternally derived antibodies may take up to 6 or 7 months to disappear. There is no way of distinguishing passively transferred antibodies from those resulting from active infection. Active infection, however, can be confirmed by the detection of BLV provirus by the PCR. Passive antibody tends to protect calves against infection. During the periparturient period, cows may have serum antibody that is undetectable by AGID because of an antibody shift from the dam’s circulation to her colostrum. Therefore, when using the AGID test, a negative test result on serum taken at this time (2–6 weeks pre- and 1–2 weeks post-partum) is not conclusive and the test should be repeated. However, the AGID can be performed at this stage with first-phase colostrum.

The antibodies most readily detected are those directed towards the gp51 and p24 of the virus. Most AGID tests and ELISAs in routine use detect antibodies to the glycoprotein gp51, as these appear earlier. Methods of performing these tests have been published (7, 9). ELISAs are usually more sensitive than the AGID tests.
Weak positive and negative OIE Standard Sera for use in ELISA are available in freeze-dried, irradiated form from the OIE Reference Laboratory in Germany (see Table given in Part 3 of this Terrestrial Manual). The calibration of these sera is based on the new, accredited OIE Standard serum, named ‘E05’, which has been validated against the former Standard serum E4 by different AGID and ELISAs. These sera can be used to establish ELISA sensitivity.

a) Enzyme-linked immunosorbent assay (a prescribed test for international trade)

Either an indirect or blocking ELISA may be used. Assays based on both of these are available commercially; different kits may be required for serum or milk samples. Some ELISAs are sufficiently sensitive to be used with pooled samples. ELISAs are carried out in solid-phase microplates. BLV antigen is used to coat the plates either directly or by the use of a capture polyclonal or monoclonal antibody (MAb). The antigen is prepared from the cell culture supernatant of persistently BLV-infected cell lines. Fetal lamb kidney (FLK) cells are most commonly used for commercial tests (20). Since 2004, a new BLV-producing cell line, PO714, which is free from other viral infections and contains a provirus of the Belgian subgroup, has been made available (4). The antigen is used at a predetermined dilution (e.g. 1/10) in phosphate buffered saline (PBS). In kit form, the plates are sometimes purchased precoated. Some preservatives may be added to milk samples to prevent souring. Preserved samples will not usually deteriorate significantly if stored for up to 6 weeks at 4°C.

• Indirect enzyme-linked immunosorbent assay – Milk ELISA

The following method is suitable for antibody detection in pooled milk samples.

• Controls

Strong positive, weak positive, negative milk and diluent controls should be included in each assay. A strong positive control should be prepared by diluting the OIE positive Standard Serum (E05) 1/25 in negative milk. A weak positive control should be prepared by diluting, in negative milk, the OIE positive Standard Serum (E05) 25 times the number of individual milk samples in the pool under test. The milk used for diluting the Standard Serum controls should be unpasteurised, cream free and preserved.

• Example test procedure

i) Milk samples must be stored, undisturbed in a refrigerator until a definite cream layer has formed (24–48 hours), or alternatively, centrifuged at 2000 rpm for 10 minutes, the cream layer should be removed prior to testing.

ii) A BLV antigen and a control negative antigen are precoated in alternate columns in the plate. 100 µl of test sample is added to 100 µl wash buffer in the plate to make a 1/2 dilution, adding to two control antigen wells and two BLV antigen wells.

iii) The plate is sealed and mixed on a shaker.

iv) The plate is incubated between 14 and 18 hours at 2–8°C.

v) 300 µl per well of wash diluent is added and discarded, and then 200 µl per well wash diluent is added, shaken for 10 seconds and discarded. Finally, 300 µl of wash diluent is added and soaked for 3 minutes and discarded.

vi) 200 µl per well of anti-bovine IgG-horseradish peroxidase affinity-purified conjugate diluted in wash diluent is added and the plate is incubated for 90 minutes at room temperature.

vii) The plate is washed by adding 300 µl of wash diluent per well; this is then discarded and a further 300 µl of wash diluent is added. This is left to soak for 3 minutes and discarded. Steps vi and vii are repeated.

viii) 200 µl of ABTS (2,2’-azino-bis-[3-ethylbenzothiazoline-6-sulphonic acid]) substrate (prewarmed to 25°C) is added and the plate is incubated for 20 minutes at room temperature in the dark. The reaction may be stopped by adding 50 µl of stopping solution.

• Reading and interpreting the results

The plate reader is blanked on air and the absorbance is read at 405 nm. All microplate wells must be read within 2 hours of addition of stopper. The absorbance readings of the wells containing negative antigen are subtracted from the readings of wells containing the positive antigen. The two net absorbance values for each test sample should be averaged. The same applies for the replicate weak positive controls. Replicates should be within 0.1 absorbance units of each other.

For the test to be considered valid, the averaged net absorbance of the weak positive (WP) controls should be 0.2–0.6 absorbance units. The net absorbance of the strong positive control should be >1.0 absorbance
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The absorbance of the negative control should be about 1.1 ± 0.4; if the absorbance is below 0.7, the colour development time in step iii above (preparation and addition of conjugates and substrate) should be increased. Conversely, the time should be shortened if the absorbance is above 1.5. The absorbance of the positive control should be less than the absorbance of the negative control × 0.25.

A sample is positive when the absorbance of each of the two test wells is identical with or less than the mean absorbance of the four negative wells × 0.5.

A sample is negative when the absorbance of each of the two test wells is identical with or higher than the mean absorbance of the four negative control wells × 0.65.

For samples giving values between the absorbance of the negative control × 0.5 and × 0.65 it is recommended to retest the animal, using a sample taken 1 month later.
• Sensitivity of the enzyme-linked immunosorbent assay

The sensitivity of pooled milk ELISAs can be evaluated using the OIE weak positive and negative Standard Sera. Assays should give a positive result on OIE standard serum E05 diluted in negative milk 250 times more than the number of individual milks in the pool (EU Directive 88/406). For example, for pools of 80 milks, E05 should be diluted 1/250 × 80 = 1/15000. For individual milk samples the positive OIE Standard Serum E05 diluted 1/250 in negative milk must be positive.

Where pooled serum samples are tested, the positive OIE Standard Serum E05 must test positive at a dilution 10 times higher than the number of individual animals in the pool. For example, for a pool of 50 individual samples, the positive OIE Standard Serum diluted 1/500 in negative serum should give a positive result. In assays where serum samples are tested individually, positive OIE Standard Serum E05 diluted 1/10 must be positive.

b) Agar gel immunodiffusion (a prescribed test for international trade)

The AGID test is a specific, but not very sensitive, test for detecting antibody in serum samples from individual animals. It is, however, unsuitable for milk samples (except first colostrums) because of lack of specificity and sensitivity. The AGID is simple and easy to perform and has proven to be highly useful and efficient as a basis for eradication schemes. Reference sera are included with commercial AGID test kits.

i) Agar gel: A 0.8–1.2% solution of agar or agarose is prepared in 0.2 M Tris buffer, pH 7.2, with 8.5% NaCl. One method of preparing the agar is to dissolve 24.23 g of Tris methylamine in 1 litre of distilled water and adjust to pH 7.2 with 2.5 M HCl. Sodium chloride (85 g) is dissolved in 250 ml Tris/HCl and made up to 1 litre. Agarose (8 g) is added and the mixture is heated in a pressure cooker or autoclave at 4.55 kg/sq. cm for 10 minutes. The mixture is dispensed in 15 ml aliquots, which can be stored at 4°C for up to approximately 6 weeks.

ii) Antigen: The antigen must contain specific glycoprotein gp51 of BLV. Antigen is prepared in a suitable cell culture system, such as permanently infected FLK cell monolayers. The cells used to produce the BLV antigen should be free from noncytopathic bovine viral diarrhoea virus and of bovine retroviruses, bovine Immunodeficiency-like virus (lentivirus), and bovine syncytial virus (spumavirus). After 3–4 days’ culture at 37°C, the growth medium is replaced with maintenance medium. The cells are harvested after 7 days using standard trypsin/versene solution. The cell suspension is centrifuged at 500 g for 10 minutes. Cells are resuspended in growth medium; 30% of the cells are returned to the culture vessel and the remainder is discarded. All culture supernatants are collected. The supernatants are concentrated 50–100-fold by available methods. This can be done by concentration in Visking tubing immersed in polyethylene glycol, or by precipitation with ammonium sulphate followed by ultrafiltration, or by precipitation in polyethylene glycol followed by desalting and size separation on a polyacrylamide bead column. The antigen contains gp51 predominantly, but may also contain p24.

The antigen may be standardised for glycoprotein gp51 by titration against the OIE standard Serum E05 as follows: a twofold dilution of the antigen preparation is made. The highest dilution that, when tested against undiluted standard serum E05, gives a precipitation line equidistant between the antigen and the serum will contain one unit. Two units of antigen are used in the test.

iii) Known positive control serum: The positive control serum comes from a naturally or experimentally infected animal (cattle or sheep). The precipitation line formed should be a sharp distinct line midway between the antigen and the control serum wells. A dilution of the control positive serum that gives a weak positive result should be included in the test as an indicator of the test’s sensitivity.

iv) Known negative control serum: Serum from uninfected animals (cattle, sheep) is used.

v) Test sera: Sera from any species of animal are suitable.

• Test procedure

i) The agar is melted by heating in a water bath and poured into Petri dishes (15 ml per Petri dish of diameter 8.5 cm). The poured plates are allowed to cool at 4°C for about 1 hour before holes are cut in the agar. A punch is used that cuts a hexagonal arrangement of six wells round a central well. Various dimensions of wells can be used; one satisfactory pattern has been produced using wells of 6.5 mm in diameter with 3 mm between wells. For best results, agar plates are used the same day that they are poured and cut.

ii) Antigen is placed in the central wells of the hexagonally arranged patterns. Test sera are placed alternately with positive control serum in the outer wells. There should be one control pattern per plate with positive control serum, weak positive control serum and negative control serum in the place of test sera.

iii) The test plates are kept at room temperature (20–27°C) in a closed humid chamber, and read at 24, 48 and 72 hours.
iv) **Interpretation of the results:** A test serum is positive if it forms a specific precipitation line with the antigen and forms a line of identity with the control serum. A test serum is negative if it does not form a specific line with the antigen and if it does not bend the line of the control serum. Nonspecific lines may occur; these do not merge with or deflect the lines formed by the positive control. A test serum is a weak positive if it bends the line of the control serum towards the antigen well without forming a visible precipitation line with the antigen; the reaction is inconclusive if it cannot be read either as negative or positive. A test is invalid if the controls do not give the expected results. Sera giving inconclusive or weak positive results can be concentrated and retested.

C. **REQUIREMENTS FOR VACCINES AND DIAGNOSTIC BIOLOGICALS**

There is no commercially available vaccine for EBL. The following is a summary of studies that have been conducted to try to produce an effective vaccine.

In young calves born to a BLV-infected mother, maternal antibodies to BLV gp51 might be important in protection against BLV infection. However, experiments using inactivated BLV, fixed infected FLK cells, and purified gp51 have indicated that these give only short-term protection. It was also found that vaccination of cattle with live cells from a cell line BL3, established from an animal with sporadic bovine leukosis, resulted in short-term protection. The nature of the antigen conferring protection was possibly a tumour-associated transplantation antigen (19). Ovine cells synthesising only the env gene products gp51 and gp30 and the main structural protein p24, induced a serological response in cattle (1); cattle were protected after repeated vaccination with these cells.

Vaccination in sheep with a recombinant vaccinia virus expressing BLV gp51 induced protection (16). However, protection was achieved without production of detectable levels of neutralising antibodies. It is therefore thought that a cell-mediated immune response may play a role in protective immunity against BLV infection. Expression of gp51 has been obtained by recombinant vaccinia virus and yeast containing the coding sequence of BLV env gene. These resulted in protection of sheep (8). Despite these advances in knowledge, there is as yet no vaccine available commercially for the control of EBL.

**REFERENCES**


* * *

**NB:** There are OIE Reference Laboratories for Enzootic bovine leukosis (see Table in Part 3 of this *Terrestrial Manual* or consult the OIE Web site for the most up-to-date list: www.oie.int).
CHAPTER 2.4.12.

HAEMORRHAGIC SEPTICAEMIA

SUMMARY

Haemorrhagic septicaemia (HS) is a major disease of cattle and buffaloes characterised by an acute, highly fatal septicaemia with high morbidity and mortality. It is caused by certain serotypes of Pasteurella multocida.

The diagnosis of HS depends on the isolation of the causative organism, P. multocida, from the blood or bone marrow of a dead animal by cultural and biological methods, and the identification of the organism by biochemical, serological and molecular methods.

Isolation and identification of the agent: Pure cultures of P. multocida can be obtained by streaking materials on to artificial media and the subsequent identification on the basis of the morphological, cultural, and biochemical characteristics of P. multocida.

Conventionally, the identification of the specific serotype is carried out using one or more serological methods. These include rapid slide agglutination, indirect haemagglutination for ‘capsular’ typing using sheep red blood cells coated with bacterial extracts, ‘somatic’ typing by agar gel immunodiffusion tests using heat-treated cell extracts, or agglutination using acid-treated cells. Confirmation of the isolates can be made using molecular techniques.

Serology: Serological tests for detecting specific antibodies are not normally used for diagnostic purposes.

Requirements for vaccines and diagnostic biologicals: Effective vaccines against haemorrhagic septicaemia are formalin-killed bacteria, or dense bacterins with adjuvants. The latter enhance the level and prolong the duration of immunity.

Seed cultures for the production of vaccines should contain capsulated organisms. Vaccines are standardised as to their bacterial density on the basis of turbidity tests and dry bacterial weight. Potency tests are carried out in mice and/or rabbits.

A. INTRODUCTION

Haemorrhagic septicaemia (HS) is a major disease of cattle and buffaloes occurring as catastrophic epizootics in many Asian and African countries, resulting in high mortality and morbidity (3, 5, 15, 21, 22, 31, 44). The disease has been recorded in wild mammals in several Asian and European countries (10, 41). In many Asian countries disease outbreaks mostly occur during the climatic conditions typical of monsoon (high humidity and high temperatures). The disease is caused by Pasteurella multocida, a Gram-negative coccobacillus residing mostly as a commensal in the upper respiratory tract of animals. The Asian serotype B:2 and the African serotype E:2 (Carter and Heddleston system), corresponding to 6:B and 6:E (Namioka-carter system), are mainly responsible for the disease (26). In wild animals, serotype B:2,5 is predominantly present. The association of other serotypes, namely A:1, A:3 with a HS-like condition in cattle and buffaloes in India has been recorded (29).

HS has been erroneously and widely used as a synonym for shipping fever and other infections. The result has been that the disease has been mistakenly reported in South America and elsewhere. There was similar confusion in the 1940s and the differences between the diseases have been clarified (1, 12). HS and shipping fever are two separate conditions caused by different bacteria (Pasteurella multocida vs Mannheimia haemolytica). Unlike HS, shipping fever is not septicaemic nor does it cause multisystemic petechial haemorrhages.

The clinical manifestations of the typical disease caused by B:2 or E:2 strains include a rise in temperature, respiratory distress with nasal discharge, and frothing from the mouth, and leads to recumbency and death.
Infection with serotypes A:1 and A:3 predominantly involves pneumonia resulting in mortality. Septicaemia is the characteristic feature in all the disease conditions. The incubation period varies from 3 to 5 days. In peracute cases, sudden death with observable clinical signs may be observed (15, 22). Buffaloes are generally more susceptible to HS than cattle and show more severe forms of disease with profound clinical signs. Subcutaneous oedema from the mandible to the brisket is one distinctive feature of the disease in endemic areas most deaths are confined to older calves and young adults.

Massive epizootics may occur in endemic as well as non-endemic areas (15, 22). In the recent past, HS has been identified as a secondary complication in cattle and buffalos following outbreaks of foot and mouth disease (FMD). Case fatality approaches 100% if treatment is not followed at the initial stage of infection (15, 22).

The diagnosis of the disease is based on the clinical signs, gross pathological lesions, morbidity and mortality patterns, and confirmation by isolation of the pathogens and their conventional and molecular characterisation.

**B. DIAGNOSTIC TECHNIQUES**

1. **Post-mortem lesions**

   Most animals succumbing to HS typically show swelling of the neck due to severe blood-tinge oedema. There are abundant petechial haemorrhages involving many tissues, and particularly serosal membranes. The thoracic, pericardial and abdominal cavities may contain serosanguinolent fluid. The lungs are congested and notably oedematous. Microscopically, there is interstitial pneumonia as well as focal infiltrates of neutrophils and macrophages in many tissues. These lesions are similar to those observed in severe sepsis.

2. **Isolation and identification of the agent**

   • **Cultural and biochemical methods**

     The septicaemia in HS occurs at the terminal stage of the disease. Therefore, blood samples taken from sick animals before death may not always contain *P. multocida* organisms. The latter are also not consistently present in the nasal secretions of sick animals.

     A blood sample or swab collected from the heart is satisfactory if it is taken within a few hours of death. If the animal has been dead for a long time, a long bone, free of tissue, can be taken. If there is no facility for post-mortem examination, blood can be collected from the jugular vein by incision or aspiration. Blood samples in any standard transport medium should be dispatched on ice and well packed to avoid any leakage.

     Blood smears from affected animals are stained with Gram, Leishman’s or methylene blue stains. The organisms appear as Gram-negative, bipolar-staining short bacilli. No conclusive diagnosis can be made on the basis of direct microscopic examinations alone.

     Blood samples, or swabs eluted into 2–3 ml sterile physiological saline, are cultured. Alternatively, the surface of a long bone is swabbed with alcohol and split open. The marrow is extracted aseptically and cultured. Direct culture is usually satisfactory only if the material is fresh and free from contaminants or post-mortem invaders that would otherwise overgrow any *Pasteurella* present.

     For biological examinations, a small volume (0.2 ml) of eluted blood swabs or a portion of bone marrow in saline is inoculated subcutaneously or intramuscularly into mice. The mouse usually serves as a biological ‘screen’ for extraneous organisms. If viable *P. multocida* is present, the mice die 24–36 hours following inoculation, and a pure growth of *P. multocida* can be seen in blood smears. Pure cultures of *P. multocida* can usually be grown from blood cultures of the mice, even when the original samples come from relatively old carcasses. The organism can be identified by its morphological and cultural characteristics, biochemical reactions and serological tests.

     A suitable medium for the growth of *Pasteurella* is casein/sucrose/yeast (CSY) agar containing 5% blood. The composition of this medium is casein hydrolysate (3 g), sucrose (3 g), yeast extract (5 g), sodium chloride (5 g), anhydrous dipotassium hydrogen orthophosphate (3 g), and distilled water to 1 litre. The pH is adjusted to 7.3–7.4, after which 1.5% agar is added. The medium is autoclaved at 1 bar for 15 minutes. After cooling to 45–50°C, 5% calf blood (antibody-free *P. multocida*) is added (51).

     Freshly isolated *P. multocida* forms smooth, greyish glistening translucent colonies, approximately 1 mm in diameter, on blood agar after 24 hours’ incubation at 37°C. Colonies grown on CSY agar are larger. Old cultures, particularly those grown on media devoid of blood, may produce smaller colonies. *Pasteurella multocida* does not grow on MacConkey agar. Gram-stained blood or tissue smears show Gram-negative, short, ovoid, bipolar-
staining coccoid forms. A degree of pleomorphism will be noted, particularly in old cultures, with longer rods of varying length. The bipolar staining will be more evident with methylene blue or Leishman’s stain.

HS organisms produce oxidase, catalase and indole, and will reduce nitrates. They do not produce hydrogen sulhide or urease, and fail to use citrate or liquefy gelatin. Glucose and sucrose are always fermented with the production of acid only. Most strains also ferment sorbitol. Some strains ferment arabinose, xylose and maltose, whereas salicin and lactose are almost invariably not fermented.

One property of HS-causing strains of *P. multocida* is the ability to produce the enzyme hyaluronidase (13). Having identified the genus and species by cultural characteristics and biochemical tests, hyaluronidase production may then be used as a specific test for HS-causing pasteurellae. It should be noted that B serotypes other than B:2 (or 6:B), and type E, are hyaluronidase negative.

A hyaluronic-acid-producing culture is streaked across the centre of a dextrose starch agar plate. The *Pasteurella* culture to be tested for hyaluronidase production is streaked at right angles. The plates are incubated at 37°C for 18 hours. Originally, hyaluronic-acid-producing *Streptococcus equi* was used, but a convenient culture for this purpose is a capsulated mucoid *P. multocida* type A culture. At the point of intersection, the mucoid growth of the hyaluronic acid producer will diminish into a thin line of growth, indicating the production of hyaluronidase by the test culture. Use of freshly prepared plates and a humidified incubator will facilitate hyaluronic acid production and, thereby, the interpretation of the test.

### Serotyping methods

Several serotyping tests are used for the identification of the HS-causing serotypes of *P. multocida*. These consist of a rapid slide agglutination test (35), an indirect haemagglutination (IHA) test for capsular typing (11), an agglutination test using hydrochloric-acid-treated cells for somatic typing (36), the agar gel immunodiffusion (AGID) test (4, 25, 52), and the counter immunoelectrophoresis test (CIEP) (14).

Hyperimmune antisera for most of these tests are prepared against specific reference strains in rabbits. Cultures in CSY broth (6–8-hours old) are seeded on to CSY blood agar medium. After overnight incubation (18–20 hours) the growth is washed into physiological saline containing 0.3% formalin. The turbidity of the cell suspension is adjusted to that of MacFarland’s tube No. 4. Rabbits are inoculated intravenously at 3–4-day intervals with 0.2, 0.5, 1.0, 1.5 and finally, 2.0 ml of this suspension. The rabbits are inoculated subcutaneously or intramuscularly 1 week after the last injection with 0.5 ml of a similar, but live, suspension. The animals are bled 10 days later. The serum is stored at –20°C, but small quantities for regular use are stored at 4°C with the addition of 1/10,000 merthiolate.

#### a) Rapid slide agglutination test (capsular typing)

A single colony is mixed with a drop of saline on a slide, a drop of antiserum is added, and the slide is warmed gently. A coarse, floccular agglutination appears within 30 seconds. Old cultures may give a fine, granular agglutination that takes longer to appear.

#### b) Indirect hemagglutination test (capsular typing)

This was originally performed using antigen-sensitised human type ‘O’ red blood cells (RBCs) (11), but more recently sheep RBCs have been used (42, 51). The antigen is prepared as follows:

A 6–8-hour broth culture of a reference strain is seeded on to CSY blood agar plates and incubated overnight at 37°C. The growth is harvested in 3 ml physiological saline containing 0.3% formalin. This suspension is then heated at 56°C for 30 minutes, centrifuged at 3000 g for 15 minutes at 4°C, and the clear supernatant fluid is stored at –20°C. If a refrigerated centrifuge is not available, centrifugation at 1500 g for 30 minutes gives a supernatant fluid. This is used as the antigen extract. A similar procedure is followed for preparing an antigen extract from an unknown strain that is to be typed.

Sheep blood is collected aseptically into an anticoagulant and centrifuged at 500 g for 10 minutes. The packed RBCs are washed three times in sterile physiological saline. The antigen extract from an unknown strain prepared by the method described above is used to sensitise the RBCs or absorbed on to the RBCs. This is done by adding 15 volumes of the antigen extract to the RBCs and incubating the mixture for 1 hour at 37°C with frequent shaking. The sensitised RBCs are recovered by centrifugation, washed three times in sterile physiological saline, and made up to a final 1% suspension in physiological saline. The type-specific hyperimmune antiserum (three volumes) is absorbed by the addition of packed RBCs (one volume) for 30 minutes at room temperature, then centrifuged at 500 g for 10 minutes to pellet the RBCs. The absorbed antiserum is then inactivated by heating at 56°C for 30 minutes.

The test itself can be carried out in tubes or plates, and is performed in two rows. The test described below is for standard microtitre plates.
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i) The capsular extract of the unknown strain is prepared as described above and used to sensitise the sheep RBCs. The known type-specific hyperimmune sera raised in rabbits against types A, B, D and E are diluted as follows:

ii) Using four separate rows of wells, the first wells are filled with 0.72 ml saline followed by 0.4 ml in the next six wells or more.

iii) The type-specific hyperimmune sera are each separately diluted in each row by adding 0.08 ml of the serum to the first well and mixing with a pipette. From this well 0.4 ml is transferred to the next well, mixed, and the process carried on until well seven. This constitutes 1/10 dilution in the first well and a doubling dilution thereafter.

iv) All the wells are each filled with 0.4 ml of antigen-adsorbed/sensitised RBCs, shaken slightly and left at room temperature. By the addition of the sensitised blood, the serum dilutions in the wells are doubled, i.e. 1/20 in well one, 1/40 in the second, and so on. A positive, negative and saline control are included for each test run.

v) The first reading is taken after 2 hours and a final reading after 18 hours. A course agglutination of the RBCs along the sides of the concave wells is taken as a positive reading, and the formation of a button at the centre of the wells as negative. An arbitrary score of 1–4 is given depending on the size of the agglutination. An unknown strain is identified with the hyperimmune serum that has agglutination. In the absence of agglutination with all sera, the strain is considered to be untypeable.

While IHA can be used for typing unknown strains, the test itself is more efficient when dealing with serotypes B and E and is more reliable as a quantitative test against these strains.

c) Agar gel immunodiffusion tests

AGID tests are used for what is described as ‘capsular’ as well as ‘somatic’ typing, depending on the antigens and antisera used. The double-diffusion technique is employed. Wells are punched in the solid agar in a circular pattern with one centre well surrounded by six peripheral wells.

i) Capsular typing: The gel medium is 1.0% Noble agar, or equivalent product, in 0.2 M phosphate buffer containing merthiolate at a final concentration of 1/10,000 (4, 52). Antigens and antisera are the same as for capsular typing by the IHA method (11). The standard antiserum is placed in the centre well, and the test antigens are placed in the peripheral wells alternately with standard homologous antigen.

ii) Somatic typing: The gel medium consists of special Noble agar, or equivalent product, at a concentration of 0.9% in 0.85% sodium chloride solution.

iii) For antigen preparation, the growth from each plate is harvested in 1 ml of 8.5% sodium chloride containing 0.3% formalin. The suspension is heated at 100°C for 1 hour, the cells are sedimented by centrifugation, and the supernatant fluid is used as antigen.

iv) Antisera against 16 somatic types (25) are prepared in chickens. Oil-emulsified bacterin\(^1\) (1 ml) is injected subcutaneously into the mid-portions of the neck of 12–16-week-old male birds. A further injection is made 3 weeks later of 1 ml intramuscularly into the breast, 0.5 ml on each side of the sternum. The birds are bled 1 week later, and the serum is separated and preserved with 0.01% thiomersal and 0.06% phenol. Sera are tested against all somatic types and sera that cross-react are discarded. Some antisera preparations against B:2 may cross react with the somatic type 5.

v) The test antigen is placed in the centre well and antisera against the different serotypes are placed in the peripheral wells. All haemorrhagic septicaemia serotypes (Asian and African) will react with type 2 antiserum. Cross-reactions may occur with type 5.

d) Counter immunoelectrophoresis

CIEP offers a rapid method for the identification of capsular types B and E cultures.

i) Preparation of capsular substance: Capsular substance is prepared in the same manner as described for the IHA test.

ii) Preparation of hyperimmune antisera: Antisera are prepared in rabbits as for the IHA test.

---

\(^1\) The bacterial antigens in broth are covered by a light mineral oil (adjuvant) and then emulsified (stabilised) with an emulsifying agent, in this case lanolin or lanoline (wool fat). This has to be done as the watery phase with the bacteria (broth) will not mix with the oily phase (adjuvant). The proportion of oil to emulsifying agent will vary with different batches of lanolin and will have to be adjusted accordingly. The higher the percentage of lanoline, the higher the stability of the emulsion. However, a high percentage of lanoline will make the emulsion very viscous, which will greatly hinder the vaccination process in the field as well as induce local reactions in vaccinated animals.
iii) Medium for CIEP: The medium for the CIEP consists of agarose (2.0 g), barbitone sodium (2.06 g), diethyl barbituric acid (0.37 g), distilled water (180 ml), and 1/1000 merthiolate (20 ml).

iv) Veronal acetate buffer (barbitone buffer): The barbitone buffer consists of barbitone sodium (29.24 g), anhydrous sodium acetate (11.70 g), 0.1 N hydrochloric acid (180 ml), and distilled water to 3 litres. The pH should be 8.8.

v) Preparation of slides: The electrophoresis plates are prepared by precoating glass slides (57 mm × 70 mm) with 12 ml volumes of the medium. Seven wells, 4 mm in diameter and 7 mm apart, are cut in a row. A parallel set of wells is cut 6 mm (centre to centre) away from the other set of wells.

vi) Test procedure: The well on the side of the cathode is loaded with a 20 µl volume of capsular antigen, while an equal volume of type-specific antiserum is loaded on to the well on the side of the anode. Controls included in the test are 0.85% sodium chloride solution against positive antiserum, and capsular extract against negative rabbit serum as well as positive and negative control samples. The electrophoresis tank is filled with barbitone buffer, pH 8.8. The antigen and antiserum are electrophoresed for 30 minutes at 150 V (25 V/cm). The plates are then examined for precipitation lines.

vii) Interpretation of the results: The presence of a distinct line between the antigen and antiserum wells is considered to be a positive result.

e) Agglutination tests (somatic antigen)

The somatic ‘O’ antigen is prepared by a method similar to that described previously for the IHA test (34, 36). A 6–8-hour test culture is seeded on to CSY blood agar and incubated overnight. The growth is harvested in 2–3 ml of physiological saline containing 0.3% formalin per plate, and centrifuged at 3000 g for 15 minutes at 4°C (or 1200–1500 g for 30–45 minutes at room temperature). The deposited bacteria are resuspended in 25 ml normal HCl saline (0.85% saline in a normal HCl solution) to give an opacity approximately equivalent to Brown’s opacity tube No. 6, and incubated overnight. The suspension is again centrifuged, the supernatant fluid is discarded, and the cell residue is washed three times successively in phosphate buffered saline (PBS) at pH 5.0, 6.0 and 7.0, respectively.

Finally, a suspension of the residual cells, equivalent to Brown’s opacity tube No. 6, is prepared in PBS at pH 7.0. Any suspensions that autoagglutinate should be discarded.

Antisera are prepared against whole bacterial cell suspensions of the reference strains B:2 (Asian HS), E:2 (African HS) and 11:B (Australian 989, non-HS). Agglutination tests are carried out on a slide and the test antigen is used against the three types of sera. A fine granular agglutination indicates a specific somatic agglutination. Tests carried out against the standard antigens will facilitate reading and interpretation. When nonspecific partial agglutination occurs, the tests carried out with tenfold dilutions of the serum against the test and standard antigens will help to identify somatic antigen.

f) Serotype designation

Broadly, two typing systems are adopted. One is ‘capsular’ typing by Carter’s IHA test (11) or by AGID tests (4, 52). The other is ‘somatic’ typing by the method of Namioka & Murata (34, 36, 37), and by the method of Heddleston et al. (25). It is generally agreed that designation of serotypes should be based on a somatic–capsular combination. Two systems commonly in use are the Namioka–Carter and the Carter–Heddleston systems. In the former system, Asian and African HS serotypes are designated 6:B and 6:E, respectively, while in the latter system they are designated B.2 and E.2, respectively.

g) Antimicrobial susceptibility testing

Antimicrobial susceptibility testing (AST) is particularly necessary for *P. multocida* for which resistance to commonly used antimicrobial agents has been reviewed by Kehrenberg et al. (27). AST methods are described in Chapter 1.1.6 Laboratory methodologies for bacterial antimicrobial susceptibility testing. The agar disk diffusion method has been used to test common fast-growing bacterial pathogens and is recognised to work well with *P. multocida* (6). Reliable results can be obtained with disk diffusion tests that use standardised methodology and zone diameter measurement correlated with minimum inhibitory concentration (MIC) and the behaviour of strains among clinically susceptible and resistant categorisations. Selection of the most appropriate antimicrobial agents to test is a decision best made by each laboratory in accordance with the needs of veterinary practitioners and the drugs available for veterinary use in the country. The following agents have proven their clinical efficacy: penicillin, amoxicillin (or ampicillin), cephalothin, cepflio, cefquinome, streptomycin, gentamicin, spectinomycin, florfenicol, tetracycline, sulfonamides, trimethoprim/sulfamethoxazole, erythromycin, tilmicosin, enrofloxacin (or other floroquinolones) and norfloxacin.
Nucleic acid recognition methods

a) *Pasteurella multocida*-specific PCR assay

PCR technology can be applied for rapid, sensitive and specific and/or detection of *P. multocida* (28, 30, 38, 39, 49). The rapidity and high specificity of two of the *P. multocida*-specific assays (30, 49) provide optimal efficiency without the need for additional hybridisation. Although the use of hybridisation can confirm specificity, this approach is usually possible only in specialised laboratories. The *P. multocida*-specific PCRs (30, 49) identify all subspecies of *P. multocida*. The Miflin & Blackall PCR (30) was described as giving a false positive with both *P. avium* biovar 2 and *P. canis* biovar 2, while the Townsend et al. PCR (49) gave a false positive with *P. avium* biovar 2. Recently, both *P. avium* biovar 2 and *P. canis* biovar 2 have been re-named as *P. multocida* (18) – meaning that both the Townsend et al. (49) and the Miflin & Blackall (30) PCR assays are now regarded as being specific for *P. multocida*. Some difficulties remain as it is now known that sucrose-negative *P. multocida*-like organisms from large cat bite wounds form two groups. While both are positive in the Miflin & Blackall *P. multocida*-specific PCR, (30) only one group has been confirmed as true *P. multocida* by other genotypic methods (19). The Townsend et al. (49) PCR is described in the following paragraph.

A fraction of an isolated colony of the suspect organism is transferred directly into the PCR mixture. Alternatively, template DNA can be obtained from 2 µl of either a mixed or pure broth culture. All currently used methods for the preparation of template DNA produce reproducible results with the KMT1 primers (49), and allow detection of ≤10 organisms per reaction. The sensitivity and specificity of the *P. multocida*-specific PCR offer the most compelling argument for the use of PCR technology in laboratory investigation of suspected HS cases. *Pasteurella multocida* can be detected regardless of the purity of the specimen, an advantage if the specimen is from an old carcass or from tonsil or nasal swabs. In such cases, the swab should be inoculated in 2 ml CSY broth and incubated on a roller for 2–4 hours; 2 µl of the culture is then added directly to the PCR mixture prior to amplification.

Primer sequences (49):

---

P.-multocida-specific PCR:  
KMT1T7 5'-ATC-CGC-TAT-TTA-CCC-AGT-GG-3'  
KMT1SP6 5'-GCT-GTA-AAC-GAA-CTC-GCC-AC-3'

PCR conditions:

Template DNA is added to the PCR mixture (total volume of 25 µl) containing 1 × PCR buffer, 200 µM each deoxynucleotide triphosphate (dNTP), 2 mM MgCl2, 3.2 pmol of each primer and 0.5 µ Taq DNA polymerase. Cycling parameters for a Corbett FTS-320 Thermocycler (or similar) are as follows: initial denaturation at 95°C for 5 minutes; 30 cycles of 95°C for 1 minute, 55°C for 1 minute, 72°C for 1 minute; with a final extension at 72°C for 7 minutes. The reaction is held at 4°C until required for electrophoresis; 5 µl of each sample is electrophoresed on a 2% agarose gel in 1 × Tris-acetate running buffer (TAE) at 4 V/cm for 1 hour. The gel is stained with 1% ethidium bromide and DNA fragments are viewed by UV transillumination. These primers have been increasingly used for quick identification of HS isolates (23, 43).

b) *Pasteurella multocida* multiplex capsular PCR typing system

Identification of the genes involved in the biosynthesis of the *P. multocida* A:1 (20) and B:2 (8) polysaccharide capsules provided the required information to determine the biosynthetic region of the remaining three serogroups (D, E, and F) (8). Moreover, with the use of serogroup specific multiplex PCR, conflicting results as regards to typing of some strains could be confirmed (46). With this knowledge, serogroup-specific sequences were identified for use as primers in a multiplex capsular PCR-typing system (8). The *P. multocida*-specific primers are included as an internal control for species identification.

Primer sequences (8):

---

Multiplex capsular PCR:  
CAPA-FWD 5'-TGC-CAA-AAT-CGC-AGT-GAG-3'  
CAPA-REV 5'-TTG-CGA-TTA-TTG-TCA-GTG-3'  
CAPB-FWD 5'-CAT-TTA-TCA-AAG-CTC-CAC-C-3'  
CAPB-REV 5'-GCC-CGA-GAG-TTT-CAA-TCC-3'  
CAPD-FWD 5'-TTA-CAA-AAG-AAA-GAC-TAG-GAG-CCC-3'  
CAPD-REV 5'-GCT-TG-GTC-TTG-ATT-GGC-3'  
CAPE-FWD 5'-TTG-CGA-AAA-GAA-TAT-TGA-CTC-3'  
CAPE-REV 5'-GCT-TGC-TGC-TTG-ATT-TTG-TC-3'  
CAPF-FWD 5'-AAT-CGG-AGA-AAG-CAG-AAA-TTA-CTC-3'  
CAPF-REV 5'-TTC-CGC-CGT-CAA-TTA-CTC-3'  
KMT1T7 5'-ATC-CGC-TAT-TTA-CCC-AGT-GG-3'  
KMT1SP6 5'-GCT-GTA-AAC-GAA-CTC-GCC-AC-3'
Size of resulting fragments:

<table>
<thead>
<tr>
<th>Serogroup</th>
<th>PCR Primers</th>
<th>Size</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>CAPA-FWD/CAPA-REV</td>
<td>1044 bp</td>
</tr>
<tr>
<td>B</td>
<td>CAPB-FWD/CAPB-REV</td>
<td>760 bp</td>
</tr>
<tr>
<td>D</td>
<td>CAPD-FWD/CAPD-REV</td>
<td>657 bp</td>
</tr>
<tr>
<td>E</td>
<td>CAPE-FWD/CAPE-REV</td>
<td>511 bp</td>
</tr>
<tr>
<td>F</td>
<td>CAPF-FWD/CAPF-REV</td>
<td>851 bp</td>
</tr>
</tbody>
</table>

PCR conditions:

Template DNA is added to the PCR mixture (total volume of 25 µl) containing 1 × PCR buffer, 200 µM each deoxynucleotide triphosphate (dNTP), 2 mM MgCl₂, 3.2 pmol of each primer and 1 u Taq DNA polymerase. In the original publication (8) it is suggested to use a standard cycling programme as per P.-multocida-specific PCR assay. However, validation of the multiplex PCR system indicates that the following optimised cycling programme should be used for the Perkin Elmer GeneAmp PCR System 2440: initial denaturation at 95°C for 5 minutes; 30 cycles of 95°C for 30 seconds, 55°C for 30 seconds, 72°C for 90 seconds; with a final extension at 72°C for 5 minutes. Agarose gel electrophoresis is as described above.

c) **HS-causing type-B-specific PCR assay**

Presumptive identification of HS-causing type-B-specific *P. multocida* is also possible by PCR amplification (9, 49). Comparative analysis with the *Haemophilus influenzae* Rd genome indicates that DNA regions amplified in both assays reside in close proximity, yet slight differences in specificity are evident. To date, the HS-causing type-B-specific PCR (49) remains 100% specific for HS-causing type B serotypes of isolated *P. multocida*. Type B cultures with the predominant somatic antigen being either type 2 or 5 are identified by the amplification of a ~620 bp fragment with the KTSP61 and KTT72 primers.

**Primer sequences** (49):

<table>
<thead>
<tr>
<th>HS-causing type-B-specific PCR</th>
<th>Primer Sequences</th>
</tr>
</thead>
<tbody>
<tr>
<td>KTSP61</td>
<td>5'-ATC-CGC-TAA-CAC-ACT-CTC-3'</td>
</tr>
<tr>
<td>KTT72</td>
<td>5'-AGG-CTC-GTT-TGG-ATT-ATG-AAG-3'</td>
</tr>
</tbody>
</table>

Conditions for HS-causing type-B-specific PCR are as described for *P. multocida*-specific PCR. The usefulness of these primers has been reported for identification of serogroup B strains.

HS-causing type-B-specific PCR primers can also be used in a multiplex PCR with the *P.-multocida*-specific primers, dramatically decreasing the time required for *P. multocida* detection and presumptive identification of the HS-serotype. Multiplex PCR conditions are as described above except that 3.2 pmol of each of the four primers and 1 u Taq DNA polymerase are used. The use of the multiplex *P.-multocida*-specific/HS-causing type-B-specific PCR on suspect organisms can confirm the identity and provide a presumptive serotype within 3–4 hours, in comparison with biochemical analysis and conventional serotyping, which can take up to 2 weeks.

d) **Pasteurella multocida** type A specific PCR

Primers useful for typing of serogroup A strains with several somatic types have been reported to be useful in specific identification of isolates (24).

**Primers:**

| **RGPMA5:** | 5'-AAT-GT-TTG-CGA-TAG-TCC-GTT-AGA-3' |
| **RGPMA6:** | 5'-ATT-TGG-CGC-CAT-ATC-ACA-GTC-3' |

**PCR conditions:**

Template DNA (50 ng) is added to the PCR mixture (total volume of 25 µl) containing 1 × PCR buffer, 200 µM each dNTPs, 1.5 mM MgCl₂, 20 pmol of each primer and 1 unit Taq DNA polymerase. Standard amplification conditions are as follows: initial denaturation at 95°C for 5 minutes; 30 cycles of 95°C for 45 seconds, 56°C for 45 seconds, 72°C for 6 minutes. Amplified products are separated by agarose gel electrophoresis (1.5% agarose gel) in 0.5 × TBE buffer at 5 v/cm for 2 hours.

The PCR amplification yields a product of 564 bp.

The test can be applied on direct culture, boiled cell lysate and infected tissues.
e) Genotypic differentiation of isolates

Once presumptive (or definitive) identification has been made, further differentiation of isolates can be achieved by genotypic fingerprinting methods. Restriction endonuclease analysis with the enzyme HhaI has proved useful for characterisation of type B HS-serotypes. Among 71 P. multocida capsule serogroup B isolates, 20 DNA fingerprint profiles were observed. With HS-causing serogroup strains, 13 unique profile among 54 isolates resembling the profile of the somatic serotype 2 reference strain have been reported (54). In contrast, while a single HhaI profile was observed among 13 serogroup E isolates, differentiation of these strains was possible following Hpal digestion. Hpal appear to generate finer subdivisions than those achieved with the use of HhaI (53). Ribotyping and large DNA separation by pulsed-field gel electrophoresis also provide useful discrimination of serogroup B and E P. multocida isolates (47). Amplified fragment length polymorphism (AFLP) has been found to be rapid and reproducible with high indices of discrimination of P. multocida strains (2). However, these techniques are largely used for research purposes and require specialised equipment. Moreover these profiles are not unique to country of origin or host species.

PCR fingerprinting is feasible for any laboratory with PCR capability, with several methods previously used for P. multocida differentiation. Random amplified polymorphic DNA (RAPD) analysis and arbitrarily primed PCR (AP-PCR), respectively, have been shown to be useful for epidemiological studies of P. multocida isolated from rabbits (17) and for differentiating post-vaccination isolates of P. multocida obtained from turkeys (26). Repetitive sequence PCR analysis of P. multocida has provided useful for discrimination of avian and swine isolates, although all HS-causing strains analysed demonstrated similar profiles (48, 50). However, molecular variability among HS-causing strains of P. multocida belonging to serogroup B has been found recently. Using repetitive extragenic palindromic (REP)-PCR, enterobacterial repetitive intragenic consensus (ERIC)-PCR and single primer PCR, genotypic differentiation among five P. multocida serogroup B isolates have been reported (7). RAPD and AP-PCR analysis of HS-causing P. multocida isolates have not been previously described.

3. Serological tests

Serological tests for detecting antibodies are not normally used for diagnosis. The IHA test can be used for this purpose, following a method broadly similar to that described for capsular typing above. High titres detected by the IHA test are indicative of recent exposure to HS. As HS is a disease that occurs mainly in animals reared under unsophisticated husbandry conditions, where disease-reporting systems are also poor, there is often considerable delay in notification of outbreaks. Deaths occur very suddenly and no carcasses are available for examination when notification is made. In such situations, high IHA titres from 1/160 up to 1/1280 or higher among in-contact animals surviving in affected herds, are indicative of recent exposure to HS for the purpose of diagnosis.

C. REQUIREMENTS FOR VACCINES AND DIAGNOSTIC BIOLOGICALS

The three types of vaccines used against HS are bacterins, alum-precipitated vaccine (APV) and oil-adjuvanted vaccine (OAV). To provide sufficient immunity with bacterins, repeated vaccination is required. Administration of dense bacterins can give rise to shock reactions, which are less frequent with the APV and almost nonexistent with the OAV.

A live HS vaccine prepared using an avirulent P. multocida strain B:3,4 (Fallow deer strain) has been used for control of the disease in cattle and buffaloes over 6 months of age in Myanmar since 1989. It is administered by intranasal aerosol application (16, 32, 33). The vaccine has been recommended by the Food and Agriculture Organization of the United Nations (FAO) as a safe and potent vaccine for use in Asian countries. However, there is no report of its use in other countries and killed vaccines are the only preparations in use by the countries affected with HS. A trial of the vaccine has been completed in Indonesia (40).

Guidelines for the production of veterinary vaccines are given in Chapter 1.1.8 Principles of veterinary vaccine production. The guidelines given here and in Chapter 1.1.8 are intended to be general in nature and may be supplemented by national and regional requirements.

1. Seed management

a) Characteristics of the seed

A local isolate of P. multocida representing the prevalent serotype is used. A well-capsulated, stable culture that produces large colonies of approximately 2 mm in diameter on CSY blood agar must be maintained. Seed cultures should be stored as semisolid nutrient agar stab cultures at room temperature, or as lyophilised cultures.
b) Method of culture

A calf is infected with the culture, and, within 2–3 hours of its death, blood is collected aseptically from the heart and stored at –20°C in 1 ml aliquots. A fresh aliquot is used for each new batch of vaccine. It is permissible to subculture this aliquot once or twice, provided the colony size does not diminish. A blood aliquot is thawed, plated on to CSY blood agar, and the growth is tested for agglutinability by the appropriate antiserum on a slide. A good culture will give a coarse floccular agglutination in under 30 seconds. A poor culture will yield only a fine granular agglutination.

Seed lots must be shown to be:

i) Pure: Free from adventitious agents.

ii) Safe: Produce no adverse reaction in the target species when given as recommended.

iii) Efficacious: Stimulate effective immunity as indicated by potency tests.

The necessary tests are described in Section C.4, below.

2. Method of manufacture

For vaccine production, dense suspensions of bacteria are necessary. They should have a minimum bacterial content of 1.5 g dry weight per litre of suspension. There are two methods of producing dense suspensions. The first is to culture on solid medium in Roux flasks and harvest in formalinised physiological saline, by which means suspensions of any density can be achieved. This is laborious as each flask must be harvested separately and tested for purity. The second and recommended method is the use of a large vessel with aerated cultures in a medium that specifically supports \( P. \) multocida. A suitable sterilised medium is casein hydrolysate (2 g), sucrose (6 g), yeast extract (6 g), sodium chloride (5 g), anhydrous dipotassium hydrogen orthophosphate (8.6 g), anhydrous potassium dihydrogen orthophosphate (1.36 g), and distilled water to 1 litre. A denser growth is obtained if the casein, sucrose and yeast are prepared as a concentrate, filter-sterilised or autoclaved for 10 minutes at 107°C, and transferred aseptically into the tank that has previously been heat-sterilised with the rest of the ingredients.

There are two types of aeration process – by vortexing and sparging. Sterile air is provided by a compressor. In vortex aeration, the culture is stirred by an impeller shaft operating in the air stream, whereas in sparging aeration, the air is dispersed through a sparger. Intermittent aeration seems to produce denser growth (45). The more finely dispersed the air, the better is the bacterial growth. Vessels of 20–40 litres are usually employed, and incubation is at 37°C. In continuous culture systems, once a maximum density has been reached, usually within 15 hours, about 25% of the working volume is harvested and replaced hourly. The harvests of continuous cultures are collected in relatively small volumes into separate vessels, but, after several days, the density diminishes, presumably through loss of capsular antigen. For this reason, batch cultures are preferred. If batch culture vessels are inoculated at a rate of 50 ml/litre of medium, maximum turbidity is obtained within 15–18 hours, when the growth can be terminated by the addition of formalin to a final concentration of 0.5%. This procedure, where a large inoculum is employed and the growth is terminated within a short period, helps to minimise the chances of contamination. The turbidity is standardised against a reference containing the equivalent dry weight/volume of 1.5 g/litre.

Dense cultures are also obtained using fermenters, where heat sterilisation of the tanks and culture can be carried out \textit{in situ}, with automatic temperature, pH and aeration control devices. Liquid sterilisation systems by filtration, for heat-labile components, can also be built into the fermenter. A 100 litre batch fermenter will yield a minimum of 66,000 doses (each of 3 ml) of OAV, and even more doses if the density is high enough for dilution to a reference standard equivalent to 1.5 g/litre, dry weight/volume.

OAV is made by the emulsification of equal volumes of a light mineral oil and the bacterial suspension, with 5% pure anhydrous lanolin as emulsifying agent. The mineral oil and lanolin are first sterilised and, on cooling to 40°C, 0.5% formalin is added to the mixture. The bacterial suspension is added slowly and emulsification is continued for a further 10 minutes. Following overnight storage, the mixture is re-emulsified, bottled and stored at 4°C for 2 weeks prior to use.

APV is prepared by first adjusting the turbidity of the suspension to the reference standard as above, and diluting it with an equal volume of 0.5% formalinised physiological saline. The pH is adjusted to 6.5, and a hot 20% solution of potash alum is added to give a final concentration of 1% alum. After overnight storage with continuous agitation, the vaccine is bottled for use.

3. In-process control

Proper concentration of bacterial growth, the capsulation of the bacteria, purity of culture and efficient inactivation all need to be checked.
4. Batch control

a) Sterility
Tests for sterility and freedom from contamination of biological materials may be found in Chapter 1.1.9.

b) Safety
Two seronegative cattle are vaccinated with twice the recommended dose and observed for 10–14 days for adverse effects.

Five mice are inoculated intramuscularly with 0.2 ml each of the vaccine, and observed for 5 days. The blood of any mouse that dies is cultured for *P. multocida*.

c) Potency
Potency tests can be carried out by any of the following methods:

i) Vaccination of cattle followed by direct challenge or passive mouse protection tests using the bovine sera. This procedure is not very feasible as cattle take a long time to develop adequate immunity after OAV;

ii) Vaccination of rabbits followed by direct challenge or passive mouse protection test using the rabbit sera; or

iii) Potency tests in mice, the most feasible method of the three.

Each of 50 mice is vaccinated intramuscularly with 0.2 ml of vaccine, and again 14 days later. On day 21, the mice are divided into ten groups of five, each group being challenged with respective dilutions of a 6–8-hour broth culture of a field strain in the range 10^{-1}–10^{-10}; 50 unvaccinated controls are similarly challenged, and all mice are observed for 5 days. The median lethal dose (LD_{50}) can then be calculated in order to obtain an indication of the dose that is sufficient to protect cattle: vaccines prepared in the manner described give at least 10^4 units protection in the vaccinated mice.

d) Duration of immunity
A single dose of vaccine administered to young calves 4–6 months of age will protect susceptible animals for 3–4 months when APV is used, and for 6–9 months when OAV is used.

e) Stability
The OAV emulsion should be pure white, and should stick to glass like paint. If the emulsion shows signs of cracking, it should be discarded. Separation of a thin layer of oil on the surface is permissible. It can be stored at 4–8°C for 6 months without any significant loss of potency. It must not be frozen. Increase in the content of lanolin improves stability, but also increases the viscosity – a distinct disadvantage. Use of other emulsifying agents such as ‘Arlacel’ helps to produce thinner, stable emulsions.

f) Method of use
The vaccine should be administered by deep intramuscular injection. The use of nylon 5 ml volume syringes for a 3 ml dose and a gauge 14–15 needle is advised, and the recommended age for primary vaccination is 4–6 months. For routine, prophylactic vaccination, a single dose of OAV at 4–6 months, a booster 3–6 months later, and annual revaccination thereafter, is recommended. Where husbandry practices are such that reaching individual animals at appropriate times is impracticable, annual vaccination of all animals over 4 months of age, preferably before the breeding season, and vaccination of all calves under 1 year of age, 6 months later, is recommended. In the face of an outbreak in vaccinated animals, one dose of APV, followed by one dose of OAV, is recommended.

g) Precautions (hazards)
Leakage of OAV into subcutaneous tissue can occasionally give rise to fibrous lumps at sites of injection. Rarely, abscesses may develop if sterility conditions are not observed, though most animals are resistant to such infections. APV may occasionally cause shock reactions.

5. Tests on the final product

a) Safety
See Section C.4.b.
Chapter 2.4.12. — Haemorrhagic septicaemia

b) Potency

See Section C.4.c.

REFERENCES


multibiological assay for the detection of *Pasteurella multocida* in the dog [in German]). *Kleintierpraxis*, 43, 69–74.


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OIE Terrestrial Manual 2008 751
CHAPTER 2.4.13.

INFECTIOUS BOVINE RHINOTRACHEITIS/
INFECTIOUS PUSTULAR VULVOVAGINITIS

SUMMARY

Definition of the disease: Infectious bovine rhinotracheitis/infectious pustular vulvovaginitis, caused by bovine herpesvirus 1 (BoHV-1), is a disease of domestic and wild cattle. The virus is distributed world-wide, but has been eradicated from Austria, Denmark, Finland, Sweden, Italy (Province of Bolzano), Switzerland and Norway and control programmes are running in some other countries.

Description of disease: The disease is characterised by clinical signs of the upper respiratory tract, such as a (muco)purulent nasal discharge, and by conjunctivitis. Signs of general illness are fever, depression, inappetance, abortions and reduced milk yield. The virus can also infect the genital tract and cause pustular vulvovaginitis and balanoposthitis. Post-mortem examinations reveal rhinitis, laryngitis and tracheitis. Mortality is low. Many infections run a subclinical course. Secondary bacterial infections can lead to more severe respiratory disease.

Identification of the agent: The virus can be isolated from nasal swabs or genital swabs, from animals with vulvovaginitis or balanoposthitis, taken during the acute phase of the infection, and from various organs collected at post-mortem.

For virus isolation, various cell cultures of bovine origin are used, for example, secondary lung or kidney cells or the Madin–Darby bovine kidney cell line. The virus produces a cytopathic effect in 2–4 days. It is identified by neutralisation or antigen detection methods using monospecific antisera or monoclonal antibodies. The BoHV-1 isolates can be further subtyped by DNA restriction enzyme analysis into subtypes 1.1, 1.2 and 1.3. BHV 1.2 isolates can be further differentiated into 2a and 2b. The virus previously referred to as BHV 1.3, a neuropathogenic agent, is now classified at BHV-5.

Viral DNA detection methods have been developed, and the polymerase chain reaction technique is increasingly used in routine diagnosis.

Serological tests: The virus neutralisation test and various enzyme-linked immunosorbent assays (ELISA) are most widely used for antibody detection. With an ELISA antibodies can be detected in serum and with lower sensitivity in milk.

Requirements for vaccines and diagnostic biologicals: Attenuated and killed vaccines are available. The vaccines must protect cattle clinically in case of infection and markedly reduce the subsequent shedding of field virus. The vaccines must not induce disease, abortion, or any local or systemic reaction, and must be genetically stable. BoHV-1 glycoprotein E deleted mutant marker vaccines are now generally available. The use of a gE ELISA makes it possible to distinguish infected cattle from cattle vaccinated with such a marker vaccine.

A. INTRODUCTION

Infectious bovine rhinotracheitis/infectious pustular vulvovaginitis (IBR/IPV), caused by bovine herpesvirus 1 (BoHV-1), is a disease of domestic and wild cattle. BoHV-1 is a member of the genus Varicellovirus in the subfamily alphaherpesvirinae, which belongs to the Herpesviridae family. The viral genome consists of double-stranded DNA that codes for about 70 proteins, of which 33 structural and up to 15 nonstructural proteins have been demonstrated. The viral glycoproteins, which are located in the envelope on the surface of the virion, play an important role in pathogenesis and immunity. BoHV-1 can be differentiated into subtypes 1.1, 1.2a and 1.2b,
and 1.3 (25). BoHV-1.3, which is a neuropathogenic agent, has been newly classified as BoHV-5 (21). The BoHV-1.2 subtypes may be less virulent than subtype 1.1 (11).

After an incubation period of 2–4 days, a serous nasal discharge, salivation, fever, inappetance, and depression become evident. Within a few days the nasal and ocular discharges change to mucopurulent. Where natural mating is practised, genital infection can lead to pustular vulvovaginitis or balanoposthitis. Many infections run a subclinical course (41).

Uncomplicated cases of respiratory or genital disease caused by BoHV-1 last 5–10 days.

The virus enters the animal via the nose and replicates to high titres in mucous membranes of the upper respiratory tract and in the tonsils. It subsequently disseminates to conjunctivae and by neuronal axonal transport reaches the trigeminal ganglion. After genital infection, BoHV-1 replicates in mucous membranes of the vagina or prepuce, and becomes latent in sacral ganglia. The viral DNA remains in the neurons of the ganglia, probably for the entire life of the host. Stress, such as transport and parturition, can induce reactivation of the latent infection. Consequently, the virus may be shed intermittently into the environment.

An infection normally elicits an antibody response and a cell-mediated immune response within 7–10 days. The immune response is presumed to persist for life, although it may fall below the detection limit of some tests. Maternal antibodies are transferred via colostrum to the young calf, which is consequently protected against BoHV-1-induced disease (24). Maternal antibodies have a biological half-life of about 3 weeks, but may be detected occasionally in animals up to 9 months old, and rarely in animals over this age.

The virus is distributed world-wide, paralleling the distribution of domestic cattle. Other ruminants may be infected with BoHV-1. After infection, nasal viral shedding is detected for 10–14 days, with peak titres of 10^8–10^10 TCID_{50} (50% tissue culture infective doses) per ml of nasal secretion. The semen of an infected bull may contain BoHV-1 and the virus can thus be transmitted by natural mating and artificial insemination (28).

The control of BoHV-1 is based on the normal hygienic measures taken on a farm. Ideally, a 2–3-week quarantine period is imposed for newly introduced cattle. Only cattle that are BoHV-1-seronegative are then admitted to the herd. Vaccines usually prevent the development of severe clinical signs and reduce the shedding of virus after infection, but do not prevent infection. Several eradication campaigns have been or are running in different countries including test-and-removal programmes and/or vaccination campaigns (see Section C).

BoHV-1 infection may be suspected as the cause of disease on the basis of clinical, pathological and epidemiological signs. To make a definite diagnosis, however, laboratory examinations are required. A complete diagnostic procedure in the laboratory is aimed at detecting the causative virus (or viral components) and the specific antibodies they induce.

**B. DIAGNOSTIC TECHNIQUES**

1. **Identification of the agent**

a) **Collection and processing of samples**

Nasal swabs are collected from several (from five to ten) affected cattle in the early phase of the infection. These cattle still have serous rather than mucopurulent nasal discharge. In cases of vulvovaginitis or balanoposthitis, swabs are taken from the genitals. The prepuce can also be washed with saline; the washing fluid is then collected. The specimens are suspended in transport medium (cell culture medium containing antibiotics and 2–10% fetal bovine serum to protect the virus from inactivation), cooled at 4°C, and rapidly submitted to the laboratory.

During necropsy, mucous membranes of the respiratory tract, and portions of the tonsil, lung and bronchial lymph nodes are collected for virus detection. In cases of abortion, the fetal liver, lung, spleen, kidney and a placental cotyledon are examined. Samples should be sent to the laboratory as quickly as possible, on ice.

After arrival at the laboratory, swabs are agitated in the transport medium to elute virus and left at room temperature for 30 minutes. Following removal of the swabs, the transport medium is clarified by centrifugation at 1500 g for 10 minutes. Tissues are homogenised to a 10–20% (w/v) suspension in cell culture medium before centrifugation at 1500 g for 10 minutes. The supernatants of these specimens are filtered through 0.45 µm filters and used for virus isolation.

The isolation of virus from semen needs some special adaptations, because the seminal fluid contains enzymes and other factors that are toxic to the cells and inhibit viral replication (see below).
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b) Virus isolation

For virus isolation, various cell cultures can be used. Primary or secondary bovine kidney, lung or testis cells, cell strains derived from bovine fetal lung, turbinate or trachea, and established cell lines, such as the Madin–Darby bovine kidney cell line, are all suitable. Cell cultures can be grown in glass or plastic tubes, plates or dishes. When 24-well plastic plates are used, a 100–200 µl volume of the supernatants described above is inoculated into these cell cultures. After a 1-hour adsorption period, the cultures are rinsed and maintenance medium is added. The serum used as a medium supplement in the maintenance medium should be free of antibodies against BoHV-1. The cell cultures are observed daily for CPE, which usually appears within 3 days after inoculation. It is characterised by grape-like clusters of rounded cells gathered around a hole in the monolayer; sometimes giant cells with several nuclei may be observed. Experience is needed to recognise this characteristic appearance. When, after 7 days, no CPE has appeared, a blind passage must be made. The cell culture is freeze–thawed and clarified by centrifugation, and the supernatant is used for inoculation of fresh monolayers (6, 9).

To identify the virus that produces the CPE as BoHV-1, the supernatant of the culture should be neutralised with a monospecific BoHV-1 antiserum or neutralising monoclonal antibody (MAb). For this purpose, serial tenfold dilutions of the test supernatant are made, and to each dilution monospecific BoHV-1 antiserum or negative control serum is added. Following incubation at 37°C for 1 hour, the mixtures are inoculated into cell cultures; 3–5 days later, the neutralisation index is calculated. The neutralisation index is the virus titre (in \( \log_{10} \)) in the presence of negative control serum minus the virus titre in the presence of specific antiserum. If the neutralisation index is greater than 1.5, the isolate may be considered to be BoHV-1. To shorten the virus isolation procedure, two specimens may be inoculated into cell culture: one that has been preincubated with monospecific antiserum and another that has been preincubated with negative control serum. If the CPE is inhibited by the monospecific antiserum, the isolate can be considered to be BoHV-1.

An alternative method of virus identification is by direct demonstration of BoHV-1 antigen in cells around the CPE by an immunofluorescence or immunoperoxidase test (16) with conjugated monospecific antiserum or MAb.

• Virus isolation from semen (a prescribed test for international trade)

One straw, 0.5 ml, of extended semen or 0.02 ml of raw semen, should be tested, with two passages in cell culture. For extended semen, an approximation should be made to ensure that the equivalent of 0.05 ml raw semen is examined. Raw semen is generally cytotoxic and should be prediluted before being added to cell cultures. A similar problem may sometimes arise with extended semen. A suitable test procedure is given below.

• Test procedure
  i) Dilute 200 µl fresh semen in 2 ml fetal bovine serum (free from antibody against BoHV-1) with added antibiotics.
  ii) Mix vigorously and leave for 30 minutes at room temperature.
  iii) Inoculate 1 ml of the semen/serum mixture into a monolayer of susceptible cells (see virus isolation above) in a six-well tissue culture plate.
  iv) Incubate the plates for 1 hour at 37°C.
  v) Remove the mixture, wash the monolayer twice with 5 ml maintenance medium, and add 5 ml maintenance medium to each well.
  vi) Include BoHV-1 negative and positive controls in the test. Extreme caution must be taken to avoid accidental contamination of test wells by the positive control, for example always handling the control last, and using separate plates.
  vii) Observe plates under a microscope daily for the appearance of a CPE. If a CPE appears, confirmatory tests for BoHV-1 are made by specific neutralisation or immunolabelling methods (see above).
  viii) If there is no CPE after 7 days, the cultures are frozen and thawed, clarified by centrifugation, and the supernatant is used to inoculate fresh monolayers.
  ix) The sample is considered to be negative if there is no evidence of a CPE after 7 days’ incubation of the passaged cultures.

c) Viral antigen detection

Nasal, ocular or genital swabs can be directly smeared on to glass cover-slips, or, following centrifugation, the cell deposit (see Section B.1.a) may be spotted on to cover-slips. These cover-slips are subjected to a standard direct or indirect fluorescent antibody test. In a direct immunofluorescence test, the monospecific antiserum is conjugated to fluorescein isothiocyanate, whereas in the indirect procedure it is the anti-
species immunoglobulin second antibody that is conjugated to fluorescein isothiocyanate. To obtain the best results, it is necessary to sample several animals in a herd that have fever and a slight, serous nasal discharge. Smears should be air-dried and fixed in acetone within 24 hours. Smears from nasal swabs from cattle with a purulent or haemorrhagic nasal discharge are often negative (37). The advantage of this antigen-detection technique is that it can lead to a same-day diagnosis. However, the sensitivity of this procedure is lower than that of virus isolation (9). Positive and negative controls must be included in each test.

Tissues collected at post-mortem can be examined for the presence of BoHV-1 antigen by the immunofluorescence test on frozen sections. Immunohistochemistry may also be used. The advantage is that the location of the antigen can be determined. MAbs are increasingly being used for detecting BoHV-1 antigen, leading to enhanced specificity of the test. However, such MAbs must be carefully selected, because they must be directed against conserved epitopes that are present on all isolates of BoHV-1.

Another possibility for direct rapid detection of viral antigen is the use of an enzyme-linked immunosorbent assay (ELISA). Antigen can be captured by MAbs or polyclonal antibodies coated on a solid phase, usually the well of a microplate. Amounts of antigen equivalent to $10^4$–$10^5$ TCID$_{50}$ of BoHV-1 are required in order to have a high rate of positive results (7). This may not be unrealistically high, because titres of $10^5$–$10^7$ TCID$_{50}$/ml of nasal fluid can be excreted by cattle 3–5 days after infection with BoHV-1. Sensitivity can be increased by amplification systems (see ref. 10 for an example).

The advantages of antigen-detection methods versus virus isolation are that no cell culture facilities are required and a laboratory diagnosis can be made in 1 day. The disadvantages are the lower sensitivity of direct antigen detection and the extra requirement to perform virus isolation, if the isolate is required for further study.

d) Nucleic acid detection

During the past decade, various methods of demonstrating BoHV-1 DNA in clinical samples have been described, including DNA–DNA hybridisation and the polymerase chain reaction (PCR). The PCR is also increasingly used in routine diagnostic submissions (26). Compared with virus isolation, the PCR has the primary advantages of being more sensitive and more rapid: it can be performed in 1–2 days. It can also detect DNA in latently infected sensory ganglia (38). The disadvantage is that it is prone to contamination and therefore precautions have to be taken to prevent false-positive results. Risk of contamination is markedly reduced by new PCR techniques, such as the so-called real-time or quantitative PCR (QPCR) (1, 20).

So far the PCR has been used mainly to detect BoHV-1 DNA in artificially (19) or naturally (38) infected semen samples. These workers found that it was important to thoroughly optimise the PCR conditions, including the preparation of the samples, the concentration of Mg$^{2+}$, primers and Taq polymerase, and the cycle programmes. The target region for amplification must be present in all BoHV-1 strains, and its nucleotide sequence must be conserved. The TK, gB, gC, gD and gE genes have been used as targets for PCR amplification. PCRs based on detection of gE sequences can be used to differentiate between wild-type virus and gE-deleted vaccine strains (14, 35). Discrimination between infection with virulent IBR strains and infection with other live attenuated strains is not possible with the PCR technique. PCRs have been developed that discriminate between BoHV-1 and BoHV-5 (2, 33).

Experimentally, the PCR was found to be more sensitive than virus isolation: in egg yolk-extended semen samples obtained from experimentally infected bulls, PCR detected five times as many positives as did virus isolation (39). In addition, it had a detection limit of only three molecules. Nevertheless, false-negative results cannot be excluded. To identify possible false-negative results, it is recommended to spike an internal control template into the reaction tube of the semen sample to be amplified by the same primers. Such a control template may be constructed by inserting, for example, a 100 base-pair fragment into the target region. This control template also makes it possible to semi-quantify the amount of DNA that is detected (33, 38). When using an internal control, extensive testing is necessary to ensure that PCR amplification of the added internal control does not compete with the diagnostic PCR and thus lower the analytical sensitivity (see also Chapter 1.1.5 Validation and quality control of polymerase chain reaction methods used for the diagnosis of infectious diseases).

c) Real-time polymerase chain reaction (a prescribed test for international trade)

The following real-time PCR test method has been developed to detect BoHV-1 in extended bovine semen intended for trade. The method has been validated according to Chapter 1.1.5, and includes a comprehensive international inter-laboratory comparison involving six collaborating laboratories with specialist status in IBR testing.

A number of studies has shown that PCR assays are more sensitive than virus isolation (36, 39, 42, 47). Real-time PCR has been used for detection of BoHV-1 and BoHV-5 in experimentally infected cattle and
mice (1, 20) and a number of conventional PCR assays have been used for the detection of BoHV-1 DNA in artificially or naturally infected bovine semen samples (8, 15, 23, 38, 44, 45, 47, 49). Conventional detection of amplified PCR products relies on gel electrophoresis analysis (32). Sequence-specific primers have been selected to amplify different parts of conserved glycoprotein gene of BHV-1 genome, including glycoprotein B (gB) gene (15, 34), gC gene (36, 39), gD gene (36, 47), gE gene (15), and the thymidine kinase (tk) gene (26, 50).

Real-time PCR differs from standard PCR in that the amplified PCR products are detected directly during the amplification cycle using a hybridisation probe, which enhances assay specificity. Real-time PCR assays have several advantages over the conventional PCR methods. Real-time PCR assays using only one pair of primers are able to provide sensitivity close or equal to nested PCR methods with a much lower risk of contamination. The amplification and detection of target is conducted simultaneously. There is no post-amplification PCR product handling, which significantly reduces the risk of contamination, and it is possible to perform quantitative analysis with real-time PCR systems.

The real-time PCR described here uses a pair of sequence-specific primers for amplification of target DNA and a 5′-nuclease oligonucleotide (TaqMan) for detection of amplified products. The oligonucleotide is a single, sequence-specific oligonucleotide labelled with two different fluorophores, the reporter/donor, 5-carboxyfluorescein (FAM) at the 5′ end, and the acceptor/quencher 6-carboxytetramethylrhodamine (TAMRA) at the 3′ end. This real-time PCR assay is designed to detect viral DNA of all BHV-1 strains, including subtype 1 and 2, from extended bovine semen. The assay selectively amplifies a 97 basepair sequence of the glycoprotein B (gB) gene. Details of the primers and probes are given in the protocol outlined below.

**Sample preparation, equipment and reagents**

i) The samples used for the test are, typically, extended bovine semen stored in liquid nitrogen. The semen samples can be transported to the laboratory in liquid nitrogen, or shipped at 4°C, and stored in liquid nitrogen or at –70°C (for long-term storage) or 4°C (for short-term storage). Storing semen at 4°C for a short period (up to 7 days) does not appear to affect PCR test result.

ii) Three straws from each batch of semen to be tested should be processed. Duplicate PCR amplifications should be carried out for each DNA preparation (six amplifications in total) to ensure the detection of DNA in samples containing low levels of virus.

iii) A real-time PCR detection system, and the associated data analysis software, is required to perform the assay. A number of real-time PCR detection systems are available from various sources. In the procedure described below, a RotorGene 3000, Corbett Research Ltd, Australia, was used. Other real-time PCR detection systems can also be used. Other equipment required for the test includes a micro-centrifuge, a heating block, a boiling water bath, a micro-vortex, magnetic stirrer and micropipettes.

Real-time PCR assays are able to detect very small amounts of target nucleic acid molecules therefore appropriate measures are required to avoid contamination 1.

iv) The real-time PCR assay described here involves two separate procedures. Firstly, BoHV-1 DNA is extracted from semen using Chelex-100 chelating resin, along with Proteinase K and DL-Dithiothreitol (DTT). The second procedure is the amplification and detection of the extracted DNA template by a real-time PCR detection system using a PCR reaction mixture: Platinum Quantitative PCR SuperMix-UDG, Invitrogen Technologies (note that there are a number of other commercial real-time PCR amplification kits available from various sources and the kit selected needs to be compatible with the real-time PCR platform selected). The required primers and probes can be synthesised by various commercial companies. In this protocol, all the primers and probes used were synthesised by Sigma-Genosys.

**Extraction of DNA**

i) In a screw top 1.5 ml tube, add:

- Chelex 100 sodium (Sigma) (10% w/v in distilled deionised water) 100 µl.
- Proteinase K (10 mg/ml, Sigma) 11.5 µl
- DL-Dithiothreitol (1 M, Sigma) 7.5 µl
- Nuclease-free water 90 µl
- Semen sample 10 µl
- Mix gently by pipetting 2.

---

1 Sources of contamination may include product carry-over from positive samples or, more commonly, from cross-contamination by PCR products from earlier experiments. Samples and reagents should be handled in separate areas, with separate equipment for reagent and sample preparation and amplification/detection.

2 It is important that Chelex 100 sodium be distributed evenly in the solution while pipetting, as Chelex 100 sodium is not soluble. This can be done by putting the vessel containing Chelex-100 solution on a magnetic stirrer while pipetting.
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ii) The sample tubes are incubated at 56°C for 30 minutes and then vortexed at high speed for 10 seconds.

iii) The tubes are then incubated in a boiling water bath for 8 minutes and then vortexed at high speed for 10 seconds.

iv) The tubes are centrifuged at 10,000 g for 3 minutes.

v) The supernatant is transferred into a new microtube and can be used directly for PCR, or stored at –20°C for testing at a later date.

• Preparation of reagents

The real-time PCR reaction mixture (Platinum Quantitative PCR SuperMix-UDG, or other reaction mixture) is normally provided as a 2× concentration ready for use. The manufacturer’s instructions should be followed for application and storage.

Working stock solutions for primers are made with nuclease-free water at the concentration of 4.5 µM and 3 µM, respectively. The stock solution of primers and probe are stored at –20°C and the probe solution should be kept in the dark. Single-use aliquots can be prepared to limit freeze-thawing of primers and probes and extend their shelf life.

• Real-time PCR test procedure

i) Primers and probe sequences

Selection of the primers and probe are outlined in Abril et al. (2004) and described below.

Primer gB-F: 5’-TGT-GGA-CCT-AAA-CCT-CAC-GGT-3’ (position 57499–57519 GenBank®, accession AJ004801)

Primer gB-R: 5’-GTA-GTC-GAG-CAG-ACC-CGT-GTC-3’ (position 57595–57575 GenBank®, accession AJ004801)

TaqMan Probe: 5’-FAM-AGG-ACC-GCG-AGT-TCT-TGC-CGC-TAMRA-3’ (position 57525–57545 GenBank®, accession AJ004801)

ii) Preparation of reaction mixtures

The PCR reaction mixtures are prepared in a clean laboratory room. All the reagents except the test samples are mixed before distribution to each individual reaction tube. For each PCR test, appropriate controls should be included. As a minimum, a no template control (NTC, reagent only), appropriate negative controls, i.e. 1 per 10 test samples, and two positive controls (strong and weak) should be included. Each test sample and control is tested in duplicate. The PCR amplifications are carried out in a volume of 25 µl.

a) PCR reagent mixtures are added in a clean room (no viral cultures, DNA extracts or post-amplification products should be handled here)

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>2 × Platinum Quantitative PCR SuperMix-UDG</td>
<td>12.5 µl</td>
</tr>
<tr>
<td>ROX reference dye (optional)</td>
<td>0.5 µl</td>
</tr>
<tr>
<td>Forward primer (gB-F, 4.5 µM)</td>
<td>1 µl</td>
</tr>
<tr>
<td>Reverse primer (gB-R, 4.5 µM)</td>
<td>1 µl</td>
</tr>
<tr>
<td>Probe (3 µM)</td>
<td>1 µl</td>
</tr>
<tr>
<td>Nuclease free water</td>
<td>4 µl</td>
</tr>
</tbody>
</table>

b) 5 µl of the DNA template are added to the PCR reagent mixture to a final volume of 25 µl. DNA samples are prepared and added in a separate room.

iii) Real-time (TaqMan) polymerase chain reaction

The PCR tubes are placed in the real-time PCR detection system in a separate, designated PCR room.

The PCR detection system is programmed for the test as follows:

PCR Reaction Parameters

One cycle: Hold 50°C 2 minutes

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3 Some DNA samples can become cloudy and a thin white membrane may form occasionally after freezing and thawing. This appears to have no influence on the PCR performance. No heating or re-centrifuging of the samples is necessary.

4 These PCR parameters are based on those most suitable for the RotorGene 3000, Corbett Research Ltd, Australia, and may vary with different PCR platforms.
One cycle: Hold 95°C 2 minutes \(^5\)
45 cycles: Hold 95°C 15 seconds
Hold 60°C 45 seconds

iv) Analysis of real-time PCR data

The threshold level is usually set according to the manufacturer's instructions for the selected analysis software used. Alternatively, virus isolation negative semen samples, from sero-negative animals, can be run exhaustively (e.g. up to 60 amplification cycles) to determine the background reaction associated with the detection system used.

- **Interpretation of results**
  - **Test controls**

  Positive and negative controls, as well as reagent controls, should be included in each PCR test. Negative semen, from virus isolation negative sero-negative bulls, can be used as a negative control. Positive semen from naturally infected bulls is preferable as a positive control. However, this might be hard to obtain. Alternatively, positive controls can be derived from negative semen spiked with known quantities of BoHV-1 virus.

  - **Test results**

    **Positive result:** Any sample that has a cycle threshold (Ct) value equal or less than 45 is regarded as positive. The positive control should have a Ct value within an acceptable range (± 3 Ct values) as previously determined by repeatability testing.

    **Negative result:** Any sample that shows no Ct value is regarded as negative. Negative control and no template control should have no Ct values.

f) Differentiation of bovine herpesvirus 1 subtypes and of viruses related to bovine herpesvirus 1

By using MAbs and immunofluorescence, radioimmunoprecipitation, immunoperoxidase or immunoblot assays, BoHV-1 subtype 1 and subtype 2b can be differentiated (31, 48). Restriction HindIII endonuclease analysis makes it possible to differentiate among all the recognised BoHV-1 subtypes 1, 2a and 2b. This differentiation is based on the molecular weight of three relevant DNA fragments \((l, k\) and \(l)\) (25). Restriction endonuclease analysis includes extraction of the DNA from virions or from infected cells, digestion of the isolated DNA by restriction endonucleases, and separation of the resulting fragments by agarose gel electrophoresis. Such techniques are of limited diagnostic value, but may be useful in epidemiological studies.

When differentiation between antigenically and genetically related alphaherpesviruses (BoHV-1, BoHV-5), caprine herpesvirus (CpHV-1), and cervine herpesvirus 1 (CvHV-1 and CvHV-2) is needed, improved methods are available using monoclonal antibodies (17).

g) Interpretation of results

The isolation of BoHV-1 from an animal does not unequivocally mean that this virus is the cause of the disease outbreak. It may, for instance, be a latent virus that has been reactivated due to stressful conditions. A confirmatory laboratory diagnosis must be made from a group of animals and must be accompanied by seroconversion from negative to positive, or a four-fold or higher titre rise in antibodies to BoHV-1. Cattle from which the nasal swabs are to be collected must be bled twice, 2–3 weeks apart. These paired serum samples are examined together in a serological test for the presence of specific antibody (see Section B.2).

2. Serological tests

Serological tests can be used for several purposes:

i) To diagnose an acute infection: serum samples from the acute and convalescent stages of infection in the same animals are examined in one test. A seroconversion from negative to positive or a four-fold or higher increase in antibody titre is considered to indicate infection.

ii) To demonstrate the absence of infection, for instance, for international trade purposes.

\(^5\) PCR Taq polymerase systems from different commercial sources may require a prolonged initial denaturation (95°C) time up to 10 minutes. Please follow the manufacturer’s instructions.
iii) To determine the prevalence of infection in seroepidemiological studies.

iv) To support eradication programmes and subsequent surveillance.

v) For research purposes, for instance, the evaluation of the antibody response after vaccination and challenge infection.

Virus neutralisation (VN) tests (4) and various ELISAs (19) are usually used for detecting antibodies against BoHV-1 in serum. Because virus latency is a normal sequel to BoHV-1 infection, the identification of serologically positive animals provides a useful and reliable indicator of infection status. Any animal with antibody to the virus is considered to be a carrier and potential intermittent excretor of the virus. The only exceptions to this are young calves that have acquired passive colostral antibody from their dam, and noninfected cattle vaccinated with inactivated vaccines.

In general, BoHV-1 serological tests can be divided into conventional and marker tests. The only marker serological test available at this time is the BoHV-1 gE antibody blocking ELISAs (40). For conventional serology, VNT, BoHV1 antibody blocking ELISAs as well as indirect ELISAs are used.

ELISAs, including the gE-ELISA, are increasingly used for the detection of antibodies in (bulk) milk samples (45), but have some limitations. By testing bulk milk, a positive gB-specific test indicates that the infection has already spread in the herd (13). With the gE blocking ELISA, bulk milk gives a positive reaction when more than 10–15% of the herd is infected (46). Consequently, it is not possible to declare a herd to be free from BoHV-1 infection on the basis of bulk or pooled milk tests, and a negative bulk milk test should be followed up with individual serum samples from all cattle in the herd. For general surveillance purposes, bulk milk tank tests can give an estimate of BoHV-1 prevalence in a herd, an area or country (27). These should be supplemented by serum testing (individual or pooled) from non-milking herds.

In a recent extensive study, tests for the detection of antibodies as routinely used by national reference laboratories in Europe were evaluated (18). Twelve reference laboratories from 12 European countries participated in this study. Fifty three serum samples and 13 milk samples, originating from several countries, were sent in duplicate under code to the participating laboratories. The serum samples included the three European reference sera EU1 (antibody positive), EU2 (antibody weak positive and defined as borderline sample) and EU3 (antibody negative) (30). It was concluded that VNT and gB-specific ELISAs are the most sensitive tests for the detection of antibodies in serum. In contrast, indirect ELISAs were found to be the most sensitive for the detection of antibodies in milk. Moreover, it was observed that commercially obtained ELISAs performed better than home-made ELISAs.

Recently, new indirect BoHV-1 ELISAs have been developed that are highly sensitive and specific. The results of these ELISAs are comparable with those obtained using gB blocking ELISAs or VNT (3).

a) Virus neutralisation (a prescribed test for international trade)

VN tests are performed with various modifications. Tests vary with regard to the virus strain used in the test protocol, the starting dilution of the serum, the virus/serum incubation period (1–24 hours), the type of cells used, the day of final reading and the reading of the end-point (50% versus 100%) (29). Of these variables, the virus/serum incubation period has the most profound effect on the antibody titre. A 24-hour incubation period may score up to 16-fold higher antibody titres than a 1-hour incubation period (4), and is recommended where maximum sensitivity is required (e.g. for international trade purposes). Various bovine cells or cell lines are suitable for use in the VN test, including secondary bovine kidney or testis cells, cell strains of bovine lung or tracheal cells, or the established Madin–Darby bovine kidney cell line.

A suitable protocol for a VN test is shown below.

i) Inactivate sera, including control standard sera, for 30 minutes in a water bath at 56°C.

ii) Make doubling dilutions of test sera in cell culture medium. Start with undiluted serum and continue to 1/1024 horizontally in a 96-well flat-bottomed cell-culture grade microtitre plate, at least two wells per dilution and 50 µl volumes per well. Dilutions of a positive control serum, and of weak positive and negative internal control sera, are also included in the test. An extra well with undiluted test serum is used for toxicity control of sera.

iii) Add 50 µl per well of BoHV-1 stock at a dilution in culture medium calculated to provide 100–200 TCID50 per well. In the toxicity control wells, add 50 µl of culture medium in place of virus. Add 100 µl of culture medium to ten empty wells for cell controls.

iv) Make at least four tenfold dilutions of the residual virus stock (back titration) in culture medium, using 50 µl per well and at least four wells per dilution.

v) Incubate the plates for 24 hours at 37°C.
vi) Add 100 µl per well of the cell suspension at 3 × 10⁴ cells per well.

vii) Incubate the plates for 3–5 days at 37°C.

viii) Read the plates microscopically for CPEs. Validate the test by checking the back titration of virus (which should give a value of 100 TCID₅₀ with a permissible range of 30–300 TCID₅₀), the control sera and the cell control wells. The positive control serum should give a titre of ± 1 twofold dilution (±0.3 log₁₀ units) from its target value. The weak positive serum should be positive. The negative serum should give no neutralisation when undiluted (equivalent to a final dilution of 1/2 at the neutralisation stage). In the cell control wells, the monolayer should be intact.

ix) The test serum results are expressed as the reciprocal of the dilution of serum that neutralised the virus in 50% of the wells. If 50% of the wells with undiluted serum neutralised the virus, the (initial dilution) titre is read as 1 (1/2 using the final dilution convention). If all the undiluted and 50% of the wells with 1/2 diluted serum neutralised the virus, the (initial dilution) titre is 2 (final dilution 1/4). For qualitative results, any neutralisation at a titre of 1 or above (initial dilution convention) is considered to be positive. If cytotoxicity is observed in the serum toxicity control wells, the sample is reported to be toxic (no result) unless neutralisation of the virus without cytotoxicity is observed at higher dilutions and a titre can be read without ambiguity. Where there is cytotoxicity with a serum from which it is critical to obtain a result, changing the medium in the wells of the lowest two or three dilutions 16–24 hours after the addition of cells will remove the cytotoxic effect with many problem sera.

b) Enzyme-linked immunosorbent assay (a prescribed test for international trade)

ELISAs for the detection of antibody against BoHV-1 appear to be gradually replacing VN tests. A standard procedure for ELISA has not been established. Several types of ELISA are commercially available, including indirect and blocking ELISAs, some of which are also suitable for detecting antibodies in milk (18). For reasons of standardisation in a country or state, it may be desirable to compare the quality of the kits and to perform batch release tests by previously defined criteria in one national reference laboratory, before it is used by other laboratories in the country.

There are a number of variations in the ELISA procedures. The most common are: antigen preparation and coating, the dilution of the test sample, the incubation period of antigen and test sample, and the substrate/chromogen solution. Before being used routinely, an ELISA should be validated with respect to sensitivity, specificity and reproducibility (see Chapter 1.1.4 Principles of validation of diagnostic assays for infectious diseases). For this purpose, a panel of well defined (e.g. by VN test) strong positive, weak positive and negative sera should be tested.

• Indirect enzyme-linked immunosorbent assay

The principle of an indirect ELISA is based on the binding of BoHV-1-specific antibodies present in the test sample to immobilised BoHV-1 antigen. The bound antibodies are detected using enzyme-labelled antobody-specific antibodies. The presence of antibodies in the test sample will result in colour development after addition of the substrate/chromogen solution.

• Blocking enzyme-linked immunosorbent assay

The principle of a blocking or competitive ELISA is based on blocking the binding of antigen to an enzyme-labelled BoHV-1 antigen and anti-BoHV-1 MAbs in the test sample. The presence of antibodies in the test sample will block binding, resulting in reduced colour development after addition of the substrate/chromogen solution. An example of a gB blocking ELISA procedure is given below:

i) Prepare the antigen by growing BoHV-1 in cell cultures. When extensive CPE is observed, cells and medium are frozen at –20°C. After thawing, the resulting cellular lysate is centrifuged for 4 hours at 8500 g. The virus-containing pellet is suspended in a small volume of phosphate buffered saline (PBS), cooled on ice and disrupted using an ultrasonic disintegrator. The antigen preparation is then centrifuged for 10 minutes at 800 g and inactivated if needed by adding detergent (final concentration of 0.5% Nonidet P 40). The antigen preparation is used at an appropriate dilution to coat the microtitre plates. Many alternative methods of antigen production may be found in the published literature.

ii) Coat the microtitre plates with antigen by adding 100 µl of diluted antigen (in 0.05 M carbonate buffer, pH 9.6) to each well. Seal the plates with tape, incubate at 37°C overnight, and store at –20°C.

iii) Before the test is performed, wash the plates with 0.05% Tween 80. Add 100 µl negative serum (fetal calf serum, FCS), 100 µl of each of the serum test samples and 100 µl of positive, weak positive and negative control sera. Usually, serum samples are tested undiluted. Shake, seal the plates and incubate overnight at 37°C. With some ELISAs, it is necessary to heat sera for 30 minutes at 56°C before testing in order to avoid weak nonspecific responses.
iv) Wash the plates thoroughly and add 100 µl of an anti-BoHV-1-gB-monoclonal antibody/horseradish peroxidase conjugate at a predetermined dilution, and incubate again for 1 hour at 37°C. The monoclonal antibody must be selected carefully for its specificity to gB of BoVH-1.

v) Wash the plates and add freshly prepared substrate/chromogen solution (e.g. 0.05 M citric acid buffer, pH 4.5, containing 2,2'-azino-bis-[3-ethylbenzothiazoline]-6-sulphonic acid [ABTS; 0.55 mg/ml] and a 3% solution of freshly added H₂O₂ [5 µl/ml]), and incubate for the appropriate time (1–2 hours at room temperature).

vi) Measure the absorbance of the plates on a microplate photometer at 405 nm.

vii) A test sample is considered to be positive if it has a blocking percentage of e.g. 50% or higher. The test is valid if the positive and weak positive control sera are positive and the negative control serum is negative. The acceptable limits for control and cut-off values must be determined for the individual assay.

c) Standardisation

In each serological test, appropriate controls of strong positive, weak positive and negative serum should be included. A scientific group in Europe, initiated by the group of artificial insemination veterinarians of the European Union (EU), has agreed on the use of a strong positive (EU1), a weak positive (EU2) and negative serum (EU3) for standardisation of BoHV-1 tests in laboratories that routinely examine samples from artificial insemination centres (30). These sera have been adopted as OIE international standards for BoHV-1 tests and are available in limited quantities at the OIE Reference Laboratories for IBR/IPV. Prescribed tests for international trade purposes (VN or ELISA) must be capable of scoring both the strong and weak positive standards (or secondary national standards of equivalent potency) as positive. Because of the limited availability of the international standard sera, there is a need to prepare a new extended panel of reference lyophilised serum (and milk) samples taken from infected as well as from vaccinated animals. This panel should be used to validate newly developed tests and to harmonise tests between laboratories.

C. REQUIREMENTS FOR VACCINES AND DIAGNOSTIC BIOLOGICALS

Several attenuated and inactivated BoHV-1 vaccines are available currently. The vaccines contain virus strains that have usually undergone multiple passages in cell culture. Some of the vaccine virus strains have a temperature-sensitive phenotype, i.e. they do not replicate at temperatures of 39°C or higher. Attenuated vaccines are administered intranasally or intramuscularly. Inactivated vaccines contain high levels of inactivated virus or portions of the virus particle (glycoproteins) supplemented with an adjuvant to stimulate an adequate immune response. Inactivated vaccines are given intramuscularly or subcutaneously.

Marker or DIVA (differentiation of infected from vaccinated animals) vaccines are now available in various countries. These attenuated or inactivated marker vaccines are based on deletion mutants or on a subunit of the virion, for instance glycoprotein D. The use of such marker vaccines in conjunction with companion diagnostic tests makes possible the distinction between infected and vaccinated cattle, and may thus provide the basis for eradication programmes of BoHV-1. Intensive vaccination programmes can reduce the prevalence of infected animals (5, 22), which could be monitored by using a companion diagnostic test. In situations where it is economically justifiable, the residual infected animals could then be culled, if necessary, resulting in a region free from BoHV-1. Control and eradication of BoHV-1 was started in some countries in the early 1980s. Different policies have been used due to differences in herd prevalence, breeding practices and disease eradication strategies. In the European Union at this time, only gE-deleted DIVA vaccines (live as well as killed) have been marketed and used for these control or eradication programmes.

Guidelines for the production of veterinary vaccines are given in Chapter 1.1.8 Principles of veterinary vaccine production. The guidelines given here and in Chapter 1.1.8 are intended to be general in nature and may be supplemented by national and regional requirements.

1. Seed management

a) Characteristics of the seed

The vaccine is prepared using a seed-lot system. The origin, passage history and storage conditions of the master seed virus (MSV) must be recorded. A virus identity test must be performed on the MSV. The seed...
lot contains BoHV-1 strains that have been attenuated to yield a live vaccine strain. The strains can be attenuated by multiple passages in cell cultures, by adapting virus to grow at low temperatures (temperature-sensitive mutants), or by genetic engineering, for example, by deleting one or more viral genes (e.g. the BoHV-1 glycoprotein E) that are nonessential for replication. There should be some means of distinguishing the live vaccine virus from field viruses (for example temperature-specific growth patterns or restriction fragment length polymorphisms). Strains used for the preparation of inactivated vaccines need not be attenuated. The seed lot must be free from contaminants.

b) Method of culture

The cells used for vaccine production are prepared using a seed-lot system. The virus should be cultured on established cell lines that have been shown to be suitable for vaccine production, for example the Madin–Darby bovine kidney cell line. The history of the cell line must be known. The cell line must be free from extraneous agents and may be tested for tumorigenicity.

c) Validation as a vaccine

Irrespective of the method of preparation of the seed-lot vaccine virus, the seed-lot virus destined for incorporation in a live vaccine must be shown to be efficacious, safe and pure.

i) Efficacy

This must be shown in a vaccination challenge experiment under laboratory conditions. Example guidelines are given in a monograph of the European Pharmacopoeia (12). Briefly, the vaccine is administered to five 2–3-month-old BoHV-1 seronegative calves. Two calves are kept as controls. All the calves are challenged intranasally 3 weeks later with a virulent strain of BoHV-1 that gives rise to typical signs of a BoHV-1 infection. The vaccinated calves should show no or only mild signs. The maximum virus titre found in the nasal mucus of vaccinated calves should be at least 100 times lower than that found in control calves. The virus excretion period should be at least 3 days fewer in vaccinated than in control calves.

ii) Safety

A quantity of virus equivalent to ten doses of vaccine should (a) not induce significant local or systemic reactions in young calves; (b) not cause fetal infection or abortion, and (c) not revert to virulence during five serial passages in calves. For inactivated vaccine, a double dose is usually administered. The reversion to virulence test is not applicable to inactivated vaccines.

iii) Purity

The seed lot is tested for absence of extraneous viruses and absence from contamination with bacteria, fungi or mycoplasmas. The following extraneous viruses should be specifically excluded in BoHV-1 vaccines: adenovirus, Akabane virus, bovine coronavirus, bovine herpesviruses 2, 4 and 5, bovine parvovirus, bovine respiratory syncytial virus, bovine viral diarrheaea virus, bovine rotavirus, vaccinia virus, and the viruses of Aujeszky’s disease, bluetongue, bovine ephemeral fever, bovine leukaemia, bovine papilloma, bovine papular stomatitis, cowpox, foot and mouth disease, lumpy skin disease, malignant catarrhal fever, parainfluenza 3, rabies, rinderpest, and vesicular stomatitis. As bovine viral diarrheaa virus (either CPE and/or non-CPE) has regularly been found to be a contaminant of vaccines, special attention should be paid to ensure that it is absent.

2. Method of manufacture

All substances used for the manufacture of vaccines must be free from contaminants. Cells should be used that are not further than 20 passages from the master cell seed. The seed virus should not be more than five passages from the MSV. Genetically engineered vaccine virus strains are treated in the same way as conventionally attenuated vaccine virus strains. When sufficient cells are grown, infection of the cell line with the vaccine virus takes place. The addition of antibiotics is normally restricted to cell culture fluids. The supernatant fluid is harvested at times when the virus (antigen) production peaks. For live vaccines, the supernatant is clarified, mixed with a stabiliser, freeze-dried and bottled. For the production of classical inactivated vaccines, the supernatant is homogenised before the inactivating agent is added in order to ensure proper inactivation. After the inactivation procedure, a test for detecting complete inactivation of the virus is carried out. The test should consist of at least two passages in cells. The inactivated virus suspension is then mixed with an adjuvant and bottled. The manufacture of vaccines must comply with guidelines for Good Manufacturing Practice (GMP).

3. In-process control

Working cell seed and working virus seed must have been shown to be free from contaminants. The cells must have their normal morphology before being inoculated with virus. They are checked for CPE during cultivation. Uninoculated control cells must have retained their morphology until the time of harvesting. A virus titration is
performed on the harvested supernatant. During the production of inactivated vaccines, tests are performed to ensure inactivation. The final bulk should be tested for freedom from contaminants.

4. Batch control

The following tests must normally be performed on each batch. Example guidelines for performing batch control can be found in EU directives, the European Pharmacopoeia and the United States Department of Agriculture’s Code of Federal Regulations.

a) Sterility
Bacteria, fungi, mycoplasmas and extraneous viruses must not be present. Tests for sterility and freedom from contamination of biological material may be found in Chapter 1.1.9.

b) Safety
For inactivated vaccines, a twofold dose of vaccine, and for live vaccines, a tenfold dose of vaccine, must not produce adverse effects in young BoHV-1 seronegative calves.

c) Potency
It is sufficient to test one representative batch for efficacy, as described in Section C.1.c.i. In the case of live vaccine, the virus titre of each batch must be determined and must be not higher than 1/10 of the dose at which the vaccine has been shown to be safe, and no lower than the minimum release titre. In the case of inactivated vaccines, the potency is tested using another validated method, for instance, efficacy assessment in calves.

d) Duration of immunity
It is sufficient to test this on the seed lot of vaccine virus. An efficacious BoHV-1 vaccine should induce protective immunity for at least 1 year, although many existing vaccines have not been tested to this standard.

e) Stability
For live vaccines, virus titrations should be carried out 3 months beyond the indicated shelf life. In addition, tests for determining moisture content, concentrations of preservatives, and pH are performed. For inactivated vaccines, the viscosity and stability of the emulsion are also tested.

f) Preservatives
The efficacy of preservatives should be demonstrated. The concentration of the preservative and its persistence throughout shelf life should be checked. The concentration must be in conformity with the limits set for the preservative.

g) Precautions (hazards)
No special precautions need to be taken. BoHV-1 is not pathogenic for humans.

5. Tests on the final product

a) Safety
Each product must be shown to be safe in at least two susceptible calves that receive a twofold (inactivated vaccine) or a tenfold (live vaccine) dose of vaccine.

b) Potency
See Section C.4.c.
REFERENCES


* * *

**NB:** There are OIE Reference Laboratories for Infectious bovine rhinotracheitis/infectious pustular vulvovaginitis (see Table in Part 3 of this *Terrestrial Manual* or consult the OIE Web site for the most up-to-date list: www.oie.int).
CHAPTER 2.4.14.

LUMPY SKIN DISEASE

SUMMARY

Lumpy skin disease (LSD, knopvelsiekte) is a pox disease of cattle characterised by fever, nodules on the skin, mucous membranes and internal organs, emaciation, enlarged lymph nodes, oedema of the skin, and sometimes death. The disease is of economic importance because it causes reduced production, particularly in dairy herds. It also causes damage to the hide. LSD is caused by strains of capripoxvirus that are antigenically indistinguishable from strains causing sheep pox and goat pox. However, LSD has a different geographical distribution to sheep and goat pox, suggesting that cattle strains of capripoxvirus do not infect and transmit between sheep and goats. Transmission of LSD virus is thought to be predominantly by insects, natural contact transmission in the absence of insect vectors being inefficient. Until 1988 LSD was confined to sub-Saharan Africa, but then spread into Egypt. There has been only one laboratory-confirmed outbreak of LSD outside Africa, in Israel in 1989, which was eliminated by slaughter of all infected and in-contact cattle, and vaccination. Outbreaks reported in Bahrain and Reunion in 1993 were not confirmed by virus isolation. There was an outbreak in 2000 in cattle imported into Mauritius; the diagnosis was confirmed by electron microscopy.

Identification of the agent: Laboratory confirmation of LSD is most rapid by the demonstration of typical capripox virions in biopsy material or desiccated crusts using the transmission electron microscope in combination with a clinical history of a generalised nodular skin disease and enlarged superficial lymph glands in cattle. Capripoxvirus is distinct from parapoxvirus, which causes bovine papular stomatitis and pseudocowpox, but cannot be distinguished morphologically from cowpox and vaccinia virus, both orthopoxvirus infections of cattle. Neither of these, however, causes generalised infection and both are uncommon in cattle. LSD virus will grow in tissue culture of bovine, ovine or caprine origin, although maximum yield is obtained using lamb testis cells. Capripoxvirus causes a characteristic cytopathic effect and intracytoplasmic inclusion bodies, and is distinct from the virus of pseudo-LSD (Allerton – herpes mammilitis), which is a herpesvirus producing syncytia and intranuclear inclusion bodies. The antigen of capripoxvirus can be demonstrated in tissue culture using immunoperoxidase or immunofluorescent staining and the virus can be neutralised using specific antisera.

Serological tests: The virus neutralisation test is the most specific serological test, but because immunity to LSD infection is predominantly cell mediated, the test is not sufficiently sensitive to identify animals that have had contact with LSD virus and developed only low levels of neutralising antibody. The agar gel immunodiffusion test and indirect immunofluorescent antibody test are less specific due to cross-reactions with antibody to other poxviruses. Western blotting using the reaction between the P32 antigen of LSD virus with test sera is both sensitive and specific, but is difficult and expensive to carry out. The use of this antigen, expressed by a suitable vector, in an ELISA offers the prospect of an acceptable and standardised serological test.

Requirements for vaccines and diagnostic biologicals: All strains of capripoxvirus examined so far, whether derived from cattle, sheep or goats, share immunising antigens. Attenuated cattle strains, and strains derived from sheep and goats have been used as live vaccines.
A. INTRODUCTION

Lumpy skin disease (LSD) was first seen in Zambia in 1929, spreading into Botswana by 1943 (16), and then into South Africa, where it affected over eight million cattle causing major economic loss. In 1957 it entered Kenya, associated with an outbreak of sheep pox (28). In 1970 LSD spread north into the Sudan, by 1974 it had spread west as far as Nigeria, and in 1977 was reported from Mauritania, Mali, Ghana and Liberia. Another epizootic of LSD between 1981 and 1986 affected Tanzania, Kenya, Zimbabwe, Somalia and the Cameroon, with reported mortality rates in affected cattle of 20%. However, the true extent of this epizootic was not clear, and it probably affected a considerable area of central Africa. In 2000/2001, another large outbreak spread across sub-Saharan Africa (11). In 1988 LSD became established in Egypt, and in 1989 a single outbreak was reported in Israel. LSD must be considered to have the potential to become established outside Africa. The principle method of transmission is mechanical by arthropod vector (6, 9).

The severity of clinical signs of LSD, (Neethling virus infection or knopvelsiekte), depends on the strain of capripoxvirus and the breed of host. Bos taurus is more susceptible to clinical disease than Bos indicus; the Asian buffalo has also been reported to be susceptible. Within Bos taurus, the fine-skinned Channel Island breeds develop more severe disease, with lactating cows appearing to be the most at risk. However, even among groups of cattle of the same breed kept together under the same conditions, there is a large variation in the clinical signs presented, ranging from subclinical infection to death (7). There may be failure of the virus to infect the whole group, depending on vector prevalence.

In the acutely infected animal, there is an initial pyrexia, which may exceed 41°C and persist for 1 week. The incubation period under field conditions has not been reported, but following inoculation is 6–9 days until the onset of fever. A rhinitis and conjunctivitis develop, and in lactating cattle there is a marked reduction in milk yield. Nodules of 2–5 cm in diameter develop over the body, particularly on the head, neck, udder and perineum between 7 and 19 days after virus inoculation (11). These nodules involve the dermis and epidermis and may initially exude serum, but over the following 2 weeks may become necrotic plugs that penetrate the full thickness of the hide. All the superficial lymph nodes are enlarged, the limbs may be oedematous and the animal is reluctant to move. The nodules on the mucous membranes of the eyes, nose, mouth, rectum, udder and genitalia quickly ulcerate, and by then all secretions contain LSD virus. On the appearance of clinical signs, the discharge from the eyes and nose becomes mucopurulent, and keratitis may develop. Nodules may also develop in the mouth, subcutis and muscle, in the trachea and alimentary tract, particularly the abomasum, and in the lungs, resulting in primary and secondary pneumonia. Pregnant cattle may abort, and there are reports of aborted fetuses being covered in nodules. Bulls may become permanently or temporarily infertile and the virus can be excreted in the semen for prolonged periods (18). Recovery from severe infection is slow; the animal is emaciated, may have pneumonia and mastitis, and the necrotic plugs of skin, which may have been subject to fly strike, are shed leaving deep holes in the hide (23).

LSD virus is not transmissible to humans.

B. DIAGNOSTIC TECHNIQUES

1. Identification of the agent

• Sample collection, submission and preparation

Material for virus isolation and antigen detection should be collected by biopsy or at post-mortem from skin nodules, lung lesions or lymph nodes. Samples for virus isolation and antigen-detection enzyme-linked immunosorbent assay (ELISA) should be collected within the first week of the occurrence of clinical signs, before the development of neutralising antibodies (12, 13). Samples for genome detection by polymerase chain reaction (PCR) may be collected when neutralising antibody is present. Following the first appearance of the skin lesions, the virus can be isolated for up to 35 days and viral nucleic acid can be demonstrated by PCR for up to 3 months (26, 27). Buffy coat from blood collected into heparin or EDTA (ethylene diamine tetra-acetic acid) during the viraemic stage of LSD (before generalisation of lesions or within 4 days of generalisation), can also be used for virus isolation. Samples for histology should include tissue from the surrounding area and should be placed immediately following collection into ten times the sample volume of 10% formalin.

Tissues in formalin have no special transportation requirements. Blood samples with anticoagulant for virus isolation from the buffy coat should be placed immediately on ice and processed as soon as possible. In practice, the samples may be kept at 4°C for up to 2 days prior to processing, but should not be frozen or kept at ambient temperatures. Tissues for virus isolation and antigen detection should be kept at 4°C, on ice or at −20°C. If it is necessary to transport samples over long distances without refrigeration, the medium should contain 10% glycerol; the samples should be of sufficient size that the transport medium does not penetrate the central part of the biopsy, which should be used for virus isolation.
Material for histology should be prepared by standard techniques and stained with haematoxylin and eosin (H&E) (2). Lesion material for virus isolation and antigen detection is minced using sterile scissors and forceps and then ground in a sterile pestle and mortar with sterile sand and an equal volume of sterile phosphate buffered saline (PBS) containing sodium penicillin (1000 international units [IU]/ml), streptomycin sulphate (1 mg/ml), mycostatin (100 IU/ml) or fungizone (2.5 µg/ml) and neomycin (200 IU/ml). The suspension is freeze–thawed three times and then partially clarified by centrifugation using a bench centrifuge at 600 g for 10 minutes. Buffy coats may be prepared from unclotted blood by centrifugation at 600 g for 15 minutes, and the buffy coat carefully removed into 5 ml of cold double-distilled water using a sterile Pasteur pipette. After 30 seconds, 5 ml of cold double-strength growth medium is added and mixed. The mixture is centrifuged at 600 g for 15 minutes, the supernatant is discarded and the cell pellet is suspended in 5 ml growth medium, such as Glasgow’s modified Eagle’s medium (GMEM). After centrifugation at 600 g for a further 15 minutes, the resulting pellet is suspended in 5 ml of fresh GMEM. Alternatively, the buffy coat may be separated from a heparinised sample by using a Ficoll gradient.

a) Culture

LSD virus will grow in tissue culture of bovine, ovine or caprine origin, although primary or secondary culture of lamb testis (LT) cells are considered to be the most susceptible, particularly those derived from a breed of wool sheep. Sample material prepared as above, i.e. 1 ml of clarified supernatant or buffy coat, is inoculated on to a 25 cm² culture flask at 37°C and allowed to absorb for 1 hour. The culture is then washed with warm PBS and covered with 10 ml of a suitable medium, such as GMEM, containing antibiotics and 2% fetal calf serum. If available, tissue culture tubes containing LT cells and a flying cover-slip, or tissue culture microscope slides, are also infected.

The flasks are examined daily for 14 days for evidence of cytopathic effect (CPE) and the medium is replaced if it appears to be cloudy. Infected cells develop a characteristic CPE consisting of retraction of the cell membrane from surrounding cells, and eventually rounding of cells and margination of the nuclear chromatin. At first only small areas of CPE can be seen, sometimes as soon as 2 days after infection; over the following 4–6 days these expand to involve the whole cell sheet. If no CPE is apparent by day 14, the culture should be freeze–thawed three times, and clarified supernatant inoculated on to fresh LT culture. At the first sign of CPE in the flasks, or earlier if a number of infected cover-slips are being used, a cover-slip should be removed, fixed in acetone and stained using H&E. Eosinophilic intracytoplasmic inclusion bodies, which are variable in size but up to half the size of the nucleus and surrounded by a clear halo, are diagnostic for poxvirus infection. The CPE can be prevented or delayed by inclusion in the medium of specific anti-LSD serum. The herpsivirus of pseudo-LSD produces a Cowdry type A intranuclear inclusion body. Formation of syncytia is not a feature of capripoxvirus infection, unlike the herpsivirus causing pseudo-LSD.

Strains of capripoxvirus that cause LSD have been adapted to grow on the chorioallantoic membrane of embryonated chicken eggs and African green monkey kidney (Vero) cells. This is not recommended for primary isolation.

- Electron microscopy

Before centrifugation, material from the original biopsy suspension is prepared for examination under the transmission electron microscope by floating a 400-mesh hexagon electron microscope grid, with pleoform-carbon substrate activated by glow discharge in pentylamine vapour, on to a drop of the suspension placed on paraaffin or a wax plate. After 1 minute, the grid is transferred to a drop of Tris/EDTA buffer, pH 7.8, for 20 seconds and then to a drop of 1% phosphotungstic acid, pH 7.2, for 10 seconds. The grid is drained using filter paper, air-dried and placed in the electron microscope. The capripox virion is brick shaped, covered in short tubular elements and measures approximately 290 × 270 nm. A host-cell-derived membrane may surround some of the virions, and as many as possible should be examined to confirm their appearance (21).

The virions of capripoxvirus are indistinguishable from those of orthopoxvirus, but, apart from vaccinia virus and cowpox virus, which are both uncommon in cattle and do not cause generalised infection, no other orthopoxvirus causes lesions in cattle. However, vaccinia virus may cause generalised infection in young immunocompromised calves. In contrast, orthopoxviruses are a common cause of skin disease in domestic buffalo causing buffalo pox, a disease that usually manifests as pock lesions on the teats, but may cause skin lesions at other sites, such as the perineum, the medial aspects of the thighs and the head. Orthopoxviruses that cause buffalo pox cannot be readily distinguished from capripoxvirus by electron microscopy. The virions of parapoxvirus that cause bovine papular stomatitis and pseudocowpox are smaller, oval in shape and each is covered in a single continuous tubular element that appears as striations over the virion. The capripoxvirus is also distinct from the herpsivirus that causes pseudo-LSD (Allerton – herpes mammillitis).
b) Immunological methods

- Fluorescent antibody tests
  Capripoxvirus antigen can also be identified on the infected cover-slips or tissue culture slides using fluorescent antibody tests. Cover-slips or slides should be washed and air-dried and fixed in cold acetone for 10 minutes. The indirect test using immune cattle sera is subject to high background colour and nonspecific reactions. However, a direct conjugate can be prepared from sera from convalescent cattle (or from sheep or goats convalescing from capripox) or from rabbits hyperimmunised with purified capripoxvirus. Uninfected tissue culture should be included as a negative control as cross-reactions, due to antibodies to cell culture, can cause problems.

- Agar gel immunodiffusion
  An agar gel immunodiffusion (AGID) test has been used for detecting the precipitating antigen of capripoxvirus, but has the disadvantage that this antigen is shared by parapoxvirus. Agarose (1%) is prepared in borate buffer, pH 8.6, dissolved by heating, and 2 ml is poured on to a glass microscope slide (76 × 26 mm). When the agar has solidified, wells are cut to give a six-well rosette around a central well. Each well is 5 mm in diameter, with a distance of 7 mm between the middle of the central well and the middle of each peripheral well. The wells are filled as follows: 18 µl of the 10% lesion suspension is added to three of the peripheral wells, alternately with positive control antigen, and 18 µl of positive capripoxvirus control serum is added to the central well. The slides are placed in a humid chamber at room temperature for 48 hours, and examined for visible precipitation lines using a light box. The test material is positive if a precipitation line develops with the control serum that is confluent with that produced by the positive control antigen.

To prepare antigen for the AGID, one of two 125 cm² flasks of LT cells is infected with capripoxvirus, and harvested when there is 90% CPE (8–12 days). The flask is freeze–thawed twice, and the cells are shaken free of the flask. The contents are centrifuged at 4000 g for 15 minutes, most of the supernatant is decanted and stored, and the pellet is resuspended in the remaining supernatant. The cells should be lysed using an ultrasonic probe for approximately 60 seconds. This homogenate is then centrifuged as before, the resulting supernatant being pooled with that already collected. The pooled supernatant is then added to an equal volume of saturated ammonium sulphate at pH 7.4 and left at 4°C for 1 hour. This solution is centrifuged at 4000 g for 15 minutes, and the precipitate is collected and resuspended in a small volume of 0.8% saline for use in the AGID test. The uninfected flask is processed in an identical manner throughout, to produce a tissue culture control antigen (20).

- Enzyme-linked immunosorbent assay
  Following the cloning of the highly antigenic capripoxvirus structural protein P32, it is possible to use expressed recombinant antigen for the production of diagnostic reagents, including the raising of P32 monospecific polyclonal antiserum and the production of monoclonal antibodies (MAbs). These reagents have facilitated the development of a highly specific ELISA (5). Using hyperimmune rabbit antiserum, raised by inoculation of rabbits with purified capripoxvirus, capripox antigen from biopsy suspensions or tissue culture supernatant can be trapped on an ELISA plate. The presence of the antigen can then be indicated using guinea-pig serum, raised against the group-specific structural protein P32, commercial horseradish-peroxidase-conjugated rabbit anti-guinea-pig immunoglobulin and a chromogen/substrate solution.

c) Nucleic acid recognition methods

The PCR is a fast and sensitive method for the detection of capripoxvirus genome in EDTA blood, biopsy or tissue culture samples. However, it does not allow differentiation between LSD and sheep and goat pox viruses. Primers for the viral attachment protein gene and the viral fusion protein gene (17) are specific for all the strains within the genus Capripoxvirus. By the use of sequence and phylogenetic analysis; strains of virus can be identified; this work should be done in a Reference Laboratory. Virus isolates can also be characterised by comparing the genome fragments generated by HindIII digestion of their purified DNA (1, 3, 19). This technique has identified differences between isolates from the different species, but these are not consistent and there is evidence of the movement of strains between species and recombination between strains in the field (14, 15).

The LSD virus genome contains 156 putative genes (25). An example of a published PCR method is described below (26).

- Polymerase chain reaction
  - Test procedure
    i) Freeze and thaw 200 µl of blood in EDTA and suspend in 1000 µl of lysis buffer.
    ii) Cut skin and other tissue samples into fine pieces using sterile scissors and forceps or disposable scalpel blade. Grind with a pestle in a mortar. Suspend the sample in 1000 µl of lysis buffer. (Lysis
buffer: 60 g guanidine thiocyanate; 0.378 g potassium chloride (KCl); 1 ml Tris (1 M, pH 8); and 0.5 ml Tween 20 in 100 ml of nuclease-free water).

iii) Add 1 µl of proteinase K (20 mg/ml, Invitrogen) to blood samples and 10 µl of proteinase K to tissue samples. Incubate with a dry block heater at 56°C for 2 hours, followed by heating at 100°C for 10 minutes, to inactivate the enzyme. Add 300 µl of a mixture of phenol: chloroform: isooamylalcohol (25:24:1) to blood samples and 1000 µl to tissue samples). Vortex and incubate at room temperature for 10 minutes. Centrifuge the samples at 10,000 g for 15 minutes at 4°C. Carefully collect the upper, aqueous phase and transfer into a clean 2.0 ml tube. Add two volumes of ice cold ethanol (100%). Place the samples at –20°C for 1 hour to precipitate the DNA. Centrifuge again at 10,000 g for 15 minutes at 4°C and discard the supernatant. Wash the pellets with 70% ethanol (100 µl) and centrifuge at 10,000 g for 1 minute at 4°C. Discard the supernatant and dry the pellets thoroughly. Suspend the pellets in 30 µl of nuclease-free water (26).

iv) The primers developed from the viral attachment protein encoding gene, are described by Ireland & Binepal (1998). The size of the amplicon is 192 bp. The primers have following gene sequences:

Forward primer 5’-TTT-CCT-GAT-TTT-TCT-TAC-TAT-3’
Reverse primer 5’–AAA-TTA-TAT-ACG-TAA-ATA-AC-3’.

v) DNA amplification is carried out in a final volume of 50 µl containing: 5 µl of 10 × PCR buffer, 1.5 µl of MgCl2 (50 mM), 1 µl of dNTP (10 mM), 1 µl of forward primer, 1 µl of reverse primer, 1 µl of DNA template, 0.5 µl of Taq DNA polymerase and 39 µl of nuclease-free water. The volume of DNA template required may vary and the volume of nuclease-free water must be adjusted to the final volume of 50 µl.

vi) Incubate the samples in a thermal cycler: first cycle: 2 minutes at 95°C (initial denaturation step), second cycle: 45 seconds at 95°C, 50 seconds at 50°C and 1 minute at 72°C. Repeat the second cycle 34 times. Last cycle: 2 minutes at 72°C (final elongation step) and hold at 4°C until analysis.

vii) Mix 10 µl of each sample with dye solution and load on 1.5% agarose gel in TBE (Tris-Borate-EDTA) buffer. Load a parallel lane with a 100 bp DNA-marker ladder. Separate the products at 80 V for 30–40 minutes and visualise.

2. Serological tests

All the viruses in the Capripoxvirus genus share a common major antigen for neutralising antibodies and it is not possible to distinguish strains of capripoxvirus from cattle, sheep or goats using serological techniques.

a) Virus neutralisation

A test serum can either be titrated against a constant titre of capripoxvirus (100 TCID50 [50% tissue culture infective dose]) or a standard virus strain can be titrated against a constant dilution of test serum in order to calculate a neutralisation index. Because of the variable sensitivity of tissue culture to capripoxvirus, and the consequent difficulty of ensuring the use of 100 TCID50, the neutralisation index is the preferred method. The test is described using 96-well flat-bottomed tissue-culture grade microtitre plates, but it can be performed equally well in tissue culture tubes with the appropriate changes to the volumes used, although it is more difficult to read an end-point in tubes. The use of Vero cells in the virus neutralisation test has been reported to give more consistent results.

• Test procedure

i) Test sera including a negative and a positive control are diluted 1/5 in Eagle’s/HEPES (N-2-hydroxyethylpiperazine, N-2-ethanesulphonic acid) and inactivated at 56°C for 30 minutes.

ii) Next, 50 µl of the first inactivated serum is added to columns 1 and 2, rows A to H of the microtitre plate. The second serum is placed in columns 3 and 4, the third in columns 5 and 6, the positive control serum is placed in columns 7 and 8, the negative control serum is placed in columns 9 and 10, and 50 µl of Eagle’s/HEPES without serum is placed in columns 11 and 12 and to all wells in row H.

iii) A reference strain of capripoxvirus, usually a vaccine strain known to grow well in tissue culture, with a titre of over log10 6 TCID50 per ml is diluted in Eagle’s/HEPES in bijoux bottles to give a log dilution series of log10 5.0; 4.0; 3.5; 3.0; 2.5; 2.0; 1.5 TCID50 per ml (equivalent to log10 3.7; 2.7; 2.2; 1.7; 1.2; 0.7; 0.2 TCID50 per 50 µl).

iv) Starting with row G and the most diluted virus preparation, 50 µl of virus is added to each well in that row. This is repeated with each virus dilution, the highest titre virus dilution being placed in row A.

v) The plates are covered and incubated for 1 hour at 37°C.

vi) LT cells are prepared from pregrown monolayers as a suspension of 105 cells/ml in Eagle’s medium containing antibiotics and 2% fetal calf serum. Following incubation of the microtitre plates, 100 µl of
cell suspension is added to all the wells, except wells H11 and H12, which serve as control wells for the medium. The remaining wells of row H are cell and serum controls.

vii) The microtitre plates are covered and incubated at 37°C for 9 days.

viii) Using an inverted microscope, the monolayers are examined daily from day 4 for evidence of CPE. There should be no CPE in the cells of row H. Using the 0240 KSOF vaccine strain of capripoxvirus, the final reading is taken on day 9, and the titre of virus in each duplicate titration is calculated according to Kärber (1931). If left longer, there is invariably a ‘breakthrough’ of virus in which virus that was at first neutralised appears to disassociate from the antibody.

ix) Interpretation of the results: The neutralisation index is the log titre difference between the titre of the virus in the negative serum and in the test serum. An index of ≥1.5 is positive. The test can be made more sensitive if serum from the same animal is examined before and after infection. Because the immunity to capripox is predominantly cell mediated, a negative result, particularly following vaccination in which the response is necessarily mild, does not imply that the animal from which the serum was taken is not protected.

A constant-virus/varying-serum method has been described using serum dilutions in the range 1/5 to 1/500 and fetal calf muscle cells. Because these cells have a lower sensitivity to capripoxvirus than LT cells, the problem of virus ‘breakthrough’ is overcome.

Antibodies to capripoxvirus can be detected from day 2 after the onset of clinical signs. These remain detectable for about 7 months, but a significant rise in titre is usually seen between days 21 and 42.

b) Agar gel immunodiffusion

The AGID test cannot be recommended as a serological test for the diagnosis of LSD because of the cross-reaction with antibody to bovine papular stomatitis and pseudocowpox virus. A consequence of this cross-reaction is false-positive results. Lack of sensitivity of the test can also lead to false-negative results.

c) Indirect fluorescent antibody test

Capripoxvirus-infected tissue culture grown on flying cover-slips or tissue culture microscope slides can be used for the indirect fluorescent antibody test. Uninfected tissue culture control, and positive and negative control sera, should be included in the test. The infected and control cultures are fixed in acetone at –20°C for 10 minutes and stored at 4°C. Dilutions of test sera are made in PBS, starting at 1/20 or 1/40, and positives are identified using an anti-bovine gamma-globulin conjugated with fluorescein isothiocyanate. Antibody titres may exceed 1/1000 after infection. Sera may be screened at 1/50 and 1/500. Cross-reactions can occur with orf (contagious pustular dermatitis of sheep virus), bovine papular stomatitis and perhaps other poxviruses.

d) Western blot analysis

Western blotting of test sera against capripoxvirus-infected cell lysate provides a sensitive and specific system for the detection of antibody to capripoxvirus structural proteins, although the test is expensive and difficult to carry out.

Capripoxvirus-infected LT cells should be harvested when 90% CPE is seen, freeze–thawed three times, and the cellular debris pelleted by centrifugation. The supernatant should be decanted, and the proteins should be separated by SDS/PAGE (sodium dodecyl sulphate/polyacrylamide gel electrophoresis). A vertical discontinuous gel system, using a stacking gel made up of acrylamide 5% in Tris (125 mM), pH 6.8, and SDS (0.1%), and a resolving gel made up of acrylamide (10–12.5%) in Tris (560 mM), pH 8.7, and SDS (0.1%), is recommended for use with a glycine running buffer containing Tris (250 mM), glycine (2 M), and SDS (0.1%). Samples of supernatant should be prepared by boiling for 5 minutes with an appropriate lysis buffer prior to loading. Alternatively, purified virus or recombinant antigens may replace tissue-culture-derived antigen.

Molecular weight markers should be run concurrently with the protein samples. The separated proteins in the SDS/PAGE gel should be transferred electrophoretically to a nitrocellulose membrane (NCM). After transfer, the NCM is rinsed thoroughly in PBS and blocked in 3% bovine serum albumin (BSA) in PBS, or 5% skimmed milk powder in PBS, on a rotating shaker at 4°C overnight. The NCM can then be separated into strips by employing a commercial apparatus to allow the concurrent testing of multiple serum samples, or may be cut into strips and each strip incubated separately thereafter. The NCM is washed thoroughly with five changes of PBS for 5 minutes on a rotating shaker, and then incubated at room temperature on the shaker for 1.5 hours, with the appropriate serum at a dilution of 1/50 in blocking buffer (3% BSA and 0.05% Tween 20 in PBS; or 5% milk powder and 0.05% Tween 20 in PBS). The membrane is again thoroughly washed and incubated (in blocking buffer) with anti-species immunoglobulin horseradish-peroxidase-conjugated immunoglobulins at a dilution determined by titration. After further incubation at room temperature for 1.5 hours, the membrane is washed and a solution of diaminobenzidin tetrahydrochloride
(10 mg in 50 ml of 50 mm Tris/HCl, pH 7.5, and 20 µl of 30% [v/v] hydrogen peroxide) is added. This is then incubated for approximately 3–7 minutes at room temperature on a shaker with constant observation, and the reaction is stopped by washing in PBS before excessive background colour is seen. A positive and negative control serum should be used on each occasion.

Positive test samples and the positive control will produce a pattern consistent with reaction to proteins of molecular weights 67, 32, 26, 19 and 17 kDa – the major structural proteins of capripoxvirus – whereas negative serum samples will not react with this pattern. Hyperimmune serum prepared against parapoxvirus (bovine papular stomatitis, pseudocowpox) will react with some of the capripoxvirus proteins, but not the 32 kDa protein that is specific for capripoxvirus.

e) **Enzyme-linked immunosorbent assay**

A capripoxvirus antibody ELISA has been developed using the expressed structural protein P32 of capripoxvirus and MAbs raised against the P32 protein (8).

### C. REQUIREMENTS FOR VACCINES AND DIAGNOSTIC BIOLOGICALS

Two live attenuated strains of capripoxvirus have been used as vaccines specifically for the control of LSD (3, 4): a strain of Kenya sheep and goat pox virus passaged 18 times in LT or fetal calf muscle cells, and a strain from South Africa, passaged 60 times in lamb kidney cells and 20 times on the chorioallantoic membrane of embryonated chicken eggs. All strains of capripoxvirus examined so far, whether of bovine, ovine or caprine origin, share a major neutralising site, so that animals recovered from infection with one strain are resistant to infection with any other strain. Consequently, it is possible to protect cattle against LSD using strains of capripoxvirus derived from sheep or goats (10). In 1969 and 1990 the Romanian strain of sheep pox vaccine was used to help control the LSD outbreak in Egypt (22). However, it is essential to carry out controlled trials, particularly using the most susceptible breeds in peak lactation, prior to introducing a vaccine strain not usually used in cattle. Protection following vaccination is probably lifelong, although as immunity wanes, local capripoxvirus replication will occur at the site of inoculation, but the virus will not become generalised. Both strains of capripoxvirus used routinely as vaccines can produce a large local reaction at the site of inoculation in the sheep (11), which some stock owners find unacceptable. This has discouraged the use of vaccine even though the consequences of an outbreak of LSD are invariably more severe.

A new generation of capripox vaccines is being developed that uses the capripoxvirus genome as a vector for the genes of other ruminant pathogens, for instance genes of rinderpest and peste des petits ruminants viruses. The recombinant vaccine will provide protection against LSD and rinderpest in a single vaccination (24, 27).

1. **Seed management**

a) **Characteristics of the seed**

A strain of capripoxvirus used for vaccine production must be accompanied by a history describing its origin and tissue culture or animal passage. It must be safe to use in all breeds of cattle for which it is intended, including pregnant animals. It must also be nontransmissible, remain attenuated after further tissue culture passage, and provide complete protection against challenge with virulent field strains for a minimum of 1 year. A quantity of master seed vaccine virus should be prepared and stored in order to provide a consistent working seed for regular vaccine production.

b) **Method of culture**

Vaccine seed should be lyophilised and stored in 2 ml vials at –20°C. It may be stored wet at –20°C, but when wet, it is more stable at –70°C or lower. The virus should be cultured in primary or secondary LT cells of wool sheep origin for maximum yield. Vero cells may also be used.

c) **Validation as a vaccine**

Seed lots must be shown to be:

i) **Pure**: Free from adventitious viruses, in particular pestiviruses, such as border disease and bovine viral diarrhoea virus, and free from contamination with bacteria, fungi and/or mycoplasmas.

ii) **Safe**: Produce minimum clinical reaction in all breeds of cattle when given by the recommended route.

iii) **Efficacious**: Stimulate complete immunity to LSD in all breeds of cattle for at least 1 year.

The necessary tests are described in Section C.4.
2. Method of manufacture

Vaccine batches are produced on fresh monolayers of secondary LT cells. A vial of seed virus is reconstituted with GMEM and inoculated onto an LT monolayer that has been previously washed with warm PBS, and allowed to absorb for 15 minutes at 37°C before being overlaid with additional GMEM. After 4–6 days, there will be extensive (50–70%) CPE. The culture is freeze–thawed three times, and the suspension is removed and centrifuged at 600 \( \text{g} \) for 20 minutes. Before harvest, the culture should be examined for any evidence of nonspecific CPE, medium cloudiness or change in medium pH. A second passage may be required to produce sufficient virus for a production batch (to produce enough for \( 10^6 \) doses, the yield from five 175 cm\(^2\) flasks is required).

The procedure is repeated and the harvests from individually numbered flasks are each mixed separately with an equal volume of sterile, chilled 5% lactalbumin hydrolysate and 10% sucrose, and transferred to individually numbered bottles for storage at –20°C. Prior to storage, 0.2 ml is removed from each bottle for sterility control. An additional 0.2 ml is removed; 2 ml pools composed of 0.2 ml samples taken from ten bottles are used for virus titration. A written record of all the procedures must be kept for all vaccine batches.

3. In-process control

**Cells:** Cells should be obtained from the testes of a healthy young lamb from a scrapie-free flock of a wool sheep breed. During cultivation, cells must be observed for any evidence of CPE, and for normal morphology (predominantly fibroblastic). They can usually be passaged successfully up to ten times. When used for vaccine production, uninfected control cultures should be grown in parallel and maintained for at least one additional passage for further observation. They should be checked for the presence of noncytopathic strains of bovine viral diarrhoea or border disease viruses by immunofluorescence or immunoperoxidase techniques. If possible, cells should be prepared and screened prior to vaccine production, and a stock stored in sterile DMSO (dimethyl sulphoxide) in liquid nitrogen (1–2 ml aliquots containing \( 20 \times 10^6 \) cells/ml). Serum used in the growth medium must be free from antibody to capripoxvirus and contamination with pestivirus.

**Virus:** Seed virus and final vaccine must be titrated in tissue culture tubes or microtitre plates. The minimum recommended field dose of the Kenyan and South African vaccines is \( \log_{10} 3.5 \) TCID\(_{50}\), although the minimum protective dose is \( \log_{10} 2.0 \) TCID\(_{50}\). Capripoxvirus is highly susceptible to inactivation by sunlight, and allowance should be made for loss of activity in the field. The recommended field dose of the Romanian sheep pox vaccine for cattle is \( \log_{10} 2.5 \) sheep infective doses (SID\(_{50}\)), and the recommended dose for cattle of the RM65-adapted strain of Romanian sheep pox vaccine is \( \log_{10} 3 \) TCID\(_{50}\) (11). Vaccine samples must be examined for the presence of adventitious viruses including cytopathic and noncytopathic strains of pestivirus, and should be mixed with a high titre capripoxvirus-immune serum that has previously tested negative for antibodies to pestiviruses to prevent the vaccine virus itself interfering with the test. The vaccine can be held at –20°C until all sterility tests and titrations have been completed, at which time it should be freeze-dried. A further titration is carried out on five randomly chosen vials of the freeze-dried preparation to confirm the titre.

4. Batch control

**a) Sterility**

Tests for sterility and freedom from contamination with biological materials may be found in Chapter 1.1.9.

**b) Safety and efficacy**

Six cattle of known susceptibility to LSD are placed in a high containment level large animal unit and serum samples are collected. Five randomly chosen vials of the freeze-dried vaccine are reconstituted in sterile PBS and pooled. Two cattle are inoculated with 100 times the field dose of the vaccine, the remaining vaccine is diluted with sterile PBS and two cattle are inoculated subcutaneously with the recommended field dose. The remaining two cattle are control animals. The animals are clinically examined daily and rectal temperatures are recorded. On day 21 after vaccination, the six animals are again serum sampled and challenged with a known virulent capripoxvirus strain by intradermal inoculation. The clinical response is recorded during the following 14 days. Control animals should develop the typical clinical signs of LSD, whereas there should be no local or systemic reaction in the vaccines other than a delayed-type hypersensitivity reaction, which should disappear after 4 days. Serum samples are again collected on day 30 after vaccination. The day 21 serum samples are examined for seroconversion to selected viral diseases that could have contaminated the vaccine, and the days 0 and 30 samples are compared to confirm the absence of antibody to pestivirus. Because of the variable response in cattle to LSD challenge, generalised disease may not be seen in the control animals, although there should be a large local reaction.

The fully reconstituted vaccine is also tested in mice and guinea-pigs. Two guinea-pigs are inoculated intramuscularly with 0.5 ml into the hind leg, and two guinea-pigs and six mice are inoculated...
intraperitoneally with 0.5 ml and 0.1 ml, respectively. Two guinea-pigs and four mice are kept as uninoculated controls. The animals are observed for 3 weeks, humanely killed and a post-mortem examination is carried out. There should be no evidence of pathology due to the vaccine.

c) Potency tests
Potency tests in cattle must be undertaken for vaccine strains of capripoxvirus if the minimum immunising dose is not known. This is usually carried out by comparing the titre of a virulent challenge virus on the flanks of vaccinated and control animals. Following vaccination, the flanks of at least three animals and three controls are shaved of hair. Log₁₀ dilutions of the challenge virus are prepared in sterile PBS and six dilutions are inoculated intradermally (0.1 ml per inoculum) along the length of the flank; four replicates of each dilution are inoculated down the flank. An oedematous swelling will develop at possibly all 24 inoculation sites on the control animals, although preferably there will be little or no reaction at the four sites of the most dilute inocula. The vaccinated animals should develop an initial hypersensitivity reaction at sites of inoculation within 24 hours, which should quickly subside. Small areas of necrosis may develop at the inoculation site of the most concentrated challenge virus. The titre of the challenge virus is calculated for the vaccinated and control animals; a difference of log titre $>\log_{10} 2.5$ is taken as evidence of protection.

d) Duration of immunity
Immunity to virulent field virus following vaccination lasts 2 years with the Kenyan strain and 3 years with the South African vaccine, and protection against generalised infection following intradermal challenge is effectively lifelong. The duration of immunity produced by other vaccine strains should be ascertained in cattle by undertaking controlled trials in an environment in which there is no possibility of field strains of capripoxvirus confusing the results.

e) Stability
Properly freeze-dried preparations of LSD vaccine, particularly those that include a protectant, such as sucrose and lactalbumin hydrolysate, are stable for over 25 years when stored at –20°C and for 2–4 years when stored at 4°C. There is evidence that they are stable at higher temperatures, but no long-term controlled experiments have been reported.

f) Preservatives
No preservatives other than a protectant, such as sucrose and lactalbumin hydrolysate, are required for the freeze-dried preparation.

g) Precautions (hazards)
There are no precautions other than those described above for sterility and freedom from adventitious agents. Strains of LSD virus are not a hazard to human health.

5. Tests on the final product

a) Safety
Safety tests should be carried out on the final product of each batch as described in Section C.4.b.

b) Potency
Once the potency of the particular strain being used for vaccine production has been determined in terms of minimum dose required to provide immunity, it is not necessary to repeat this on the final product of each batch, provided the titre of virus present has been ascertained.

REFERENCES


* * *

**NB:** There are OIE Reference Laboratories for Lumpy skin disease (see Table in Part 3 of this *Terrestrial Manual* or consult the OIE Web site for the most up-to-date list: www.oie.int).
CHAPTER 2.4.15.

MALIGNANT CATARRHAL FEVER

SUMMARY

Malignant catarrhal fever (MCF) is an acute, generalised and usually fatal disease affecting many species of Artiodactyla. The disease has been most often described as affecting species of the subfamily Bovinae and family Cervidae, but is also recognised in domestic pigs as well as giraffe and species of antelope belonging to the subfamily Tragelaphinae. MCF is defined by the recognition of characteristic lymphoid cell accumulations in nonlymphoid organs, vasculitis and T-lymphocyte hyperplasia in lymphoid organs, the main cause of which is either of two gammaherpesviruses. The alcelaphine herpesvirus-1 (AIHV-1), which is maintained by inapparently infected wildebeest, causes the disease in cattle in regions of Africa and in a variety of ruminant species in zoological collections world-wide. Ovine herpesvirus-2 (OvHV-2), which is prevalent in all varieties of domestic sheep as a subclinical infection, is the cause of MCF in most regions of the world. This form of the disease is also known as sheep-associated MCF. In both forms of the disease, animals with clinical disease are not a source of infection as virus is only excreted by the natural hosts – wildebeest and sheep, respectively.

MCF usually appears sporadically and affects few animals, though both AIHV-1 and OvHV-2 can give rise to epizootics. There is a marked gradation in susceptibility to the OvHV-2 form of MCF ranging from the relatively resistant Bos taurus and B. indicus, through water buffalo, North American bison and many species of deer, to the extremely susceptible Père David's deer, and Bali cattle. The disease may present a wide spectrum of clinical manifestations ranging from the acute form, when minimal changes are observed prior to death, to the more florid cases characterised by high fever, bilateral corneal opacity, profuse catarrhal discharges from the eye and nares, necrosis of the muzzle and erosion of the buccal epithelium. Infectivity from animals with the AIHV-1 form of MCF can be recovered only by employing techniques that retain the viability of host cells, while OvHV-2 has never been recovered from affected animals. Diagnosis is normally achieved by observing the characteristic histopathological changes, though detection of viral DNA in either form of the disease has become the preferred option.

Identification of the agent: AIHV-1 may be recovered from clinically affected animals using peripheral blood leukocytes or cell suspensions prepared from lymph nodes and spleen, but cell viability must be preserved during processing, as infectivity cannot be recovered from dead cells. Virus can also be recovered from wildebeest, either from peripheral blood leukocytes or from cell suspensions of other organs. Most monolayer cultures of ruminant origin are probably susceptible and develop cytopathic effect (CPE), although bovine thyroid cell cultures have been used extensively for recovery of virus. Primary isolates typically produce multinucleated CPE in which viral antigen can be identified by immunofluorescence or immunocytochemistry using suitable antisera or monoclonal antibodies. The OvHV-2 agent has never been identified formally, although lymphoblastoid cell lines propagated from affected animals contain OvHV-2-specific DNA. Both agents have been transmitted experimentally to rabbits and hamsters, which develop lesions characteristic of MCF.

Viral DNA has been detected in clinical material from cases of MCF caused by both AIHV-1 and OvHV-2 using the polymerase chain reaction, and this is becoming the method of choice for diagnosing the OvHV-2 form of the disease.

Serological tests: Infected wildebeest, the natural host, consistently develop antibody to AIHV-1, which can be detected in a variety of assays including virus neutralisation, immunoblotting, enzyme-linked immunosorbent assay (ELISA) and immunofluorescence. However, the antibody response of clinically affected animals is limited, with no neutralising antibody developing, so that...
detection relies on the use of immunofluorescence, ELISA or immunoblotting. Antibody to OvHV-2 has only been detected by using AIHV-1 as the source of antigen. Domestic sheep consistently have antibody that can be detected by immunofluorescence, ELISA or immunoblotting. While antibody often can be detected by immunofluorescence and ELISA in cattle with MCF, in more acutely affected animals, such as deer, antibody is not always present. The competitive inhibition enzyme-linked immunosorbent assay (CI-ELISA) appears to have a sensitivity and specificity that are equal to or better than the other tests, although a recently described ELISA gave good concordance with this test.

Requirements for vaccines and diagnostic biologicals: No vaccine has been developed for this disease.

A. INTRODUCTION

Malignant catarrhal fever (MCF) is a generally fatal disease of cattle and many other species of Artiodactyla, which occurs following infection with either alcelaphine herpesvirus-1 (AIHV-1) or ovine herpesvirus-2 (OvHV-2). Wildebeest (Connochaetes spp. of the subfamily Alcelaphinae), the natural hosts of AIHV-1, experience no clinical disease following infection. Likewise, infection of domestic sheep, the natural host of OvHV-2, has not been associated with any clinical reaction following natural infection, although experimentally, large doses of virus produced clinical signs of MCF, when inoculated into susceptible sheep (11). Disease caused by AIHV-1 is restricted to those areas of Africa where wildebeest are present and to zoological collections elsewhere, and has been referred to as wildebeest-associated MCF. The OvHV-2 form of the disease occurs world-wide wherever sheep husbandry is practised and has been described as sheep-associated (SA) MCF. Both forms of the disease may present a wide spectrum of clinical entities, though the characteristic histopathological changes are very similar in all cases. These two viruses belong to a subgroup of closely related ruminant rhadinoviruses that infect three subfamilies of Bovidae (Alcelaphinae, Hippotraginae and Caprinae); all probably have a potential to cause typical MCF. On rare occasions members of this group of viruses other than AIHV-1 and OvHV-2 have been identified as a cause of MCF.

- Clinical and pathological changes

The clinical signs of MCF are highly variable and range from peracute to chronic, with, in general, the most obvious manifestations developing in the more protracted cases. In the peracute form, either no clinical signs are detected, or depression followed by diarrhoea and dysentery may develop for 12–24 hours prior to death. In general, the onset of signs is associated with the development of a high fever, increased serous lachrymation and nasal exudate, which progresses to profuse mucopurulent discharges. Animals may be inappetent and milk yields may drop. Characteristically, progressive bilateral corneal opacity develops, starting at the periphery. In some cases skin lesions appear (characterised by ulceration and exudation), which may form hardened scabs associated with necrosis of the epidermis, and are often restricted to the perineum, udder and teats. Salivation associated with hyperaemia may be an early sign, progressing to erosions of the tongue, hard palate, gums and, characteristically, the tips of the buccal papillae. Superficial lymph nodes may be enlarged and limb joints may be swollen.

Nervous signs such as hyperaesthesia, incoordination, nystagmus and head pressing may be present in the absence of other clinical signs or as part of a broader more characteristic syndrome.

There is a wide spectrum of susceptibility to OvHV-2-induced disease, ranging from Bos taurus and B. indicus, which are relatively resistant, through most species of deer, bison (Bison bison) and water buffalo (Bubalus bubalis), which are much more susceptible, to the extremely susceptible Bali cattle (Bos javanicus) and Père David’s deer (Elaphurus davidianus). The more resistant species tend to experience a more protracted infection and florid lesions, while in the more susceptible species the disease course tends to be shorter and the clinical signs less dramatic.

Reports from several countries, and in particular from Norway, that the disease affects domestic pigs have recently been confirmed (14). Signs are very similar to those seen in acutely affected cattle.

A mild form of the disease described in 1930 was regarded with some scepticism because the disease could be confirmed only by histological changes observed at post-mortem. However, recent investigations using molecular and serological methods would appear to confirm that a few infected animals may recover following mild or even quite severe clinical reactions (15). Some studies indicate that substantial numbers of animals may become infected without developing clinical disease.
Pathology

Gross pathological changes reflect the severity of clinical signs, but are generally widespread and may involve most organ systems. Erosions and haemorrhages may be present throughout the gastrointestinal tract, and in the more acute cases can be associated with haemorrhagic intestinal contents. In general, lymph nodes are enlarged, although the extent of lymph node involvement varies within an animal. Lymph nodes can often be firm and white when cross-sectioned, while others, in particular submandibular and retropharyngeal, may be haemorrhagic and even necrotic. Catarrhal accumulations, erosions and the formation of a diphtheritic membrane are often observed in the respiratory tract.

Within the urinary tract characteristic echymotic haemorrhages of the epithelial lining of the bladder are often present, while the renal cortex may be affected with multiple raised white foci, each 1–5 mm in diameter and sometimes surrounded by a thin zone of haemorrhage.

Histological changes have been the basis for confirming cases of MCF and are characterised by epithelial degeneration, vasculitis, hyperplasia and necrosis of lymphoid organs, and widespread interstitial accumulations of lymphoid cells in nonlymphoid organs. Epithelial lesions may be present at all epithelial surfaces and are characterised by erosion and ulceration, frequently with subepithelial and intraepithelial lymphoid cell infiltration, which may be associated with vasculitis and haemorrhages.

Vasculitis is generally present and may be pronounced in the brain, affecting veins, arteries, arterioles and venules. It is characterised by lymphoid cell infiltration of the tunica adventitia and media, often associated with fibrinoid degeneration. In the lumen there may be ‘pavementing’ by lymphoid cells, and in severe cases, endothelial damage and subendothelial accumulations by lymphoid cells can sometimes lead to occlusion.

Lymph-node hyperplasia is characterised by an expansion of lymphoblastoid cells in the paracortex, while degenerative lesions are generally associated with the follicles. Oedema with lymphoid inflammation often affects the perinodal tissue.

The interstitial accumulation of lymphoid cells in nonlymphoid organs, in particular the renal cortex and periporal areas of the liver, is typical, and in the case of the kidney may be very extensive. In the brain there may be a nonsuppurative meningoencephalitis with lymphocytic perivascular cuffing and a marked increase in the cellularity of the cerebrospinal fluid.

The macroscopic lesions observed in the cornea are reflected histologically by lymphoid cell infiltration originating in the limbus and progressing centrally, with oedema and erosion developing in the more advanced cases. Vasculitis, hypopyon and iridocyclitis also may be present.

The pathological features of MCF irrespective of the agent involved are essentially similar. However, apart from histological examination, the methods available for diagnosing AIHV-1- and OvHV-2-induced disease are very different and are thus considered separately.

B. DIAGNOSTIC TECHNIQUES

B1. Alcelaphine herpesvirus-1

This form of the disease occurs in the cattle-raising regions of eastern Africa where pastoralists use areas grazed by wildebeest, and in southern Africa in areas where wildebeest and cattle are grazed together. The disease, however, can also affect a variety of other ruminant species in zoological collections world-wide and so, apart from antelope of the subfamilies Alcelaphinae and Hippotraginae, it is advisable to regard all ruminants as susceptible. Most laboratory-based tests have relied on one attenuated isolate (WC11) that has been subjected to many laboratory passages as a source of viral antigen and DNA (17). The full nucleotide sequence of the virulent low passage virus (C500) is now available and will form the basis of further studies of this virus (4).

1. Identification of the agent

   a) Isolation

      The striking feature of AIHV-1-induced MCF is the lack of detectable viral antigen or herpesvirus-specific cytology within lesions. Confirmation of infection thus relies on virus recovery. Generally, infectivity is strictly cell associated and thus isolation can be achieved only from cell suspensions either of peripheral blood leukocytes, lymph nodes or other affected tissues. Cell suspensions are prepared in tissue culture fluid, approximately $5 \times 10^6$ cells/ml, and inoculated into preformed monolayer cell cultures. Bovine thyroid cells have been used extensively, but most primary and low passage monolayer cell cultures of ruminant origin
will probably provide a suitable cell substrate for isolating the virus. Following 36–48 hours’ incubation, culture medium should be changed and monolayers should be examined microscopically (×40) for evidence of cytopathic effects (CPE). These appear characteristically as multinucleate foci within the monolayers, which then progressively retract forming dense bodies with cytoplasmic processes that may detach. This is followed by regrowth of normal monolayers. A CPE may take up to 21 days to become visible and is seldom present before day 7. Infectivity at this stage tends to be largely cell associated and thus any further passage or storage must employ methods that ensure that cell viability is retained. Specificity of the isolate should be determined using specific antisera or monoclonal antibodies (MAbs) in fluorescence or immunocytochemical tests.

b) Viral DNA

Characteristically, very little viral DNA can be detected within affected tissues, hence it is necessary to amplify the viral genome either by conventional culture or the polymerase chain reaction (PCR).

The full sequence of the C500 isolate has been published permitting the design of primers for PCR reactions from conserved regions of the genome. The polymerase gene sequence has been employed for phylogenetic comparison of AlHV-1 and related viruses (9).

- **Natural hosts**

It is almost certain that all free-living wildebeest are infected with AlHV-1 by 6 months of age, virus having been spread as an intense epizootic during the perinatal period. The species *Connochaetes taurinus taurinus*, *C. t. albojubatus* and *C. gnu* are all assumed to be infected with the same virus. Infection also appears to persist in most groups of wildebeest held in zoological collections. However, it is possible that infection may be absent in animals that have been isolated during calf-hood or that live in small groups. Natural infection has been successfully demonstrated by in-situ hybridisation on lung sections from *C. t. taurinus* calves in South Africa (16).

Following infection there is a brief period when virus is excreted in a cell-free form and can be isolated from nasal swabs. Virus can also be isolated from blood leukocytes at this time, but in older animals this is less likely to be successful unless the animal is immunosuppressed either through stress or pharmacological intervention. In addition, virus may be isolated by establishing cultures of tissues from apparently normal animals, and this has been achieved in monolayer cultures of both kidney and thyroid cells from adult animals.

Other large antelope of the subfamilies Alcelaphinae and Hippotraginae are also infected with antigenically closely related gammaherpesviruses, but there is no evidence that they can spread to other species and cause MCF, except rarely in captive populations.

2. Serological tests

- **Clinically affected animals**

The antibody response of clinically affected animals is limited, with no neutralising antibody developing. Antibody in clinical cases can be demonstrated consistently by immunofluorescence or the immunopersoxidase test (IPT) using WC11-infected cell cultures as substrate. A competitive inhibition enzyme-linked immunosorbent assay (CI-ELISA) was first developed for detecting antibody to OvHV-2 (12) using an MAb (15-A) that targets an epitope that appears to be conserved among all MCF viruses and is probably also applicable to AlHV-1 infected animals.

- **Natural hosts**

Antibody appears to develop consistently in wildebeest following infection and can be identified by neutralisation assays using the cell-free isolate WC11, or by immunofluorescence, again using the WC11 isolate and anti-bovine IgG, which has been shown to react with wildebeest IgG. The Minnesota MCF virus strain, which is indistinguishable from the WC11 strain of AlHV-1, is used for CI-ELISA antigen production.

There has been no attempt so far to standardise the indirect fluorescent antibody (IFA) test and the IPT, but the two methods below are given as examples. The CI-ELISA is available as a commercial kit

a) **Indirect fluorescent antibody test**

The IFA is less specific than virus neutralisation (VN); it can be used to demonstrate several varieties of ‘early’ and ‘late’ antigens in AIHV-1-infected cell monolayers. Antibodies reacting in the IFA test or the IPT develop in cattle and experimentally infected rabbits during the incubation period, and later in the clinical course of the disease, though cross-reactions with some other bovine herpesviruses, as well as OvHV-2, reduce the differential diagnostic value. Detection of such cross-reacting antibodies can sometimes be useful in supporting a diagnosis of SA-MCF.
• Preparation of fixed slides

Inoculate nearly or newly confluent cell cultures (see Section B1.2.c) with AlHV-1 (strain WC11). Uninoculated control cultures should be processed in parallel. At about 4 days – when the first signs of CPE are expected to appear but before overt CPE is visible – treat the cultures as follows: discard the medium, wash with PBS, remove the cells with trypsin–versene solution, spin down cells at approximate 800 g for 5 minutes, discard the supernatant fluid, and resuspend the cells in 10 ml of phosphate buffered saline (PBS) for each 800 ml plastic bottle of cell culture.

Make test spots of the cell suspension on two wells of a polytetrafluoroethylene-coated multiwell slide; air-dry and fix in acetone. Stain the spots with positive standard serum and conjugated anti-IgG to the appropriate species. Examine the incidence of positive and negative cells under a fluorescent microscope. Adjust the cell suspension by adding noninfected cells and/or PBS to give a suitable concentration that will form a single layer of cells when spotted on to the slide, with clearly defined positive cells among a background of negative cells.

Spot the adjusted positive cell suspension and the control negative suspensions on to multiwell slides in the desired pattern, and air-dry. Fix in acetone for 10 minutes. Rinse, dry and store over silica gel in a sealed container at −70°C.

An alternative procedure, which is easier to evaluate, is to prepare monolayers of infected and noninfected cells in Leighton tubes or chamber slides. The cell monolayers are infected with from 150 to 200 TCID\(_{50}\) (50% tissue culture infective dose) of virus that has been diluted in cell culture medium. The infected and noninfected slides are fixed in acetone and stored, as above, at −70°C.

• Test procedure

i) Rehydrate the slides for 5 minutes with PBS, rinse in distilled water and air-dry.

ii) Dilute sera 1/20 in PBS. Samples that give high background staining may be retested at higher dilutions. Apply diluted fluids to one MCF virus-positive cell spot and one negative control spot for each sample. Include positive and negative serum controls. Ideally, the test should be validated by titrating the control positive to determine its end-point.

iii) Incubate at 37°C for 30 minutes in a humid chamber.

iv) Drain the fluids from the spots. Wash the slides in two changes of PBS, for 5 minutes each.

v) Wash in PBS for 1 hour with stirring, and then air-dry the slides.

vi) Apply rabbit anti-bovine IgG fluorescein isothiocyanate (FITC) conjugate at a predetermined working dilution.

vii) Incubate at 37°C for 20 minutes, drain the slides, and wash twice in PBS for 10 minutes each.

viii) Counterstain in Evans blue 1/10\(^4\) for 30 seconds, and wash with PBS for 2 minutes. Dip in distilled water, dry and mount in PBS/glycerol (50/50).

ix) Examine by fluorescence microscopy for specific binding of antibody to the infected cells.

b) Immunoperoxidase test

A dilution of bovine turbinate (BT) cell-cultured AlHV-1 containing approximately 10\(^3\) TCID\(_{50}\) is made in a freshly trypsinised suspension of BT cells and seeded into Leighton tubes containing glass cover-slips, 1.6 ml per tube, or four-chambered slides, 1.0 ml per chamber.

Observe the cell cultures at 4–6 days for CPE and fix the cultures with acetone when signs of CPE begin. Remove the plastic chambers, but not the gaskets, from the slide chambers before fixation, and use acetone (e.g. UltimAR) that will not degrade the gasket. Store the fixed cells at −70°C.

• Test procedure

i) Prepare IPT diluent (21.0 g NaCl and 0.5 ml Tween 20 added to 1 litre of 0.01 M PBS, pH 7.2) and washing fluid (0.5 ml Tween 20 added to 1 litre of 0.01 M PBS, pH 7.2).

ii) Dilute the serum to be tested 1/20 in IPT diluent and overlay 150–200 µl on to a fixed virus-infected cover-slip or slide chamber.

iii) Incubate the cover-slip in a humid chamber at 37°C for 30 minutes.

iv) Dip the cover-slip three times in washing fluid.

v) Overlay 150–200 µl of diluted (1/5000 in IPT diluent) peroxidase-labelled anti-bovine IgG on to the cover-slip or slide chamber.
vi) Incubate the cover-slip or slide chamber in a humid chamber at 37°C for 30 minutes.

vii) Dip the cover-slip three times in washing fluid.

viii) Dilute the AEC substrate (3-amino-9-ethylcarbazole) in distilled water (5 ml of distilled water, 2 drops buffer, 2 drops hydrogen peroxide, and 3 drops AEC) and apply to the cover-slip or slide chamber.

ix) Incubate in a humid chamber at 37°C for 8–10 minutes.

x) Dip the cover-slip in distilled water, air-dry, and mount on a glass slide. Slide chambers are read dry.

xi) The slide is read on a light microscope. The presence of a reddish-brown colour in the nuclei of the infected cells indicates a positive reaction.

c) Virus neutralisation

Tests have been developed for detecting antibodies to AlHV-1 in both naturally infected reservoir and indicator hosts. The first of these is a VN test using cell-free virus of the WC11 strain, and another uses a hartebeest isolate (AlHV-2). AlHV-1 and AlHV-2 have cross-reactive antigens and therefore either strain can be used in the test. The test is laborious, but can be performed in microtitre plates using low passage cells or cell lines. The main applications have been in studying the range and extent of natural gammaherpes viruses infection in wildlife, captive species in zoos and, to a lesser extent, sheep populations. It has also been useful in attempts to develop vaccines, all of which have had limited success. The VN test is of no value as a diagnostic test in clinically affected animals as no VN antibody develops in clinically susceptible species.

AlHV-1 stock (strain WC11) is grown in primary or secondary cell cultures of bovine kidney, bovine thyroid, low passage bovine testis, or another permissive cell type. The virus is stored in aliquots at –70°C. The stock is titrated to determine the dilution that will give 100 TCID50 in 25 µl under the conditions of the test.

- Test procedure
  i) Inactivate the sera for 30 minutes in a water bath at 56°C.
  
  ii) Make doubling dilutions of test sera in cell culture medium from 1/2 to 1/16 using a 96-well flat-bottomed cell-culture grade microtitre plate, four wells per dilution and 25 µl volumes per well. Positive and negative control sera are also included in the test. No standard sera are available, but internal positive standards should be prepared and titrated in an appropriate range.

  iii) Add 25 µl per well of WC11 virus stock at a dilution in culture medium calculated to provide 100 TCID50 per well.

  iv) Incubate for 1 hour at 37°C. The residual virus stock is also incubated.

  v) Back titrate the residual virus in four tenfold dilution steps, using 25 µl per well and at least four wells per dilution.

  vi) Add 50 µl per well of bovine kidney cell suspension at 3 × 10^5 cells/ml.

  vii) Incubate the plates in a humidified CO₂ atmosphere at 37°C for 7–10 days.

  viii) Read the plates microscopically for CPE. Validate the test by checking the back titration of virus (which should give a value of 100 TCID50 with a permissible range 30–300) and the control sera. The standard positive serum should give a titre within 0.3 log₁₀ units of its predetermined mean.

  ix) The test serum results are determined by the Spearman–Kärber method as the dilution of serum that neutralised the virus in 50% of the wells.

  x) A negative serum should give no neutralisation at the lowest dilution tested (1/2 equivalent to a dilution of 1/4 at the neutralisation stage).

d) Competitive inhibition enzyme-linked immunosorbent assay (CI-ELISA)

A CI-ELISA was first developed for detecting antibody to OvHV-2 (12) using a MAb (15-A) that targets an epitope on a complex of glycoproteins that appears to be conserved among all MCF viruses. The MAb was raised against the Minnesota isolate of virus, which is indistinguishable from the WC11 strain of AlHV-1. The test has been employed to detect antibody in serum of wild and domestic ruminants in North America and antibody to the following pathogenic viruses has been detected: AlHV-1, AlHV-2, OvHV-2, CpHV-2 and the herpesvirus of unknown origin observed to cause classic MCF in white-tailed deer, as well as the MCF-group viruses not yet reported to be pathogenic, such as those carried by the oryx, muskox, and others. The test has recently been reformed to increase sensitivity (8). This change was made to enable the detection antibody in newly infected lambs and animals in the acute stage of the disease, which were sometimes not detected in the previous format. The CI-ELISA has the advantage of being faster and more efficient than the
IFA or IPT. Additional validation data will become available as its use is expanded to more laboratories in other parts of the world.

The complete reagent set for the CI-ELISA, including pre-coated plates, labelled MAb and control sera, is commercially available. For laboratories wishing to prepare their own antigen-coated plates, the following protocol is provided. Immuno 4 ELISA plates (Dynatech Lab, Chantilly, Virginia) are coated at 4°C (39°F) for 18–20 hours with 50 µl of a solution containing 0.2 µg per well of semi-purified MCF viral antigens (Minnesota or WC11 isolates of AlHV-1) in 50 mM carbonate/bicarbonate buffer (pH 9.0). The coated plates are blocked at room temperature (21–25°C, 70–77°F) for 2 hours with 0.05 M PBS containing 2% sucrose, 0.1 M glycine, 0.5% bovine serum albumin and 0.44% NaCl (pH 7.2). After blocking, wells are emptied and the plates are then dried in a low humidity environment at 37°C for 18 hours, sealed in plastic bags with desiccant, and stored at 4°C (39°F) (10). MAb 15-A is conjugated with horseradish peroxidase by the VMRD, Inc. using a standard periodate method.

- **Test procedure**
  i) Dilute positive and negative controls and test samples (either serum or plasma) 1/5 with dilution buffer (PBS containing 0.1% Tween 20, pH 7.2).
  ii) Add 50 µl of diluted test or control samples to the antigen-coated plate (four wells for negative control and two wells for positive control). Leave well A1 empty and for use as a blank for the plate reader.
  iii) Cover the plate with parafilm and incubate for 60 minutes at room temperature, (21–25°C, 70–77°F).
  iv) Using a wash bottle, wash the plate three times with wash buffer (same as dilution buffer: PBS containing 0.1% Tween 20, pH 7.2).
  v) Prepare fresh 1 × antibody-peroxidase conjugate by diluting one part of the 100 × conjugate with 99 parts of dilution buffer.
  vi) Add 50 µl of diluted antibody-peroxidase conjugate to each sample well. Cover the plate with parafilm and incubate for 60 minutes at room temperature (21–25°C, 70–77°F).
  vii) Wash the plate with wash buffer three times.
  viii) Add 100 µl of substrate solution (TMB Microwell, BioFX Laboratories, Owings Mills, Maryland) to each sample well. Incubate for 60 minutes at room temperature (21–25°C; 70–77°F). Do not remove the solution from the wells.
  ix) Add 100 µl of stop solution (0.18 M sulphuric acid) to each well. Do not remove the solution from the wells.
  x) Read the optical densities (OD) on an ELISA plate reader at 450 nm.
  xi) Calculating % inhibition:
      \[
      \text{Sample OD (Average)} \times \frac{100}{\text{Mean negative control OD}} = \% \text{ Inhibition}
      \]
  xii) Interpreting the results: If a test sample yields equal to or greater than 25% inhibition, it is considered positive. If a test sample yields less than 25% inhibition, it is considered negative.

- **B2. Ovine herpesvirus-2**

This form of the disease occurs world-wide in cattle and other species, normally appearing sporadically and affecting only one or a few animals. However, on occasion, incidents occur in which several animals become affected, and this appears to be associated with certain sheep flocks that may continue to transmit disease for a number of years. The disease can also spread and cause substantial losses in North American Bison (*Bison bison*), red deer (*Cervus elaphus*), other deer species and water buffalo (*Bubalus bubalis*) and even more readily to Père David’s deer (*Elaphurus davidianus*) and Bali cattle (*Bos javanicus*). OvHV-2 is also responsible for causing MCF in zoological collections, where disease has been reported in a variety of species including giraffe. Disease in pigs has been reported from several countries, but is most frequently recognised in Norway where incidents involving several animals regularly occur.

Diagnosis based on clinical signs and gross pathological examination cannot be relied on as these can be extremely variable. Histological examination of a variety of tissues including, by preference, kidney, liver, urinary bladder, buccal epithelium, cornea/conjunctiva and brain, has been the only method of reaching a more certain diagnosis. However, detection of antibody to the virus and/or viral DNA can now also be attempted and are rapidly becoming the methods of choice.
It must be emphasised that the viral cause of SA-MCF cannot be reliably isolated and evidence for OvHV-2 relies on: (a) the presence of antibody in sera of all domestic sheep that cross-reacts with AIHV-1 antigens in the IFA test and immunoblots (5), but not in neutralisation assays; (b) the development of antibody that cross-reacts with AIHV-1 in the IFA test and CI ELISA in most cattle with SA-MCF and in all experimentally infected hamsters; (c) the detection and cloning of DNA from lymphoblastoid cell lines derived from natural cases of SA-MCF that cross-hybridises with, but is distinct from, AIHV-1 DNA; (d) the detection by PCR of amplicons unique to OvHV-2 in peripheral blood and affected tissues.

1. Identification of the agent

• Clinically affected animals

Attempts to recover the disease-causing virus from clinical cases have failed consistently. There are, however, several reports of the recovery of different viral agents from clinical cases, none of which has established any causal relationship; their isolation is certainly fortuitous or due to laboratory contamination. However, lymphoblastoid cell lines have been generated from affected cattle and deer, some of which transmit MCF following inoculation into experimental animals (18). Such cell lines contain viral sequences that hybridise with clones of AIHV-1 DNA (3). A virus sequence was cloned from such a cell line that coded for a tegument protein that was distinct from AIHV-1. Subsequently the whole length of the viral genome has been cloned and the nucleotide sequence determined (5). Primers were identified within this sequence that were suitable for use in the PCR, and a sensitive protocol was designed in which a fragment of 422 base pairs (bp) is amplified initially, followed by amplification of a truncated internal fragment of 238 bp. It has been proven that this test is able to detect as few as 35 viral genome equivalents and that no product is amplified from AIHV-1 or other bovid herpesviruses (1). This PCR is thus both highly specific and sensitive for OvHV-2 and has been employed worldwide in studies of the disease in clinically affected animals and the natural host. It is emerging as a robust test that can be employed to detect viral DNA in peripheral blood leukocytes of clinically affected animals as well as fresh tissues and paraffin-embedded samples collected at post-mortem. The use of magnetic particles to purify DNA prior to amplification has been reported to be an additional improvement to the test, but is yet to be evaluated. A quantitative fluorogenic PCR assay for OvHV-2 has also been established and validated using the semi-nested PCR (1) as a gold standard (7) and is likely to have valuable future application.

While early studies indicated that infection of MCF-susceptible species would normally result in death, some prospective studies in high incidence herds of animals suggest that animals may become infected without developing a clinical response. Factors that predispose animals to infection and development of disease are not understood and it is likely to be a complex interaction of environmental, host factors and the infecting virus. That MHC class 11a polymorphism may contribute to resistance of American bison as suggested in one study (19) is of interest and should be further examined.

• Polymerase chain reaction

Extraction of DNA from clinical material is performed according to the protocol defined in an appropriate extraction kit (e.g. Quiagen DNeasy Tissue Kit). Amplification reactions are performed in 50 µl volumes containing not more than 2 µg test DNA in 10 mM Tris HCl, pH 8.3, 50 mM KCl, 2 mM MgCl2, 0.01% (v/v) gelatine, 10% (v/v) dimethyl sulfoxide (DMSO), 200 µm dATP, dCTP, dGTP and dTTP (Pharmacia), 1 µM of each primer and 2 units Taq DNA-polymerase overlaid with 50 µl mineral oil (Sigma) to prevent evaporation.

The programme consists of a precycle at 99°C for 3 minutes, after which dNTP and enzyme mix are added. This is followed by 25 cycles of 94°C for 20 seconds, 35°C for 30 seconds and 72°C for 30 seconds. A 2 µl aliquot of the primary amplification product, specified by the primer pair 556/755, is transferred directly to a new reaction mixture and amplified using the primer pairs 556/555 under identical conditions for a further 25 cycles with a final extension at 72°C for 5 minutes.

Final amplification products (10 µl) are analysed directly by 1.8% agarose gel electrophoresis and ethidium bromide fluorescence. With each batch of test samples a known positive control and distilled water are also amplified and analysed.

• Natural hosts

The domestic sheep is the natural host of OvHV-2 and probably all sheep populations are infected with the virus in the absence of any clinical response. Studies of the dynamics of infection within sheep flocks have however, generated conflicting results with some suggesting productive infection occurs in the first weeks of a lamb’s life while others suggest infection of most lambs does not occur until 3 months of age with excretion of infectious virus occurring between 5 and 6 months (13). There is also evidence that some lambs may become infected in utero while other studies suggest that removal of lambs from their dams during the first week permits the establishment of virus-free animals. There may therefore be considerable variation in the dynamics of infection in different flocks. However, circumstantial evidence of the occurrence of MCF in susceptible species does suggest
that the perinatal sheep flock is the principal source of infection, but that periodic recrudescence of infection may occur in sheep of all ages.

Factors that predispose to virus shedding and transmission to MCF-susceptible hosts remain speculative.

In addition to domestic sheep, domestic goats and other members of the subfamily Caprinae have antibody that reacts with AIHV-1 in a similar pattern to sheep serum. This implies that these species are infected with viruses similar to OvHV-2, and some goats have been found to be positive to an OvHV-2 PCR, though their potential role in causing MCF would appear to be very limited.

2. Serological tests

Antibody to OvHV-2 has only been detected using AIHV-1 as the source of antigen. Antibody to AIHV-1 can be detected in 70–80% of clinically affected cattle by IFA or IPT procedures, but may not be present in affected deer or animals that develop acute or peracute disease. Antibody is detected by IFA using tissue culture cells infected with AIHV-1. Cell monolayers grown on cover-slips exhibiting 10–50% CPE are harvested, washed, fixed in acetone and used in the assay. Cover-slips are mounted with DPX, the side containing the cells facing uppermost, on microscope slides and treated with 10% normal horse serum before progressing with a conventional IFA test. The IPT procedure can be carried out as for AIHV-1. The only virus of cattle that has been reported to cross-react with AIHV-1 is bovine herpesvirus-4 (BHV-4). Thus the negative control for this test should be similarly infected monolayers of BHV-4. Sera are only considered to be positive when foci show characteristic intranuclear distribution of antigen with little or no cytoplasmic staining being detected in the AIHV-1-infected cells and no reaction in the BHV-4-infected cells. Sera that react to antigens of both viruses are considered to be inconclusive. A CI-ELISA has been developed for detecting antibody to OvHV-2 (12) using a MAb (15-A) raised against the so-called Minnesota isolate of virus, which is indistinguishable from AIHV-1. The test has been employed to detect antibody in serum of wild and domestic ruminants in North America and appears to have some merit (Section B1.2.d) (10). In a study on the reaction of sheep serum to the structural proteins of AIHV-1 in immunoblots, the reactivity of different sera varied strikingly, indicating that individual sheep responded differently with regard to antibody recognition of cross-reacting epitopes of AIHV-1.

B3. Control

Control at present relies on segregating natural hosts from susceptible species, the extent to which this is enforced depending on the species involved. With AIHV-1, it would appear that MCF-affected animals never or rarely transmit infection, hence it is only the natural hosts that can act as a source of infection. Wildebeest would appear to be relatively efficient transmitters of infection to most other categories of ruminant, and hence their segregation in mixed collections is important. Likewise, pastoralists must ensure that cattle are entirely segregated from the vicinity of wildebeest and pastures recently grazed by them, particularly around the time of wildebeest calving.

With OvHV-2, the requirement to segregate sheep depends on the susceptibility of the species involved. Thus with Père David’s deer and Bali cattle, strict separation and avoidance of contact through fomites must be ensured. Equally, with bison and farmed deer every reasonable effort must be taken to segregate the management of sheep, although fallow deer (Dama dama) appear to be more resistant to MCF. Cattle only rarely develop SA-MCF, and thus are generally managed with sheep without taking precautions to guard against disease transmission. However, if multiple cases do occur, it is essential to segregate the sheep flock as far as possible from cattle. As such flocks may continue to be sources of infection for some years, disposal of these flocks for slaughter should be considered.

Virus also appears to have been transmitted over substantial distances thus it is not possible to define the distance that sheep should be segregated.

The possibility that very long incubation periods may occur, up to 9 months, further necessitates a guarded prognosis when advising on the control of such outbreaks.

C. REQUIREMENTS FOR VACCINES AND DIAGNOSTIC BIOLOGICALS

Numerous attempts to produce a protective vaccine against the AIHV-1 form of the disease have met with disappointing results. However, recent trials which have focussed on stimulating high titres of neutralising antibody in nasal secretions of cattle have produced encouraging results and should be the target for further research.

As OvHV-2 cannot be successfully propagated in the laboratory no attempts at developing a vaccine have been attempted.
REFERENCES


* * *
Chapter 2.4.16.

Theileriosis

Summary

Tick-transmitted Theileria parasites of cattle are a major constraint to the improvement of the livestock industry in large parts of the Old World. Theileria annulata and T. parva, the most economically important species, are responsible for mortality and losses in production. Bovine theileriosis is generally controlled by the use of acaricides to kill ticks, but this method is not sustainable. Acaricides are expensive, they cause environmental damage, and over time ticks develop resistance to them requiring newer acaricides to be developed. More sustainable and reliable methods for the control of theileriosis that deploy a combination of strategic tick control and vaccination are desirable. However, these are yet to be successfully applied on a large scale in endemic areas.

Identification of the agent: Diagnosis of a variety of disease syndromes caused by the parasites is principally based on clinical signs, knowledge of disease and vector distribution, and identification of parasites in Giemsa-stained blood and lymph node smears. The presence of multinucleate intracytoplasmic and free schizonts, in lymph node biopsy smears, is a characteristic diagnostic feature of acute infections with T. parva and T. annulata. Animals infected with T. parva show enlarged lymph nodes, fever, a gradually increasing respiratory rate, dyspnoea and occasional diarrhoea. Post-mortem lesions observed are pulmonary oedema with froth in the trachea, enlargement of lymph nodes and spleen, haemorrhages in internal organs, abomasal erosions, the presence of parasitised lymphocytes and lympho-proliferative infiltrations in visceral tissues. The gross pathology caused by schizonts of T. annulata resembles that of T. parva, while the piroplasm stages may also be pathogenic, causing anaemia and jaundice.

Serological tests: The most widely used diagnostic test for Theileria species is the indirect fluorescent antibody (IFA) test. For the IFA test, both schizont and piroplasm antigens may be prepared on slides or in suspension and preserved by freezing at ≤ –20°C, except in the case of the piroplasm suspension, which is stored at 4°C. Test sera are diluted with bovine lymphocyte lysate and incubated with the antigen in suspension, and anti-bovine immunoglobulin conjugate is then added. Using the test as described, the fluorescence is specific for the causative agent. The IFA test is sensitive, fairly specific, and usually easy to perform. However, because of the problems of cross-reactivity among some Theileria species, the test has limitations for large-scale surveys in areas where species distribution overlaps. The IFA test for T. parva, does not distinguish among the different immunogenic stocks. The new indirect enzyme-linked immunoabsorbent assays for T. parva, and T. mutans, based on recombinant parasite-specific antigens, have demonstrated higher sensitivity and specificity and have largely replaced the IFA tests previously used in Africa. In addition, newer molecular diagnostic tests, particularly those based on the polymerase chain reaction and reverse line blot hybridisation are proving to be powerful tools for characterising parasite polymorphisms, defining population genetics and generating epidemiological data.

Requirements for vaccines and diagnostic biologicals: Reliable vaccines of known efficacy have been developed for T. parva and T. annulata. For T. annulata, the vaccine is prepared from schizont-infected cell lines that have been isolated from cattle and attenuated during in-vitro culture. The vaccine must remain frozen until shortly before administration. Vaccination against T. parva is based on a method of infection and treatment in which cattle are given a subcutaneous dose of tick-derived sporozoites and a simultaneous treatment with a long-acting tetracycline formulation. This treatment results in a mild or inapparent East Coast fever reaction followed by recovery. Recovered

1 In this chapter, the term ‘New World’ refers to the Americas and the term ‘Old World’ refers to Europe, Africa and Asia.
animals demonstrate a robust immunity to homologous challenge, which usually lasts for the lifetime of an animal. Immunisation of animals with a stock(s) engendering a broad-spectrum immunity is desirable to cover a range of immunological T. parva strains that exist in the field. Immunised animals usually become carriers of the immunising parasite stock. Safety precautions must be taken in the preparation and handling of T. parva vaccines to protect the workers and to avoid contamination of the stabilates. Consideration should also be given to the risk of introducing new isolates into an area where they may then become established through a carrier state.

**A. INTRODUCTION**

Theileriae are obligate intracellular protozoan parasites that infect both wild and domestic Bovidae throughout much of the world (some species also infect small ruminants). They are transmitted by ixodid ticks, and have complex life cycles in both vertebrate and invertebrate hosts. There are six identified *Theileria* spp. that infect cattle; the two most pathogenic and economically important are *T. parva* and *T. annulata*. *Theileria parva* occurs in 13 countries in sub-Saharan Africa causing East Coast fever (ECF), Corridor disease and January disease. *Theileria annulata*, the cause of tropical theileriosis, occurs in large parts of the Mediterranean coast of North Africa, extending to northern Sudan, and southern Europe. South-eastern Europe, the near and Middle East, India, China and Central Asia are also affected. Endemic regions of *T. annulata* and *T. parva* do not overlap. *Theileria taurotragi* and *T. mutans* generally cause no disease or mild disease, and *T. velifera* is non-pathogenic. These latter three parasites are mainly found in Africa, and overlap in their distribution complicating the epidemiology of theileriosis in cattle. The parasite group referred to as *T. sergenti/T. buffeli/T. orientalis* complex is now thought to consist of two species — *T. sergenti*, occurring in the Far East, and *T. buffeli/T. orientalis* (referred to as *T. buffeli*) with a global distribution (15).

Most *T. parva* stocks produce a carrier state in recovered cattle, and studies using DNA markers for parasite strains have shown that *T. parva* carrier animals are a source of infection and can be transmitted naturally by ticks in the field (R. Bishop, R. Skilton, D. Odongo and S. Morzaria, unpublished data). The severity of ECF may vary depending on factors such as the virulence of the parasite strain, sporozoite infection rates in ticks and genetic background of infected animals. Indigenous cattle in East Coast fever-endemic areas are often observed to experience mild disease or subclinical infection, while introduced indigenous or exotic cattle usually develop severe disease.

The most practical and widely used method for the control of theileriosis is the chemical control of ticks with acaricides. However, tick control practices are not always fully effective due to a number of factors including development of acaricide resistance, the high cost of acaricides, poor management of tick control, and illegal cattle movement in many countries. Vaccination using attenuated schizont-infected cell lines has been widely used for *T. annulata*, while for *T. parva* control, infection and treatment using tick-derived sporozoites and tetracycline is being implemented in a number of countries in eastern, central and southern Africa.

Chemotherapeutic agents such as parvaquone, buparvaquone and halofuginone are available to treat *T. parva* and *T. annulata* infections. Treatments with these agents do not completely eradicate theilerial infections leading to the development of carrier states in their hosts.

The immune response to these parasites is complicated. Cell-mediated immunity is the most important protective response in *T. parva* and *T. annulata*. In *T. parva*, the principal protective responses are mediated through the bovine major histocompatibility complex (MHC) class I-restricted cytotoxic T lymphocytes. *Theileria annulata* schizonts inhabit macrophages and B cells. Innate and adaptive immune responses cooperate to protect cattle against *T. annulata* theileriosis. Infection of macrophages with *T. annulata* activates the release of cytokines, initiating an immune response and helping to present parasite antigen to CD4+ T cells. The CD4+ T cells produce interferon-γ (IFN-γ), which activates non-infected macrophages to synthesise tumour necrosis factor α (TNF-α) and nitric oxide (NO), which destroy schizont- and piroplasm-infected cells. B cells produce antibody that along with NO kill extracellular merozoites and intracellular piroplasms. On the other hand overproduction of cytokines, in particular TNF-α, by macrophages generates many of the clinical signs and pathological lesions that characterise *T. annulata* theileriosis and the outcome of the infection depends upon the fine balance between protective and pathological properties of the immune system.

**B. DIAGNOSTIC TECHNIQUES**

Diagnosis of acute theileriosis is based on clinical signs, knowledge of disease, and vector distribution as well as examination of Giemsa-stained blood, lymph node and tissue impression smears. *Theileria parva* and *T. annulata* are diagnosed by the detection of schizonts in white blood cells or piroplasms in erythrocytes. The piroplasmic stage follows the schizont stage and, in both *T. parva* and *T. annulata*, it is usually less pathogenic and is thus often found in recovering or less acute cases.
1. **Identification of the agent (a prescribed test for international trade)**

Multinucleate intralymphocytic and extracellular schizonts can be found in Giemsa-stained biopsy smears of lymph nodes, and is a characteristic diagnostic feature of acute infections with *T. parva* and *T. annulata*. Both intracellular and free-lying schizonts may be detected, the latter having been released from parasitised cells during preparation of the smears. Schizonts are transitory in *T. mutans* and the *T. sergenti--* *T. buffeli--* *T. orientalis* group, in which the piroplasm stage may be pathogenic. *Theileria taurotragi* schizonts are not readily detected in Giemsa-stained blood smears. A veil to the side of the piroplasm may distinguish *T. velifera*. The schizonts of *T. mutans*, if detected, are distinct from *T. parva*, having larger, flattened, and irregular nuclear particles. The piroplasms (intra-erythrocytic stage) of *T. parva*, *T. annulata* and *T. mutans* are similar, but those of *T. annulata* and *T. mutans* are generally larger and may be seen to divide. However, for practical purposes schizonts and piroplasms of different theilerias are difficult to discriminate in Giemsa-stained smears.

The schizont is the pathogenic stage of *T. parva* and *T. annulata*. It initially causes a lymphoproliferative, and later a lymphodestructive disease. The infected animal shows enlargement of the lymph nodes, fever, a gradually increasing respiratory rate, dyspnoea and/or diarrhoea. The most common post-mortem lesions are enlarged lymph nodes, a markedly enlarged spleen, pulmonary oedema, froth in the trachea, erosions and ulceration of the abomasum, and enteritis with necrosis of Peyer’s patches. Lymphoid tissues become enlarged in the initial stages of the disease, but then atrophy if the animal survives into the chronic stages of the disease. When examined histologically, infiltrations of immature lymphocytes are present in lung, kidney, brain, liver, spleen, and lymph nodes. Schizont-parasitised cells may be found in impression smears from all tissues: lung, spleen, kidney and lymph node smears are particularly useful for demonstrating schizonts. In longer standing cases, foci of lymphocytic infiltrations in kidneys appear as infarcts. In animals that recover, occasional relapses occur. A nervous syndrome called ‘turning sickness’ is sometimes observed in *T. parva*-endemic areas, and is considered to be associated with the presence of intravascular and extravascular aggregations of schizont-infected lymphocytes, causing thrombosis and ischaemic necrosis throughout the brain.

In *T. annulata*, both the schizont and piroplasm stages may be pathogenic. Schizonts are scarce in the peripheral blood of acutely sick animals and their presence in blood smears indicates a poor prognosis. However, schizonts can be easily detected in smears from lymph nodes, spleen and liver tissues obtained by needle biopsy of these organs. The gross pathology caused by schizonts of *T. annulata* resembles that of *T. parva*, while anaemia and jaundice are features of both schizont and piroplasm pathology. Pathogenic strains of *T. mutans* also cause anaemia, as can strains from Japan and Korea referred to as *T. sergenti*.

Piroplasms of most species of *Theileria* may persist for months or years in recovered animals, and may be detected intermittently in subsequent examinations. However, negative results of microscopic examination of blood films do not exclude latent infection. Relapse parasitaemia can be induced with some strains from Japan and Korea referred to as *T. sergenti*. Schizont antigen slides

2. **Serological tests**

- **The indirect fluorescent antibody test (a prescribed test for international trade)**

The indirect fluorescent antibody (IFA) test is the most widely used diagnostic test for *Theileria* spp.

- **Preparation of schizont antigen**
  
  i) **Schizont antigen slides**

  The antigens used for the IFA test are intracytoplasmic schizonts derived from infected lymphoblastoid cell lines for *T. parva* and from infected macrophage cell lines for *T. annulata*.

  Cultures of 200 ml to 1 litre of either *T. parva* or *T. annulata* schizont-infected cells containing $10^6$ cells/ml, of which at least 90% of the cells are infected, are centrifuged at 200 $g$ for 20 minutes at 4°C. The supernatant fluid is removed and the cell pellet is resuspended in 100 ml of cold (4°C) phosphate buffered saline (PBS), pH 7.2–7.4, and centrifuged as before. This washing procedure is repeated three times, and after the final wash the cell pellet is resuspended in PBS (approximately 100 ml) to give a final concentration of $10^7$ cells/ml.

  Thin layers of the cell suspension are spread on Teflon-coated multipot slides, or on ordinary slides using TEXPEN for separation. The smears should give between 50 and 80 intact cells per field view when examined under a ×40 objective lens. The antigens are distributed on to the slides

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2 Obtainable from, for example, Bellco Glass, Vineland, New Jersey, United States of America or Glaxo-Wellcome, United Kingdom.

3 Obtainable from TWmark-tex, Roseland, N.J. 07068, USA.
using multichannel or a 100-µl pipette. By dispensing and immediately sucking up the schizont suspension, a monolayer of schizonts remains in each well. This is performed for each enclosure until the volume is exhausted. With this method, approximately 600 good quality slides containing a total of 6000 individual antigen spots can be obtained. The slides are air-dried, fixed in acetone for 10 minutes, individually wrapped in tissue paper and then in groups of five in aluminium foil, and stored in airtight, waterproof plastic containers at either −20°C or −70°C. The antigens keep for at least 1 year at −20°C and longer at −70°C.

ii) Schizont antigen in suspension

First, 500 ml of *T. parva* or *T. annulata*-infected cells containing 10^6^ cells/ml are centrifuged at 200 g for 10 minutes at 4°C, and the cell pellet obtained is washed twice in 100 ml of cold PBS. The viability of the cells is determined by eosin or trypan blue exclusion (it should be greater than 90%). The cells are resuspended at 10^7^/ml in cold saline. To this volume, two volumes of a cold fixative solution containing 80% acetone and 0.1% formaldehyde (0.25% formalin) in PBS are added drop by drop while the cell suspension is stirred gently and continuously. The cell suspension is kept at −20°C and allowed to fix for 24 hours. The fixed cells are then washed three times in cold saline and centrifuged at 200 g for 20 minutes at 4°C. After the last wash, the cells are resuspended at 10^7^/ml saline. The fixed cells are distributed in aliquots of 0.5 ml. The antigen is stable at 4°C with 0.2% sodium azide as preservative for 2 weeks, and keeps indefinitely at −20°C. This method can also be used to prepare schizont antigen for *T. taurotragi* (J. Katende, A. Musoke and S. Morzaria, unpublished data).

### Preparation of piroplasm antigen

i) Piroplasm antigen slides

The piroplasm stage of *Theileria* spp. cannot be maintained in culture, therefore the piroplasm antigen must be prepared from the blood of infected animals. Experimental infections are induced by infecting cattle subcutaneously with sporozoites, or using ticks infected with *T. parva*, *T. annulata* or *T. taurotragi*. Infection with *T. annulata* is invariably produced by inoculation of blood drawn from cattle with acute theileriosis. Splenectomy of the recipient cattle prior to the infection considerably increases the piroplasm parasitaemia in red blood cells (RBC). Peak parasitaemias are of short duration and if animals survive the disease the percentage of infected RBC decreases considerably in a few days. Infections with the parasite group referred to as *T. sergentii/ T. buffeli/ T. orientalis, T. mutans* or *T. velifera* are usually induced by inoculating splenectomised cattle intravenously with blood from a carrier animal, or with a blood stabilate, or by application of infected ticks. When the piroplasm parasitaemia is 10% or higher, 100 ml of the infected blood is collected from the jugular vein in a heparinised or ethylene diamine tetra-acetic acid (EDTA) vacutainer, and gently mixed with 2 litres of PBS. The mixture is centrifuged at 500 g for 10 minutes at 4°C; the plasma anduffy coat are removed, the RBC are again resuspended in 2 litres of PBS, and the centrifugation step is repeated. It is important to remove the buffy coat after each wash. This washing procedure is repeated four times. After the final wash, an aliquot of the packed RBC is used to make doubling dilutions in PBS, and a 5-µl drop of each dilution is placed on slides. The dried spots are fixed in methanol and stained with Giemsa’s stain, and the concentration of RBC is examined using a light microscope. The dilution that gives a single layer of RBC spread uniformly on the spot is then selected for large-scale preparation of piroplasm antigen slides. Approximately 10,000 antigen slides (100,000 antigen spots) can be prepared from 100 ml of infected blood. The antigen smears are allowed to dry at room temperature before fixing in cold (4°C) acetone for 10 minutes. The fixed smears can be stored as for the schizont antigen slides, and kept for similar periods.

ii) Piroplasm antigen suspension

An alternative method of preparing antigens to that described above is available, and has been tested for *T. parva*. In this procedure, 100 ml of blood are taken from an animal with a high piroplasm parasitaemia and prepared as described previously, and the packed cell volume is adjusted to 5% in PBS.

One volume of the RBC suspension is added to two volumes of the fixative (see above schizont antigen in suspension) while stirring. The cells are allowed to fix at −20°C for 24 hours. The fixed cells are then washed three times with PBS and centrifuged at 1000 g for 30 minutes. The deposit is resuspended to the original volume of blood with PBS containing 0.2% sodium azide, and distributed in aliquots of 0.5 ml.

The piroplasm antigen is stable at 4°C when preserved with 0.2% sodium azide for a period of at least 3 years.

### Standardisation of antigen

Schizont or piroplasm antigen suspensions are mixed on a rotor mixer and titrated in PBS by doubling dilution starting from undiluted through to 1/16. The dilution giving a cell distribution of approximately 50–80 schizont-infected cells or 150–200 infected RBC per field view when examined under a ×40 objective
lens is recommended for use for that batch of antigen. Using this dilution, test antigen smears are prepared on slides. These antigen smears plus the antigen slides previously frozen (and thawed before use) are tested against a range of dilutions of a panel of known strong, intermediate and weak positive and negative control sera. If the positive control sera titrate to their known titres and the negative control sera give no fluorescence, the antigen is used in the routine IFA test.

Both types of antigen preparations, acetone-fixed smears stored at either –20°C or –70°C, and antigens fixed in suspension and stored at either 4°C or –20°C, are used routinely in many laboratories. The sensitivity of both types of antigen is comparable. In laboratories where adequate low temperature storage facilities and a reliable supply of electricity are available, the antigen slides can be used. However, such antigens can only be transported on dry ice or in liquid nitrogen. Antigens fixed in suspension have the advantage over antigen slides in that the initial method of preparation is simpler and quicker. A large batch of this antigen can be stored in one container, and aliquots may be taken out as necessary from which fresh smears are prepared for the IFA test. The need for a large storage facility is thereby avoided. The antigens fixed in suspension can also be stored at 4°C and can be safely transported at room temperature without loss of antigenicity.

**Preparation of bovine lymphocyte lysate**

A lymphocyte lysate is prepared according to the method described by Goddeeris et al. (16), for use in tests with antigens of *T. parva* in suspension. Briefly, a 3-month-old calf is splenectomised and maintained in a tick-free environment. To exclude the possibility of latent theilerial infections, Giemsa-stained blood smears are examined daily for a period of 4 weeks for parasites. The parasite-free animal is killed and the thymus and all the accessible lymph nodes are removed. These tissues are sliced into small pieces in cold PBS containing 0.45% EDTA as anticoagulant. Cells are teased out of the tissue, separated from the debris by passing through a muslin cloth, and washed three times with PBS/EDTA by centrifugation at 200 g for 20 minutes at 4°C. The washed lymphocytes are resuspended in PBS without EDTA, to give a final concentration of 5 × 10⁷ cells/ml. The cells are disrupted by sonication in 100-ml aliquots on ice for 5 minutes using the 3/8 probe. The sonicated material is centrifuged at 1000 g for 30 minutes at 4°C, and the supernatant, adjusted to 10 mg protein/ml, is stored at –20°C in 4-ml aliquots.

**Test procedure**

*With schizont or piroplasm slide antigen*

i) Remove antigen slides from freezer and allow to thaw for 30 minutes at 4°C and then for 30 minutes at room temperature.

ii) Inactivate the sera to be tested for 30 minutes in a water bath at 56°C

iii) Unpack the slides and label the numbers of the sera tested.

iv) Prepare 1/40 and 1/80 dilutions of sera to be tested. Validated positive and negative sera are included with each test as controls. Further doubling dilutions can be made if end-point antibody titres are desired.

v) Transfer 25 μl of each serum dilution to a spot of antigen.

vi) Incubate in a humid chamber for 30 minutes at room temperature.

vii) Remove the serum samples from the antigen wells by washing with PBS and rinse by immersing in two consecutive staining jars containing PBS for 10 minutes each time.

viii) Distribute to each well 20 μl of diluted anti-bovine immunoglobulin fluorescein isothiocyanate conjugate at appropriate dilution (generally, dilutions recommended by manufacturers are suitable; however, minor adjustments may be necessary for optimal results). Incorporate Evans blue into the conjugate at a final dilution of 1/10,000 as a counterstain and incubate in a humid chamber for 30 minutes at room temperature.

ix) Repeat step vii and mount with a cover-slip in a drop of PBS/glycerol (50% volumes of each).

x) Read the slides under a fluorescent microscope equipped with epi-Koem illumination (100 W mercury lamp), UV filter block, ×6.3 eyepieces and Phaco FL 40/1.3 oil objective lens.

*With schizont antigen stored in suspension*

i) Thaw frozen antigen at room temperature.

ii) Distribute the antigen suspension on the spots of multispot slides, using multichannel or a 100-μl pipette. By dispensing and immediately sucking up the suspension a monolayer of schizont-infected cells remains on each well.

iii) Allow slides to dry at room temperature or 37°C.
iv) Dilute test and control sera 1/40 in lymphocyte lysate (195 μl lymphocyte lysate + 5 μl serum).

v) Proceed as described in v to x in the test procedure with slide antigen.

With piroplasm antigen stored in suspension

i) Resuspend piroplasm antigen (stored at 4°C) by agitation and disperse RBC by passing the suspension through a 25-gauge needle to break the clumps.

ii) Dilute the antigen to previously standardised dilutions (see preparation of piroplasm antigen).

iii) Allow slides to dry at room temperature or 37°C.

iv) Proceed as described in iv and v in the test procedure with schizont antigen in suspension.

• Characteristics of the indirect fluorescent test

The incorporation of Evans blue provides a good contrast, enabling good differentiation of non-infected cells from the infected ones under the fluorescent microscope. Mounting the slides in 50% glycerol, at pH 8.0, reduces the rapid fading of FITC and makes photography of the preparation possible. Once prepared, slides are stable and can be read for up to 72 hours after preparation when kept at 4°C in the dark.

The sensitivity of the IFA test depends upon the period elapsed from infection. Following infection with sporozoites, antibodies to T. parva and T. annulata are first detected between days 10 and 14 using the schizont antigen. Using the piroplasma antigen, antibodies are first detected between days 15 and 21. Antibodies last for a variable period of time after recovery, depending on such factors as the establishment of a carrier state, chemotherapeutic intervention, and presence or absence of a rechallenge. Following recovery from infection with T. parva or T. annulata theileriosis, high levels of antibody are generally detected for 30–60 days. The antibody levels gradually decline and low antibody titres are still detectable 4–6 months after recovery. Later, antibody may become undetectable at a serum dilution of 1/40, but may persist for more than 1 year following a single challenge. In ECF endemic regions, the seroprevalence in cattle population fluctuates considerably depending on the level and regularity of challenge. In an epidemiological study with T. parva the overall diagnostic sensitivity of the IFA test has been evaluated as 55% at a cut off titre 1/40 and 28% at cut off 1/160. The specificity of the test for the two cut off points was 86% and 95% respectively (6).

The IFA test is useful for identifying herds that contain carriers of T. annulata, but is not always sufficiently sensitive to detect all infected individuals. Both schizont and merozoite (piroplasm) IFA antigens have failed to detect antibody in some animals despite carrying patent infection with piroplasms (11).

In T. mutans infections induced by sporozoite inoculation, antibodies are first detected between days 10 and 15 after the appearance of piroplasms. Low titres are detectable for at least 12–24 months.

The T. parva IFA test is highly sensitive for detection of antibodies in an epidemiological situation where only one species of Theileria exists. However, if the test is used to detect antibodies where mixed infections of Theileria occur, the specificity of the test needs to be carefully evaluated. For example, T. annulata and T. parva cross-react, although these cross-reactions are four- to six-fold lower than with the homologous sera. The cross-reactivity between the two species has little practical significance as the geographical distribution of these two parasites does not overlap. In the IFA test such cross-reactivity does not occur between T. parva and T. mutans or between T. annulata and T. mutans. There is a low level of cross-reactivity between T. parva and T. taurtragi, reducing the specificity of these two tests in many parts of sub-Saharan Africa where their distribution overlaps.

A panel of monoclonal antibodies (MAbs) detecting various epitopes on the polymorphic immunodominant antigen of the T. parva schizont stage has been generated. This panel can be used in the IFA test using the schizont-infected lymphoblastoid cells (see footnote 2) to detect differences between certain stocks of T. parva and between T. parva and other theilerial species. This test has been deployed as one of the several characterisation tools to differentiate various stocks of T. parva, and for quality control during sporozoite stabilate preparation (8).

• Future tests for Theileria diagnosis

The IFA test is easy to perform and provides adequate sensitivity and specificity for use in the field for detection of prior infection with T. parva and T. annulata infections under experimental situations and in a defined epidemiological environment where only one theilerial species is present. The IFA test has limitations for large-scale serological surveys due to its reduced specificity in field situations where several Theileria species co-exist. There is a need for tests that are more specific, easy to interpret, and robust enough to be used in field conditions. Serological tests based on the enzyme-linked immunosorbent assays
(ELISA) are being used increasingly for the detection of parasite-specific antibodies. ELISAs have been successfully adapted for the detection of antibodies to *T. annulata* (17), and has been shown to detect antibodies for a longer period of time than the IFA (23, 24). An ELISA for *T. mutans* has also been described (25). Two MAbs specific for *T. mutans* have been used in the ELISA system for the detection of antibodies and antigens in acute, subacute and chronic infections. The test is more specific and sensitive than the IFA test. However, the tests now most widely used for *T. parva* and *T. mutans* are indirect ELISAs based on parasite-specific antigens, PIM and p32, respectively. These tests have been extensively evaluated in the laboratory and the field, and are now being used in large parts of Africa. The antigens being used in these tests are expressed in *Escherichia coli* using pGEX as the expression vector (28, 31). The expressed products are fusion proteins with glutathione S transferase, and are directly coated on to ELISA plates. These ELISAs provide higher (over 95%) sensitivity and specificity than the IFA tests (28, 31) and are soon expected to be available commercially.

A range of probes is available to detect all the *Theileria* species that are known to infect cattle and are based on ribosomal RNA gene sequences (2, 7). DNA probes specific for *T. parva* (1, 10, 28) and *T. mutans* (29), have also been developed. The technology of the polymerase chain reaction (PCR) is available to amplify minute quantities of parasite DNA one million-fold, thereby greatly increasing the sensitivity of the DNA probes (3). A specific PCR was developed to test whole blood samples from *T. annulata*-carrier cattle (13). A reverse line blot (RLB) assay based on hybridisation of PCR products to specific oligonucleotide probes immobilised on a membrane for simultaneous detection of different *Theileria* species has been introduced (18). It is hoped that a combination of ELISA, PCR and DNA probes will greatly enhance our present capacity to identify infected animals, thus making possible accurate surveys of *Theileria* species. Eventually, the aim would be to develop these technologies for the diagnosis of all the vector-borne diseases.

PCR amplification of the p33/34 genes of the *T. sergenti/T. buffeli/T. orientalis* complex followed by restriction enzyme analysis can be used to differentiate *T. sergenti* from *T. buffeli/T. orientalis* (26).

**C. REQUIREMENTS FOR VACCINES AND DIAGNOSTIC BIOLOGICALS**

**C1. Cell culture vaccines for *Theileria annulata***

Vaccination against *T. parva* and *T. annulata* has been attempted since the causal organisms were first recognised early in the last century. However, reliable live vaccines of known potency are a more recent development. The most widely used are attenuated schizont cell culture vaccines against *T. annulata*. The procedures for production and safety testing have been described (14, 19, 35), and the vaccine is used in Israel, Iran, Turkey, Spain, India, northern Africa, central Asia and the People’s Republic of China.

Despite the fact that vaccination with the cell culture vaccine against *T. annulata* has been available for more than three decades and has shown to be effective under field conditions, the use of this vaccine has been limited. The concern about the introduction of vaccine-derived parasites into the field tick population has led to individual countries developing vaccines from local isolates (27). Some attenuated cell lines have lost the ability to differentiate to erythrocytic merozoites (piroplasms) when inoculated to cattle and in one instance, *Hyalomma* nymphs fed on vaccinated cattle did not become infected (21). However in most cases the loss of differentiation is based on macroscopic examination of blood films from vaccine inoculated cattle. This drawback, the difficulties in standardisation of the antigenic composition of the cultured parasites and the need of a cold chain for distribution of the vaccine to the field are limiting factors in commercialisation of this vaccine (27).

1. **Seed management**

   a) **Characteristics of the seed**

   Primary cultures of *T. annulata*-infected cells may be established from trypsinised lymph nodes, liver, or spleen taken aseptically from an infected animal after death, or from the buffy coat of heparinised peripheral blood separated on a density gradient (Ficoll Hypaque), or by lymphocytes harvested from lymph node biopsy material using a plastic syringe method (9, 14).

   Seed cultures are prepared from cryopreserved cell lines that have been isolated from cattle and attenuated as described below. Vaccines should be produced from a seed culture (master seed) that has been passed less than 30 times, because there is some uncertainty about the immunogenic stability of these cultures in long-term passage.

   b) **Method of culture**

   The infected cells are cultured initially in Eagle’s minimal essential medium (MEM) or Leibovitz L15 medium supplemented with 20% calf serum and containing penicillin (100 units/ml), streptomycin (50 µg/ml), and
mycostatin (75 units/ml) in 25-ml plastic screw-cap tissue-culture flasks. An alternative medium is RPMI 1640 with 10% fetal calf serum, 2 mM glutamine, penicillin and streptomycin, and is usually used with established cultures. Medium is replenished every 3–4 days. The presence of bright refractile cells free in the medium (on examination using a phase-contrast or inverted microscope) is indicative of infected cell growth. The cultures may establish as a monolayer or in suspension. Passage is effected by decanting the medium, adding 0.025% EDTA (versene) for 15 minutes to monolayer cultures, dispersing the cells, then counting and dispensing according to flask size. Approximately 10^6 cells are introduced into a 25 cm^2 flask, and the same seed rate in 100–200 ml is used in larger flasks. The general culture technique is as described by Brown (9).

Serum is essential for maintenance of these cultures, and is obtained either from calves up to the age of 6 months, or from commercial sources, and is tested for toxicity through three passages in an established cell line before use.

c) **Attenuation of virulence**

Attenuation of *T. annulata* schizonts is achieved by prolonged growth and passage in culture (35). The loss of parasite virulence appears to be due to a change in parasite gene expression. Attenuation is assessed by the inoculation of the culture into susceptible calves every 20–30 passages. A sample of culture should be cryopreserved every ten passages in case of accidental loss or contamination. Complete attenuation is achieved when cultures do not cause fever or detectable schizonts and piroplasms in susceptible cattle. An attenuated culture will reliably infect cattle at 10^5 cells and induce a serological reaction, and will not produce disease at 10^6 cells. Cultures may be cryopreserved using either dimethyl sulphoxide (DMSO) or glycerol. Two methods of storing and delivering the vaccine are described below.

### 2. Method of manufacture

Before starting to produce vaccine, seed material with known characteristics is required (36). Three types of seed material are distinguished:

**Master seed:** Schizont-infected cells from a specific passage that have been selected and permanently stored and from which all other passages are derived. The master seed should consist of a single uniform batch of seed that has been mixed and filled into containers as one batch. As *T. annulata* schizont infected cells are used for the manufacturing process, the master seed also represents the master cell stock (see Chapter 1.1.8 Principles of veterinary vaccine production). To prepare a master seed, schizont-infected cells that have proved to be safe for cattle are propagated to obtain in a single culture passage approximately 5 × 10^6 cells. The cells are cryopreserved in about 100 cryotubes each containing 5 × 10^6 cells. A viability check of the master seed should be performed once the master seed has been cryopreserved for at least 24 hours by reviving one of the cryotubes.

**Working seed:** Schizont-infected cells at a passage level between the master seed and the production seed. To prepare a working seed, the contents of a single cryotube of master seed are transferred to a 10 ml centrifuge tube containing 8 ml complete medium. The tube is centrifuged at 600 g for 15 minutes at 4°C and the pellet is transferred into a 75 cm^2 culture flask containing 15–20 ml medium. The medium is replaced the next day, and 4 days later the cells are dispersed and subcultured in larger vessels. After 5–6 subcultivations, a sufficient number of infected cells is available to start the production run.

**Production seed:** Schizont-infected cells from a specific passage level are used without further propagation for the preparation of a batch of vaccine. The production seed is obtained by propagating large numbers of cells in monolayer or suspension cultures. Monolayer cultures are grown in flasks, 150 cm^2 to 175 cm^2, which usually provide an average of from 7 × 10^7 to 8 × 10^7 cells per vessel. About 80 ml of complete medium per flask is required. In a roller bottle culture system, 1.2–1.5 × 10^8 cells can be obtained in a conventional roller bottle (700 cm^2) containing 100–120 ml of medium. To obtain optimal yield of cells, stationary cultures or roller bottles cultures are incubated for 6–7 days with culture media as described previously, see Section C1.1.b.

The schizont-infected cells from all vessels are harvested and pooled together and the total number is computed. Alternatively, about 20% of the cells may be seeded again to prepare another batch of vaccine. Several batches of vaccine can be produced using a portion of the production seed as working seed. As prolonged cultivation may generate alteration in the futures of the schizonts, such as immunogenic capacity, after several batches, subsequent vaccine is produced by making fresh production seed from the master seed.

Schizont-infected cells are mixed with DMSO at a final concentration of 7% or glycerol at a final concentration of 10%, and dispensed in 1.8-ml aliquots into 2-ml plastic vials, each vial containing ten doses of concentrated vaccine. As DMSO immediately penetrates the cell membranes, the time spent in dispensing the vaccine into the vials should be as short as possible. When glycerol is used, an equilibration time of 30–40 minutes is required before freezing the vaccine. There is no consensus on how many schizont-infected cells should constitute one
dose of the vaccine. A recommended practical approach is to prepare doses of $10^6$–$10^7$ infected cells in order to counteract variable environmental conditions in the field. However, considerable protection against sporozoite-induced infection has been achieved by vaccination with $10^5$ infected cells (22).

The vaccine is frozen by introducing the vials in an ultracold deep freezer (–70°C) and 24 hours later transferred to liquid nitrogen containers. Alternatively vials can be introduced in gas phase liquid nitrogen for 3 hours and then immersed in the liquid nitrogen for storage (35). Vaccine is transported to the field in liquid nitrogen, and diluted 1/10 in isotonic buffered saline in a screw-cap bottle with a rubber or silicone septum for aseptic withdrawal. For dilution of vaccine frozen with glycerol, isotonic buffered saline should also contain 10% glycerol in order to avoid osmotic damage to the schizonts. The vaccine is administered subcutaneously within 30 minutes of thawing (33).

The vaccination regimen in Iran consists of inoculation of two doses of vaccine prepared from two different stocks, 30–60 days apart (19). A fresh culture vaccine is used in Morocco, usually at a tenfold lower dose ($10^4$ shizont-infected cells) (22). However there are problems with quality control of vaccines with short shelf life.

- **Safety precautions**

*Theileria annulata* schizonts are not hazardous for humans or contagious for animals, therefore the main purpose in designing a vaccine production facility is to prevent contamination of the product by extraneous organisms.

3. **Batch control**

In Israel the schizont vaccines are tested using a documented procedure (34) before release.

The frozen vaccine has a practically unlimited shelf life. Usually, the schizont vaccine is produced in small individual batches (3–5 thousand doses), which makes the full testing of each batch impractical for economic reasons. It is recommended therefore that the first batch of vaccine produced from a master seed be tested for safety, efficacy, potency and sterility, while each subsequent batch be tested for sterility and potency only. This recommendation is based on the fact that once the cultured schizonts become attenuated, no reversion to virulence has ever been observed during further cultivation. As far as efficacy is concerned, no obvious alteration of the immunogenic properties has been observed during the limited number (20–30) of passages involved in producing the actual vaccine.

a) **Safety**

*Freedom from properties causing undue local or systemic reactions:* for testing the safety of the master seed, two to four susceptible calves, of the most sensitive stock available, are inoculated with a tenfold greater dose than is recommended for immunisation. This dose should not produce clinical signs beyond a transient rise in temperature. With completely attenuated master seed, no schizonts or piroplasms will be seen in lymph node and liver smears or in blood films. However, different breeds of cattle may show different sensitivities to the vaccine. This should be borne in mind when vaccine from a partially attenuated master seed is to be administered to high-grade cattle stocks.

Following a successful test for safety of a sample, all subsequent batches produced from the same master seed can be released without further testing for safety. However, if parasites are detected in the blood or tissues of vaccinated field animals, or if clinical signs develop following the inoculation of the vaccine, the batch or a parallel batch, from the same master seed, should be retested for safety.

b) **Efficacy**

*Capacity to protect against naturally transmitted theileriosis:* The batch of experimental vaccine used for the safety test can also be used for testing efficacy of the culture-derived anti-theilerial vaccine. Three or four calves are vaccinated with a conventional dose of vaccine and 6 weeks later, the vaccinated calves and the same number of unvaccinated calves are infected with sporozoites of *T. annulata*. Infection can be induced by live adult ticks issued from *T. annulata*-infected preimaginal stages or by inoculation of stablate prepared from macerated infected ticks (for techniques see Section C2.1) Experience shows that inoculation of stablate (macerated ticks) generally induces a more severe response than an equivalent number of live, infected ticks allowed to feed on the cattle. However in the long run, the results obtained by challenge with stablate appear to be more reproducible than those obtained with different batches of live ticks.

There are no internationally agreed standards for the size of a challenge dose used in testing the efficacy of *T. annulata* culture-derived vaccine. Five to ten female and the same number of infected, unfed male *Hyalomma* ticks have been used for infection of cattle. Alternatively, stablate equivalent to 2–4 macerated ticks inoculated subcutaneously in the neck area will invariably produce acute theileriosis. The responses to the challenge infection of the vaccinated and unvaccinated control calves are monitored using the following parameters: duration and severity of pyrexia, rate of schizont-infected cells in lymph node or liver biopsy.
smears, rate of piroplasm infected erythrocytes in the blood films, decrease in white and red blood cell counts, and severity of clinical manifestations such as anorexia, depression and recumbency.

The results of the efficacy test depends on factors such as immunological characteristics of the T. annulata isolate grown and attenuated in culture, the virulence and dose of the field isolate used for challenge, the species of infected ticks used to produce sporozoites. Research studies (35) show that calves vaccinated with schizont vaccine may exhibit an apparent near total protection or show a low level parasitaemia, accompanied by mild fever and insignificant alteration of the remaining parameters from their pre-vaccination values following a potentially lethal homologous challenge. A lesser degree of protection has been exhibited when cattle vaccinated with schizont vaccine were challenged with tick-derived parasites from a geographically remote area. In contrast, in most of the trials the non vaccinated control calves have exhibited a high level of parasitaemia and pancytopenia accompanied by severe clinical manifestations. In the absence of specific medication, the majority of the control animals have succumbed to the infection (35). Controversial results about the length of immunity engendered by vaccination with the cell culture vaccine have been obtained. Periods of from more than 48 months (39) to less than 13 months (32) have been reported.

Field observations have also been used for evaluation of the efficacy of anti-theilerial vaccines (34, 39). Susceptible indigenous cattle as well as high-grade exotic breeds were protected against clinical theileriosis and death in pastures on which nonvaccinated cattle succumbed to theileriosis. As completely attenuated schizont vaccine does not yield piroplasms, the presence of this theilerial stage in vaccinated cattle showing no clinical signs is considered to be the result of unapparent tick-induced infection.

c) **Potency**

*Viability of schizont-infected cells:* The potency test is conducted by quantitative *in-vitro* methods. Frozen vaccine remains stable during the storage period, even for long periods, but some loss of viability occurs during the freezing and thawing processes. Viability should be tested under conditions as similar as possible to those obtained when the vaccine is used in the field. For this reason, vaccine should be thawed and the diluted suspension of schizont-infected cells should be left at ambient temperature for 60 minutes before performing the viability tests. A simple test for evaluating viability of the infected cells is nigrosin dye exclusion counting (40). Vaccine that, after being thawed and diluted and left at room temperature for 1 hour, still contains 50% or more live cells can be released for use although in most cases 80–90% of live cells are found.

Viability of the schizonts is also reflected by the plating efficiency of the schizont-infected cells (40), as only cells containing viable schizonts multiply in culture. For this purpose, the thawed, diluted vaccine is transferred from the bottle to a centrifuge tube. A sample for counting is taken and the suspension is centrifuged for 15 minutes at 600 \( g \). Meanwhile, the total number of cells (live and dead) is determined in order to ascertain that the frozen vaccine had the necessary initial concentration of cells. After centrifugation, the supernatant is discarded and the cells are resuspended to the original volume using complete culture medium. Serial tenfold dilutions of cells in complete medium are performed in sterile 10 ml tubes so that the last two dilutions contain 5 × 10, and 5 cells per ml. Twelve replicates of 200 µl from each of the last two dilutions are introduced into a 96-well culture plate. The plates are incubated at 37°C in a 5% CO\(_2\) atmosphere and cultures are checked with an inverted microscope 6 and 9 days after seeding. The number of wells theoretically containing 1 cell each in which growth is observed is counted. Vaccine showing a plating efficiency <2 (cells) are adequate for field use.

d) **Sterility**

Tests for sterility and freedom from contamination of biological materials may be found in Chapter 1.1.9.

e) **Method of use**

The frozen vaccine is viably preserved in large liquid nitrogen refrigerators at production facility and transported to farms in smaller liquid nitrogen containers. Field centres for storage and supply of vaccine can be set up in theileriosis-enzootic areas. The basic equipment required for field application of frozen vaccine includes a wide mouthed jar for preparing a 40°C water bath, a thermometer for measuring the temperature of water, long forceps, face shield and temperature-resistant gloves. Application of the frozen vaccine to field cattle begins by donning the face shield and temperature-resistant gloves. The required numbers of vials are withdrawn with the forceps from the canister of the liquid nitrogen refrigerator. When withdrawing the vials, the canister should be kept as deep as possible in the neck of the refrigerator to avoid quick warming of the remaining vials. Each withdrawn vial should be checked in order to ascertain that liquid nitrogen has not leaked inside. The liquid nitrogen does not alter the vaccine, but may cause the vial to explode when introduced in the water bath. Such a vial should be held at ambient temperature for 1–2 minutes to allow the nitrogen to escape and then processed in the usual way. Leaking of liquid nitrogen into a vial containing frozen vaccine has raised questions to about the sterility of the frozen vaccine. However the system has
been used for decades with no significant problem observed. The vaccine is administered subcutaneously within 30 minutes of thawing (33).

These vaccines produce no adverse effects in healthy cattle. However, animals with existing infections, particularly viral infections, may not tolerate vaccination well. The administration of a viral vaccine, such as for foot and mouth disease, during the immunisation period (reaction period) is not recommended as the immune response may be compromised (19). In Iran, it is not recommended to vaccinate cows that are over 5 months pregnant, although studies in pregnant cattle with the vaccine stocks used in Israel found no effect on pregnancy (34). The immunity engendered is long lasting.

In general, cattle should be immunised in the first few months of life, and tick challenge under natural conditions reinforces the immunity. Although antigenically different strains of *T. annulata* have been identified (33), it is generally considered that there is sufficient cross-protection among strains to provide adequate protection against field challenge as observed in Israel. In the vast infected areas of central Asia, a single stock has proved immunologically effective in 1.5 million cattle (12, 40). However, as described previously, two stocks are used routinely in Iran (19).

### C2. Immunisation of cattle against *Theileria parva* by the infection and treatment method

Vaccination against *T. parva* is based on a method of infection and treatment in which an aliquot of viable sporozoites is inoculated subcutaneously, and the animals are simultaneously treated with a formulation of a long-acting tetracycline (37). Tetracyclines reduce the severity of the infection, and the resulting mild infection is usually controlled by the host's immune response, so that a carrier state is achieved. There are always risks associated with the use of live parasites for immunisation, however, with appropriate quality control and careful determination of a safe and effective immunising dose, the method can and is being used successfully in the field. This method has also been applied effectively for *T. annulata*, but cell culture vaccination, which is not practical for immunisation against *T. parva*, is preferred. Some *T. parva* stocks have been shown to infect cattle reliably without inducing disease, and these can be used without tetracycline treatment. One such stablate is being applied in the field and offers considerable advantages over potentially lethal stablate infections and savings in the cost of vaccination. However, different stablates of these stocks can produce severe disease in cattle, emphasising the importance of a carefully controlled immunising dose.

#### 1. Stablate preparation

For consistency in immunisation in field, it is essential that tick-derived sporozoite stablates of an immunising stock are prepared from a fully characterised ‘working seed stablate’. The ‘working seed stablate’ should be derived directly from the reference ‘master seed stablate’, which is available in suitable quantity for future preparation of immunising stablates. Immunising stablates can be prepared according to a proposed set of standards (30).

Infection is established, with the working seed stablate of *T. parva*, by inoculation of healthy cattle serologically negative for tick-borne diseases. During the parasitaemic phase of the ensuing disease reaction, clean laboratory-raised nymphs of *Rhipicephalus appendiculatus* are fed on the animals, and the engorged infected ticks are collected. The resultant adult ticks, within 3 weeks to 4 months after moulting, are applied in ear-bags to healthy rabbits. About 600 ticks are applied to each ear and unattached ticks are removed after 24 hours. After 4 days, the ticks are removed and samples (usually 60 ticks) taken to determine infection rates in dissected salivary glands. The remaining ticks are counted into batches of approximately 1000. An estimate of the total number of ticks can be obtained by counting and weighing a given number of ticks and then weighing the total number of ticks. The ticks are washed in a sieve under fast flowing tap water and may be surface disinfected in 1% benzalkonium chloride, or in 70% alcohol, and then rinsed again in distilled water.

The ticks are placed (~1000) in heavy glass specimen jars or plastic beakers, and 50 ml MEM with Hank’s or Earle’s salts and 3.5% bovine plasma albumin (BPA) is added. The jars are kept on ice, and the ticks are ground using a tissue homogeniser (for instance Silverson LR2) for 2 minutes using a large aperture disintegrating head, and for 3 minutes using a small aperture head (emulsor screen). The ground-up tick material is made up to 50 ml for every 1000 ticks, then centrifuged at 50 g for 5 minutes, and the supernatant is harvested. An equal volume of cold 15% glycerol in MEM/BPA is added dropwise while the tick material is maintained chilled on ice and stirred by a magnetic stirrer. The final volume will contain sporozoites from the equivalent of ten ticks/ml. The number of tick-equivalents/ml can be adjusted if parasite infection rates in a particular tick batch were either very high or very low. The final concentration of glycerol in the sporozoite stablate is 7.5%.

The ground-up tick material is then dispensed into glass vials by syringe or pipette for small total volumes, or by automatic syringe for larger volumes. Alternatively, artificial insemination equipment, as used to dispense semen, has been used with pre-labelled plastic straws. This latter system is ideal for large volume stablates, and colour
coding and labelling provide additional check on the identity of the immunising stabilate. An equilibration time of 30–45 minutes should be allowed for small-volume stabilates before they are placed in a deep freezer (−70°C). Once frozen, the stabilate may be transferred to permanent storage in liquid nitrogen taking care not to allow any significant increase in temperature during transfer.

The evaluation of the number of acini infected with *T. parva* in dissected tick salivary glands, before grinding, is a useful indicator of the level of infection but does not take into account the variable loss of viability during stabilate preparation caused by the intensity of grinding and the freeze–thaw processes. Furthermore, the state of maturation of the sporozoites is difficult to estimate by histological examination of the tick salivary glands. Therefore, the infectivity of the stabilate is determined by inoculation of a standard dose of 1.0 ml into susceptible cattle. The contents of 2–4 randomly selected tubes is mixed and then titrated in cattle, and its infectivity and lethality at different dilutions are established for use in immunisation. As the response of cattle to the infection and treatment method is dependent upon their susceptibility to the infection, it is important to titrate stabilates in cattle of the same type as those to be immunised. The sensitivity to tetracyclines is also determined, essentially to provide a dose of stabilate that is controlled, preferably by a single dose of long-acting tetracycline administered at the same time as inoculation. The immunising dose should induce a very mild or unapparent infection (4), and the animal should develop a serological titre and be immune to lethal homologous challenge. Should a single treatment with tetracycline fail to suppress the infection in all cattle, then either a lower dose of the immunising stabilate or two treatments of tetracycline (on days 0 and 4) may be used. A single dose of 30 mg/kg long-acting oxytetracycline has been found to be effective in field immunisations, when used with an appropriate stabilate dilution. An alternative method that has been used involves stabilate infection and treatment with parvaquone at 20 mg/kg on day 8 (depending on the stabilate). This method can be applied where tetracyclines are not reliable, but it requires that the animal be handled more than once. A single treatment with buparvaquone at 2.5 mg/kg at the time of infection has also been shown to be effective with stabilate infections that were not controlled with a single treatment at 20 mg/kg of a long-acting formulation of tetracycline.

Once the procedure which results in a safe and effective immunising dose is established, it must be adhered to strictly in the field, or breakdown of immunisation may occur. It is also important that the stabilate dilution and drug/dose regimen be determined in the most susceptible cattle in which it is likely to be used. The infection and treatment method is usually applied using long-acting tetracycline, and it is recommended that the tetracycline be administered first, in case an animal escapes having received stabilate only.

2. **Safety precautions**

At a meeting in Malawi in 1988, the following recommendations on safety in the preparation, handling and delivery of *T. parva* infection and treatment vaccines were adopted (4).

a) **Field collection of ticks**

It is important that well characterised laboratory strains of *Rhipicephalus appendiculatus* be used during preparation of immunising stabilates.

If field ticks are collected for experimental purposes, then consideration should be given to the possible hazard to humans from pathogens present in these ticks. The most important pathogen that has been recognised is Crimean–Congo haemorrhagic fever virus, usually associated with ticks of the genus *Hyalomma* and widely prevalent within the geographical distribution of *R. appendiculatus*. Those handling field tick collections should, therefore, be made aware of potential hazards. Ticks of *Hyalomma* species generally should not be removed from hosts; engorged or partially engorged ticks should not be crushed between the fingers. If removed, ticks should be handled with a forceps.

b) **Tick-handling facilities**

The handling of field-collected ticks in the laboratory must be controlled in order to avoid accidental attachment to personnel. Field-collected ticks should be fed on rabbits and cattle in isolation facilities. Animals on which laboratory-infected or field-collected ticks have fed should be destroyed. Following engorgement of field-collected ticks on laboratory animals, aliquots should be homogenised and tested for extraneous human pathogens by inoculation in baby hamster kidney (BHK) and Vero cells. The effects of these inoculations should be studied through three passages. Any unused ticks should be destroyed by chemical means or by incineration.

c) **Stabilate preparation**

Care should be taken during the preparation of sporozoite stabilates to avoid aerosol infection of personnel with extraneous pathogens when ticks are being ground. Those grinding ticks should be educated in the potential hazards involved; access to areas where ticks are homogenised should be restricted to specified and informed personnel; personnel should wear protective clothing, including gloves and masks; and tick
grinding should be carried out in a microbiological safety cabinet (see Chapter 1.1.2 Biosafety and biosecurity in the veterinary microbiology laboratory and animal facilities).

3. Purity of stabilates

Both ticks and experimental mammals are potential sources of contamination of stabilates with extraneous pathogens. In both cases, potential contaminants include *Ehrlichia bovis*, bovine *Borrelia* sp., orbiviruses, bunyaviruses, and others. Field-collected ticks should therefore not be used for the preparation of stabilates to be used for immunisation. Well characterised and pathogen-free laboratory colonies of ticks should be used for this purpose. Only healthy cattle and rabbits, free from tick-borne parasites, should be used for tick feeding. Stabilates should be prepared under aseptic conditions. In some circumstances, the use of antibiotics at concentrations appropriate for tissue culture may be indicated. Prepared stabilates should be subjected to routine tests for any viral infections in BHK and Vero cells (as above). Stabilates should be subjected to routine characterisation *in vivo*, which should involve infectivity testing in intact susceptible cattle, sensitivity to tetracyclines and other antitheilerial drugs, and cross-immunity studies. A characterised ‘working seed stabilate’ should be prepared to ensure the purity of the *T. parva* stocks in the daughter immunising stabilate.

During stabilate preparation care must also be taken to avoid extraneous contamination of the stock being used with other *T. parva* stocks. Quality assurance procedures must be enforced, for example for the handling of infected ticks, and the rules should be adhered to rigidly. Tick unit facilities should allow for strict separation of infected and uninfected ticks. Tick unit personnel should use separate overall for each batch of ticks used in stabilate preparation, and the overalls should be sterilised daily. Simultaneous work on many different stocks should be avoided. Stabilate storage systems should incorporate clear labelling of each stabilate tube or straw.

Quality control checks on the stabilate should determine the similarity to the parent seed stock and also detect any extraneous *T. parva* contamination.

4. Vaccination risks

The introduction of an immunising stock into an area/country from which it does not originate may result in that parasite, or a component parasite(s) of that stock, becoming established through a carrier state in cattle and transmission by ticks. The long-term effect of the introduction of new (and potentially lethal) parasites on the disease epidemiology should be considered before introduction, and should be monitored carefully following immunisation.

The characterisation of parasites in target populations should be carried out before immunisation, and at intervals following immunisation. At present the characterisation of parasite stocks with reference to vaccination relies primarily on immunisation and cross-challenge experiments in cattle. However a number of methods for characterising parasite stocks *in vitro* have been attempted in laboratories possessing a high degree of expertise. Preliminary studies have shown that parasite stocks that differ in MAb profile may not cross-protect, whereas stocks showing similar profiles give cross-protection (20). However, in more recent experiments using other *T. parva* stocks, this observation has been proven to be wrong. Another method to detect antigenic differences has used T cell clones specific for parasitised cell lines, as T cell responses are believed to be important in mediating immunity against *T. parva* (20). Currently there are no *in vitro* assays that correlate with protection *in vivo*. Statistically derived disease reaction index, based on parasitological, clinical and haematological measurements, was proposed for characterising levels of infectivity and virulence of different parasite stocks and assessing the impact of control intervention against theileriosis (38).

5. Vaccination strategy

Unlike *T. annulata*, where a considerable cross-protection is observed among different strains in the field, a more complex situation exists for *T. parva*. Two strategies are used to try to overcome this antigenic complexity. A combination of three stocks, which provides a broad spectrum of protection, has been tested in a number of countries. A large volume of a trivalent stabilate was prepared for the FAO by the International Livestock Research Institute (ILRI) between 1998 and 2000. This stabilate was prepared to the latest proposed standards and is used safely and effectively in Tanzania. A further batch is being prepared at ILRI with increasing demand for the infection and treatment method of immunisation in *T. parva*-endemic areas in sub-Saharan Africa. If an immunising stabilate fails to protect against a ‘breakthrough stock’, this should be isolated, characterised, tested and considered for use, either alone, or as an addition to the current immunising stabilate. Another strategy is to prepare stabilates of national or local stocks for use within defined areas. This latter strategy is more costly in time and resources, but it avoids, to some extent, the introduction of new stocks into an area. With movement of cattle, there is a risk of the introduction of different stocks into an area, which may breakthrough the immunity provided by the local stock. Therefore the use of local or introduced stocks for immunisation needs to be carefully evaluated.
The infection and treatment method of immunisation is effective provided the appropriate quality assurance measures are enforced. In the longer term, the attendant delivery problems and the risk of induction of carrier states and disease transmission, emphasise the need for the identification of protective antigens for development of subunit vaccines.

REFERENCES


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CHAPTER 2.4.17.

TRICHOMONOSIS

SUMMARY

Bovine venereal trichomonosis is caused by Tritrichomonas foetus, a flagellate protozoan parasite. It is world-wide in distribution and at one time was of major economic importance as a cause of abortion and infertility, especially in dairy cattle. The widespread use of artificial insemination in many areas of the world has contributed to reduced prevalence. Nevertheless, trichomonosis is still of importance in herds or where artificial insemination is not used.

Transmission of the disease is primarily by coitus, but mechanical transmission by insemination instruments or by gynaecological examination can occur. The organism can survive in whole or diluted semen at 5°C. Bulls are the main reservoir of the disease as they tend to be long-term carriers, whereas most cows clear the infection spontaneously. For these reasons samples from bulls are usually preferred for diagnosing and controlling the disease in herds.

Identification of the agent: Tritrichomonas foetus is a flagellate, pyriform protozoan parasite, approximately 8–18 µm long and 4–9 µm wide, with three anterior and one posterior flagellae and an undulating membrane. The organisms move with a jerky, rolling motion and are seen in culture tests of preputial samples of infected bulls and vaginal washings or cervico-vaginal mucus of infected cows, or sometimes in aborted fetuses. Tritrichomonas foetus can be cultured in vitro, and may be viewed in a wet mount or stained slide. The standard diagnostic method for bulls involves the appropriate collection, examination and culture of smegma from the prepuce and penis. Smegma can be collected by a variety of means including preputial lavage or scraping the preputial cavity and glans penis at the level of the fornic with a dry insemination pipette. A number of in-vitro culture media exist, but more recently a commercially available field culture test has been introduced that allows for trichomonad growth and direct microscopic examination.

Alternative tests: Bovine trichomonosis may also be detected by polymerase chain reaction amplification. In the past, an agglutination test using mucus collected from the cervix and an antigen made from cultured organisms has been used as a herd test. Similarly, an intradermal test using a trichloracetic acid precipitate of the organism has been used in herds.

Requirements for vaccines and diagnostic biologicals: A partially efficacious, killed whole-cell vaccine is commercially available as either a monovalent, or part of a polyvalent vaccine containing Campylobacter and Leptospira.

A. INTRODUCTION

Bovine venereal trichomonosis is caused by the flagellate protozoan parasite, Tritrichomonas foetus. The normal hosts of T. foetus are cattle (Bos taurus, B. indicus). Non-pathogenic species of trichomonads occur in the intestine of cattle; T. suis of pigs is indistinguishable morphologically, serologically and, with modern molecular analysis, genetically from T. foetus (15, 49). Further genetic characterisation is required to determine the taxonomic status of isolates from cattle and pigs.

Tritrichomonas foetus is pyriform, 8–18 µm long and 4–9 µm wide, with three anterior and one posterior flagellae, and an undulating membrane. Live organisms move with a jerky, rolling motion, and can be detected by light microscopy. Phase-contrast dark-field microscopy or other methods must be used to observe the details needed.

1 Nomenclature of parasitic diseases: see the note in Chapter 2.4.18. Trypanosomosis (tsetse-transmitted).  
2 InPouch™ TF Test, BioMed Diagnostics, White City, Oregon, United States of America (USA).  
3 Trich Guard or Trich Guard V5-L, Fort Dodge Laboratories, Fort Dodge, Iowa, USA.
for identification. Detailed morphological descriptions, including electron microscopy studies, have been published by Warton & Honigberg (52). It is important to differentiate *T. foetus* from other contaminant flagellated protozoa that may be present in samples from the bovine reproductive tract (4, 7, 38, 50). Under phase contrast illumination, the number of flagellae observed is an important characteristic as this can help to differentiate *T. foetus* from some bovine flagellates that appear similar. A staining technique has been described that can be used to more clearly observe the morphology and facilitate a definitive identification (30).

*Tritrichomonas foetus* multiplies by longitudinal binary fission; sexual reproduction is not known to occur, and environmentally resistant stages of the parasite have not been observed.

In a few early studies, three serotypes were recognised based on agglutination (47): the ‘belfast’ strain, reportedly predominated in Europe, Africa and the USA (23); the ‘brisbane’ strain in Australia (13); and the ‘manley’ strain, which has been reported in only a few outbreaks (47). Further work needs to be done in the area of comparing the growth characteristics, genetic and antigenic variation and pathogenesis of isolates of *T. foetus* from different areas before ‘strain’ and ‘serotype’ designations can be reliably established.

*Tritrichomonas foetus* may be cultured in vitro, preferably in Diamond’s medium (11), Clausen’s medium (33) or *Trichomonas* medium, which is available commercially (45). A field culture test that allows for growth of the trichomonads and direct microscopic examination without aspiration of the inoculated media has been developed in the USA (46, 51) (InPouch™ TF, see footnote 2).

Transmission of infection occurs by coitus, by artificial insemination, or by gynaecological examination of cows. The site of infection in bulls is primarily the preputial cavity (1, 40), and little or no clinical manifestation occurs. For bulls older than 3–4 years, spontaneous recovery rarely occurs, resulting in a permanent source of infection in herds. In bulls under 3–4 years old, infection may be transient.

*Tritrichomonas foetus* is present in small numbers in the preputial cavity of bulls, with some concentration in the fornix and around the glans penis (24). Chronically infected bulls show no gross lesions. In the infected cow, the initial lesion is a vaginitis, which can be followed in animals that become pregnant by invasion of the cervix and uterus. Various sequelae can result, including a placentitis leading to early abortion (1–16 weeks), uterine discharge, and pyometra. In some cases, despite infection, pregnancy is not terminated by abortion and a normal, full-term calf is born. On a herd basis, cows may, following infection, exhibit irregular oestrous cycles, uterine discharge, pyometra, or early abortion (1, 18, 47). Cows usually clear their infection and generally become immune, at least for that breeding season (1, 18, 49).

### B. DIAGNOSTIC TECHNIQUES

1. **Identification of the agent**

   a) **Agent identification by direct examination or culture (the prescribed test for international trade)**

A tentative diagnosis of trichomonosis as a cause of reproductive failure in a herd is based on the clinical history, signs of early abortion, repeated returns to service, or irregular oestrous cycles. Confirmation of infection depends on the demonstration of organisms in placental fluid, stomach contents of the aborted fetus, uterine washings, pyometra discharge, vaginal mucus or preputial smegma. In infected herds, the most reliable material for diagnosis is either preputial or vaginal washings or scrapings (14, 29, 34, 36, 46).

The number of organisms varies in different situations. They are numerous in the aborted fetus, in the uterus several days after abortion, and, in recently infected cows, they are plentiful in the vaginal mucus 12–20 days after infection. In the infected bull, *T. foetus* organisms are present on the mucosa of the prepuce and penis, apparently not invading the submucosal tissues. It is generally recommended to allow at least 1 week to pass after the last service before taking a preputial sample.

- **Sample collection**

A number of techniques for collecting preputial samples from bulls or vaginal samples from cows have been described. It is important to avoid faecal contamination, as this may introduce intestinal protozoa that may be confused with *T. foetus* (50). Contamination of samples should be minimised by removal of extraneous material and soiled hair from around the preputial orifice or vulva; however, cleansing of the area, particularly with disinfectants, is to be avoided, as this may reduce diagnostic sensitivity. Samples can be collected from bulls by scraping the preputial and penile mucosa with an artificial insemination pipette (36, 46) or metal brush (35, 36), by preputial lavage (46) or by washing the artificial vagina after semen collection (23). The latter technique is not recommended as its sensitivity may be lower (23). Samples from cows are collected by washing the vagina, or by scraping the cervix with an artificial insemination pipette or metal brush (29, 32).
Where samples must be submitted to a laboratory and cannot be delivered within 24 hours, a transport medium, preferably containing antibiotics, should be used (e.g. a thioglycollate broth media with antibiotics [6, 51], the field culture plastic pouch, Winters’ medium, buffered saline solution with 5% fetal bovine serum, or skim milk, with or without antibiotics [42]). During transportation, the organisms should be protected from exposure to daylight and extremes of temperature, which should remain above 5°C and below 38°C (6).

- **Culture**

Where organisms are too few to allow for direct detection and accurate identification, cultures should be prepared. Culture of the organisms is usually required because, in most cases, the number of organisms is not large enough to make a positive diagnosis by direct examination. Several media can be used. Diamond’s trichomonad medium, the commercial culture kit, CPLM (cysteine/peptone/liver-infusion maltose) medium, BGPS (beef-extract/glucose/peptone serum) medium, Clausen’s medium (Neopeptone-Lemco-liver extract glucose) and Oxoid’s *Trichomonas* medium are the media of choice (12, 33, 37, 45). Inoculation of samples into culture media should be done as soon as possible after collection. For samples collected by preputial wash it is necessary to process the sample by centrifugation. The sediment is then inoculated into culture media. Some protocols recommend direct viewing of the aspirate or sediment before inoculation but this does not increase diagnostic sensitivity. It is also important to make sure that the culture media are used before their established expiry date, as many media are not stable. The quality of the water used is important and an antifungal can be added to the media to control yeast growth.

Initial detection of organisms can be done by light microscopy, on a wet mount slide prepared directly from the sample or culture, or through the plastic wall of the InPouch™ (InPouch™ TF system, see footnote 2) using the specially provided plastic clip. The motile organisms may be seen under a standard compound microscope using a magnification of 100 or more. An inverted microscope may be useful for examining tubes containing culture medium. Culture media should be examined microscopically at intervals from day 1 to day 7 after inoculation (31). The organisms may be identified on the basis of characteristic morphological features. The pear-shaped organisms have three anterior and one posterior flagellae and an undulating membrane that extends nearly to the posterior end of the cell. They also have an axostyle that usually extends beyond the posterior end of the cell. Phase-contrast microscopy is very valuable in revealing these features or a recently developed rapid-staining procedure may also be used (30). Both these techniques work best when relatively high numbers of organisms are present, especially the staining technique.

- **Culture procedures**

  - **Modified Diamond’s medium**

Glassware used for culture should be washed in distilled water (avoiding the use of detergents). The modified Diamond’s medium consists of: 2 g trypctase peptone, 1 g yeast extract, 0.5 g maltose, 0.1 g L-cysteine hydrochloride, and 0.02 g L-ascorbic acid and is made up with 90 ml distilled water containing 0.08 g each of K₂HPO₄ and KH₂PO₄, and adjusted to pH 7.2–7.4 with sodium hydroxide or hydrochloric acid. Following the addition of 0.05 g agar, the medium is autoclaved for 10 minutes at 121°C, allowed to cool to 45°C, and then 10 ml inactivated bovine serum (inactivated by heating to 56°C for 30 minutes), 100,000 units crystalline penicillin C and 0.1 g streptomycin sulphate are added aseptically. The medium is aseptically dispensed in 10 ml aliquots into sterile 16 × 125 mm screw-top vials and refrigerated at 4°C until use. Media should be cultured for up to 7 days, samples being examined at daily intervals (1, 31). The incorporation of agar into the medium confines contaminating organisms largely to the upper portion of the culture medium, while helping to maintain microaerophillic conditions at the bottom where the trichomonads occur in largest numbers.

  - **Field culture test**

Where a combination of convenience and sensitivity is required, the field culture test (see footnote 2) may be used (1, 5, 37, 46, 51). The kit consists of a clear flexible plastic pouch with two chambers. The upper chamber contains special medium into which the sample is introduced. Field samples for direct inoculation into the culture pouch would normally be collected by the preputial scraping technique (1, 46). Samples collected by preputial washing require centrifugation before introduction of the sediment into the upper chamber. Following mixing, the medium is forced into the lower chamber, and the pouch is then sealed and incubated at 37°C. Microscopic examination for trichomonads can be done directly through the plastic pouch (5). Diagnostic results with samples from bulls using either Diamond’s medium or the field kit have shown that the two methods give comparable results but there are some advantages (in convenience and in test results) with the kit (5, 6, 29, 37, 46).

- **Overall sensitivity and specificity of the culture and identification test**

Any estimate of the diagnostic sensitivity and specificity of the culture and identification test will be dependent on the efficacy of sample collection, handling and processing, as well as the composition and quality of the culture medium. In bulls, the sensitivity of the InPouch™ TF kit has been estimated to be 92%
Chapter 2.4.17. – Trichomonosis

(95% confidence interval, 84–96%) (36). Estimates for Diamond’s and related media have been variable, possibly due to variation in composition and preparation, but range from 78% to 99%. Until recently, it has been assumed that the specificity of the culture test was 100%, but this is likely to be an overestimation.

Not every sample taken from a particular bull, known to be infected, will necessarily give a positive culture result. Even with optimum conditions of sampling, transport, culture and identification, more than one negative sample should be obtained before there is reasonable assurance that the animal is uninfected. To estimate the probability that an animal is uninfected, negative predictive values should be calculated using an estimate of diagnostic test sensitivity and the animal’s pretest probability of infection (36). The infection in females is usually cleared within 90–95 days, so it may be difficult to isolate organisms from animals in the late stages of their infection. In experimentally infected young cows, using the InPouch™ TF method of culture, an apparent sensitivity of 88% was achieved through a 10-week period after infection (29).

The diagnosis of abortion induced by *T. foetus* may be relatively easy where an aborted fetus is recovered, because of the large number of organisms demonstrable in the fetal abomasal contents or placental fluids. Additionally, immunohistochemical techniques and DNA methods can be used to demonstrate tissue-invasive *T. foetus* organisms in aborted fetuses.

b) Polymerase chain reaction

Molecular-based techniques that use polymerase chain reaction (PCR) technology have been developed for the identification of *T. foetus* (7, 17, 26, 39). Development of a PCR diagnostic test offers a number of potential advantages, including increased analytical sensitivity, faster diagnostic turnaround time, and the fact that the organisms in the collected sample are not required to be viable. A diagnostic PCR assay includes both a specific extraction technique and DNA amplification using PCR techniques with specific primers. The sensitivity and specificity of the assay will be affected by the choice of extraction, choice of PCR conditions and the choice of primers. Initial research has demonstrated that PCR assays are capable of detecting very low numbers of parasites from laboratory cultures of the organism with no preputial material present (17, 26) and in the presence of preputial material (17, 26, 39). However, in the presence of preputial material, a higher number of parasites is required to yield a positive PCR result; this is most likely due to inhibition by components of the preputial smegma. Several DNA extraction techniques have been described (17, 26, 39) and it is likely that the sensitivity of the diagnostic test will be influenced by the efficiency of the extraction method and the procedures to overcome contaminating inhibitors. Diagnostic specificity of the PCR test will depend largely on the specificity of the primers. One set of primers (26) yielded similar sized nonspecific products in approximately one-third of negative control samples (16) and should not be considered for diagnostic use. A set of primers based on the 5.8s rRNA sequence demonstrated good diagnostic specificity in samples from negative animals (TFR3 and TFR4, 17) and are the primer set most frequently cited in published literature. These primers do however produce amplification products from some closely related flagellates (*Trichomonas suis*, *T. mobilensis* and a trichomonad from cats) that are indistinguishable from those of *T. foetus* (17, 21). These species also cannot be differentiated by microscopy and it is possible that some of these species are synonymous with *T. foetus*. Recent work has demonstrated that these primers can be used to differentiate between *T. foetus* and a non-*T. foetus* trichomonad sometimes found in preputial samples (4, 7, 38).

The diagnostic sensitivity and specificity of these assays has yet to be determined in an adequate sample of positive and negative animals, although research to date suggests good specificity (7, 43). The diagnostic sensitivity of PCR tests has been estimated to be similar to that of the InPouch™ TF culture kit (7) but with very few animals. PCR techniques are an attractive alternative to microscopy in that they have a faster turnaround time, and they also allow the detection of dead organisms. The validation of PCR techniques should not be continued and a large number of known positive and negative samples should be tested. DNA-based techniques have potential as an ancillary or primary test (4, 7, 17) and play a key role in differentiating trichomonad protozoa recovered from bovine samples from the reproductive tract. In recent work several different approaches to continue on from earlier work that used the one set of primers (TFR3 and TFR4; [17]) specifically diagnose *T. foetus*. One study used two sets of primers together, one set amplifying DNA from the trichomonad group (TFR1 and TFR2; [15]) and one set specific to *T. foetus* (TFR3 and TFR4; [17]), to differentiate between organisms considered to be feline contaminants of the bovine reproductive system and *T. foetus* (7). Alternatively, the generic primers (TFR1 and TFR2; [15]) were used to amplify DNA and then different protozoal species were differentiated using RFLP analysis (25). In a third study, another set of primers was designed to amplify different sized amplicons from trichomonad protozoa, allowing different species to be distinguished (22). It has also been demonstrated that a PCR assay can be used to detect *T. foetus* DNA in formalin fixed endometrial and aborted fetal tissue (2).

2. Immunological tests

Several immunochemical tests have been used in the past or have been recently developed for the diagnosis of bovine trichomonosis. However, they are limited in use and are not recommended for the detection of *T. foetus* in individual animals. In the 1940s, mucus agglutination tests and intradermal diagnostic tests were developed, but
problems with sensitivity and specificity restrict their usefulness. Other immunological tests based on the antigen-trapping enzyme-linked immunosorbent assay (ELISA) are now being developed (1, 20). Immunohistochemical techniques using monoclonal antibodies have been shown to reveal T. foetus organisms in formalin-fixed tissues (43).

a) Mucus agglutination test

A mucus agglutination test was developed in the 1940s (28, 41) that detects about 60% of naturally infected cows, antibody levels varying according to stage of oestrus. Mucus samples are collected from the cervical region of the vagina, preferably a few days after oestrus. Antibodies appear in cervical mucus about 6 weeks after infection, and persist for several months. Antibodies may also be found in preputial secretions (44, 48). The mucus agglutination test is most useful as a herd test, being capable of detecting latent or recently cleared infections.

A sterile glass tube, 30 cm in length, 9 mm in diameter, and bent at an angle of 150° approximately 9 cm from one end, or an artificial insemination pipette, should be used for taking the cervical sample. Any mucus containing blood should not be used and the animal should be re-sampled. Serum contains nonspecific antibodies and will cause agglutination to occur. The mucus is diluted 1/5 with physiological saline and emulsified in a Griffith’s tube. Duplicate samples, diluted to 1/10 and 1/20, are prepared by pipetting 2 ml of mucus and 2 ml of melted agar (56°C in a water bath) into tubes for the 1/10 dilution, and 1 ml mucus, 1 ml saline and 2 ml melted agar for the 1/20 dilution. Duplicate controls containing 2 ml saline and 2 ml melted agar are also prepared. All tubes are kept in a water bath at 56°C during mixing and then poured individually into 5 cm Petri dishes and allowed to cool. The test antigen is made up by slowly adding a trichomonad culture to a 2/1 mixture of saline and 1% glucose broth to achieve a concentration of approximately 100,000 organisms/ml (approximately six trichomonad/microscope field at ×400). Next, 1.5 ml of antigen is added to each Petri dish, the dishes are incubated for 1.5 hours at 37°C and then left at room temperature for a further 1.5 hours. Agglutination at a dilution of 1/10 is considered to be positive.

b) Intradermal ‘Tricin’ test

An intradermal test for diagnosis of bovine trichomonosis has been reported (27). The injection site is in the skin of the neck, similar to the site used for the tuberculin test. A dose of 0.1 ml of the ‘Tricin’ antigen is injected intradermally and the reaction is measured 30–60 minutes later. The reaction consists of a shallow plaque observed visually and showing an increase of >2 mm in skin thickness.

c) Immunohistochemistry on tissues

There are no specific macroscopic or microscopic lesions in the aborted fetus, and identification of the organisms is necessary for diagnosis. An immunohistochemical technique using a monoclonal antibody (MAb) to detect T. foetus in formalin-fixed paraffin-embedded placenta and fetal lungs from bovine abortions has been reported (43). Immunohistochemical staining is done using a commercially available labelled streptavidin/biotin system and an MAb (34.7C4.4) to T. foetus. In the procedure, deparaffinised 4 µm sections are incubated with the MAb following blocking with non-immune goat serum. After three rinses in buffer, the sections are incubated with biotinylated goat anti-mouse and anti-rabbit immunoglobulin for 30 minutes at 37°C. Following three additional rinses in buffer, peroxidase-labelled streptavidin is applied for 30 minutes at 37°C, and the enzyme activity is diluted with 3% AEC (3-amino-9-ethylcarbazole) in N,N dimethylformamide. Sections are counterstained with Gill II haematoxylin for 3 minutes, rinsed, and blued in buffer for 1 minute. This method has been used to diagnose abortions caused by T. foetus.

C. REQUIREMENTS FOR VACCINES AND DIAGNOSTIC BIOLOGICALS

Whole cell vaccines for cows have been shown to offer protection and are available commercially (10) as either a monovalent vaccine or part of a polyvalent vaccine also containing Campylobacter and Leptospira spp. (CL-vaccine) (1) (see footnote 3). These products have shown efficacy in the female but not in the bull (3). This is in contrast to earlier studies in Australia in which protection or even clearance was afforded to bulls receiving membrane or glycoprotein fractions of T. foetus (8, 9). Specific antibodies have been demonstrated in serum and vaginal mucus of young cows inoculated with a vaccine containing T. foetus (20). In this study a partially effective killed whole-cell vaccine did not prevent infection, but appeared to allow clearance of the infection from vaccinated females before the time in gestation when the fetus is generally most at risk from abortion. Vaccines that make use of membrane surface antigens from T. foetus are being sought and offer the potential of increased efficacy or of a recombinant vaccine (10, 19).

4 DAKO Corporation, Carpinteria, California, USA
One example of a method of whole cell vaccine production is by growing *T. foetus* (culture VMC-84) in modified Diamond’s medium (10) and freezing the culture at –20°C for 60 minutes. After thawing, a suspension of 5 × 10⁷ organisms/ml in phosphate buffered saline is added to the CL-vaccine.

REFERENCES


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CHAPTER 2.4.18.
TRYPANOSOMOSIS
(tsetse-transmitted)

SUMMARY

Definition of the disease: Tsetse-transmitted trypanosomosis\(^1\) is a disease complex caused by several species of protozoan parasites of the genus Trypanosoma, mainly transmitted cyclically by the genus Glossina (tsetse flies), but also transmitted mechanically by several biting flies (tabanids, stomoxes, etc.). The disease can affect various species of mammals but, from an economic point of view, tsetse-transmitted trypanosomosis, is particularly important in cattle. It is mainly caused by Trypanosoma congolense, T. vivax and, to a lesser extent, T. brucei brucei.

Description of disease: Tsetse-transmitted trypanosomosis is a classically acute or chronic disease that causes intermittent fever and is accompanied by anaemia, oedema, lacrimation, enlarged lymph nodes, abortion, decreased fertility, loss of appetite and weight, leading to early death in acute forms or to digestive and/or nervous signs with emaciation and eventually death in chronic forms.

Identification of the agent: Several parasite detection techniques can be used, including the microscopic examination of the wet and stained thick or thin blood films. Diagnostic sensitivity is increased significantly by concentrating the parasites prior to examination in combination with a phase-contrast or dark-ground microscope. The parasite concentration techniques have the added advantage that the packed cell volume, and hence the level of anaemia, can be determined at the individual animal and/or herd level. A highly specific and more sensitive test, used in an increasing number of laboratories, is the polymerase chain reaction (PCR), which can identify parasites at the genus, species or subspecies level, depending on the cases.

Serological tests: Two trypanosomal antibody detection tests, the indirect fluorescent antibody test and the antibody-detection enzyme-linked immunosorbent assay (ELISA), are routinely used for the detection of antibodies in cattle. They have high sensitivity and specificity but can only be used for the presumptive diagnosis of trypanosomosis. The antibody-detection ELISA, in particular, lends itself to automation and will allow a high degree of standardisation when recombinant antigens have been developed and validated. However, they are at the present time carried out with native soluble antigens of trypanosomes grown in rodents with satisfying sensitivity and specificity.

Requirements for vaccines and diagnostic biologicals: No vaccines are in use at the present time. Diagnostic biological products produced from cultured parasites are available and in use for the processing of the indirect fluorescent antibody test and the indirect-ELISA.

A. INTRODUCTION

Trypanosomes are flagellate protozoans that inhabit the blood plasma, the lymph and various tissues of their hosts. The genus Trypanosoma belongs to the protozoan branch, order Kinetoplastida, family

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\(^{1}\) Note on nomenclature of parasitic diseases: The World Association for Advances in Veterinary Parasitology has recommended a 'Standardised Nomenclature of Animal Parasitic Diseases' (Kassai T., Cordero del Campillo M., Euzeby J., Gasfar S., Hepe Th. & Himonas C.A. [1988]. Vet. Parasitol., 29, 299–326). In principle, the disease name is constructed by adding the suffix ‘-osis’ to the stem of the name of the parasite taxon. This terminology has been followed in this Terrestrial Manual, and ‘trypanosomosis’ therefore replaces the old term of ‘trypanosomiasis’.
Trypanosomatidae. Tsetse-transmitted trypanosomes belong to the salivarian section, subgenus Nannomonas for *T. congoense*, Duttonella for *T. vivax*, and Trypanozoon for *T. brucei* ssp.

Tsetse-transmitted trypanosomosis is a disease complex caused by several of these species, mainly transmitted cyclically by the genus *Glossina* (tsetse flies), but also mechanically by biting flies. Tsetse infest 10 million square kilometres and affect 37 countries, mostly in Africa, where it is known as ‘nagana’. The disease infects various species of mammals but, from an economic point of view, tsetse-transmitted trypanosomosis is particularly important in cattle (also referred as tsetse-fly disease in southern Africa). It is mainly caused by *Trypanosoma congoense*, *T. vivax* and, to a lesser extent, *T. brucei brucei*. *Trypanosoma uniforme*, and *T. simiae* are other, less common tsetse-transmitted species. *Trypanosoma vivax* is also transmitted mechanically by biting flies, among which tabanids and stomoxes are presumed to be the most important, as exemplified by its presence in South and Central America, but also as observed in some areas of Africa free or cleared of tsetse (Ethiopia, Chad, etc.). Tsetse-transmitted trypanosomosis can affect camels and is a natural barrier preventing the introduction of this mammalian species into the southern Sahel region of West Africa. Horses are also highly sensitive. Very rare human cases have been observed caused by animal *Trypanosoma* species. However, tsetse-transmitted trypanosomosis also affects humans, causing sleeping sickness, through infection with either *T. brucei gambiense* or *T. brucei rhodesiense*. A large range of wild and domestic animals can act as reservoirs of these humans parasites; particular care must be taken for people handling biological material that can contain infective human parasites, for example in livestock.

Clinical signs of tsetse-transmitted trypanosomosis may include intermittent fever, oedema, abortion, decreased fertility and emaciation. Anaemia usually develops in affected animals and is followed by loss of body condition, reduced productivity and often mortality. Post-mortem signs may include emaciation, enlarged lymph nodes, enlarged liver and spleen, excessive fluid in the body cavities, and petechial haemorrhages. In animals that died during the chronic phase of the disease, the lymphoid organs are usually no longer enlarged and severe myocarditis is a common finding. Neither clinical nor post-mortem signs of tsetse-transmitted trypanosomosis are pathognomonic. Therefore, diagnosis must rely on direct techniques that confirm the presence of trypanosomes either by microscopic visualisation or by indirect serological techniques or by polymerase chain reaction (PCR). Clinically, trypanosomosis can be confused with babesioses, anaplasmosis, theileriosis, hemochonosis and even ehrlichioses, rabies or plant intoxications. Differential diagnosis is oriented by clinical observations, evolution, epidemiological context, but it is essentially based on laboratory diagnosis.

**B. DIAGNOSTIC TECHNIQUES**

A variety of diagnostic tests are available (30) and researchers are still trying to improve existing tests and to develop new ones. Current diagnostic tests vary in their sensitivity and specificity, the ease with which they can be applied and their cost (28). The choice of a particular test will be guided by economic principles and the availability of expertise, but especially by the diagnostic requirement. For example, different degrees of sensitivity and specificity are applied to the confirmation of the infection in an individual animal as compared to the detection of infection at a herd level. Similarly, the diagnostic test(s) to establish the parasitological prevalence of trypanosomosis are different from those required to establish the presence or absence of the disease in an area. Reliable diagnosis may be achieved by combining appropriate diagnostic tests. Reliable interpretation of results from diagnostic tests will depend on test validity as well as on proper sample selection/collection, the sample size, and the way the diagnostic tests are conducted.

1. **Identification of the agent**

Parasite detection techniques are highly specific, but their sensitivity is relatively low (i.e. the proportion of false-negative results recorded is high). Sensitivity is especially low when results are considered at the individual animal level rather than the herd level. Sensitivity is highly variable during the course of the infection: (i) in the early phase, the sensitivity is high as parasites are actively multiplying in the blood in the absence of immunological control; (ii) during the chronic phase the sensitivity is low as, due to the immune response of the host, parasites are scanty and rarely seen in the blood; (iii) finally the sensitivity is almost nil in healthy carriers, where parasites are never seen. At the population level these variations mean that parasite detection techniques are highly sensitive during epizootic outbreaks (when most of the animals are in the early stages of infection), and are of low or very low sensitivity in enzootic areas (most of the animals are in the chronic stages of infection), especially during subclinical phases of the infection (healthy carriers). Due to this low sensitivity, the apparent parasitological prevalence of trypanosomosis is a little or much lower than the true parasitological prevalence. The low diagnostic sensitivity also makes it difficult to detect trypanosomosis when present at low parasitological prevalence and it is impossible to establish the absence of the disease with a high degree of confidence. Moreover, in areas where trypanocidal drugs are used extensively, parasites may not be detected.

Several parasite detection techniques are available, each with varying sensitivity. The choice will depend on the laboratory facilities available and the aim of the diagnosis.
Chapter 2.4.18. – Trypanosomosis (tsetse-transmitted)

- **Direct examination techniques**

The simplest techniques are examination of wet, thick or thin films of fresh blood, usually obtained from the ear vein, jugular vein or the tail. Amongst the direct examination techniques, stained thin blood films are generally regarded as more specific but less sensitive than the other two. The actual specificity and sensitivity of these techniques is directly dependent on the volume of blood actually examined and the skill and experience of the microscopist.

a) **Wet blood films**

These are made by placing a droplet of blood (about 2 µl) on a clean microscope slide and covering with a cover-slip (22 × 22 mm). The blood is examined microscopically at ×400 total magnification with condenser aperture, phase-contrast or interference contrast. Approximately 50–100 fields are examined. Trypanosomes can be recognised by their movement among the red blood cells (RBCs).

The method is simple, inexpensive and gives immediate results. Depending on the trypanosome size and movements a presumptive diagnosis can be made of the trypanosome species. Final confirmation of the species is made by the examination of the stained preparation.

The diagnostic sensitivity of the method is generally low but depends on the examiner’s experience and the level of parasitaemia. Sensitivity can be improved significantly by lysing the RBCs before examination using a haemolytic agent such as sodium dodecyl sulfate (SDS).

b) **Thick blood films**

These are made by placing a drop of blood (5–10 µl) on a clean microscope slide and spreading it over an area of approximately 2 cm in diameter, using the corner of another slide. The thickness of the resultant film should be such that, when dry, the figures on a wristwatch dial can just be read through it. The film is dried thoroughly by rapidly waving in the air and, without fixation, is dehaemoglobinised by immersion in distilled water for a few seconds and dried before staining. A dry smear should be kept dry and protected from dust, heat, flies and other insects. It is stained for 30 minutes with 4% diluted Giemsa stain in phosphate buffered saline, pH 7.2. Staining time and stain dilution may vary with stain and individual technique. Therefore, it is important to start with the manufacturer’s directions and to vary staining time and stain concentration to obtain the optimal result. The stained smear is then washed with buffered water and examined at ×500 to ×1000 total magnification.

The method is simple and relatively inexpensive, but results are delayed because of the staining process. Trypanosomes are easily recognised by their general morphology, but may be damaged during the staining process. This may make it difficult to identify the species.

c) **Thin blood smear films**

Thin blood smears are made by placing a small drop of blood (about 5 µl), for example from a microhaematocrit capillary tube, on a clean microscope slide approximately 20 mm from one end (allowing for space to apply the thick smear) and spreading with the edge of another slide. This slide is placed at an angle of approximately 30° to the first slide and drawn back to make contact with the blood droplet. The blood is allowed to run along the edge of the spreader, which is then pushed to the other end of the slide in a fairly rapid but smooth motion. If the correct amount of blood is used, the slide should be covered with a film of blood with no surplus before the end of the slide is reached, and the smear should take the shape of a bullet. Ideally, thin films should be prepared so that the RBCs are fairly close to each other but not overlapping. The slide is dried quickly by waving in the air and protected from dust, flies and other insects. The slide is fixed for 3 minutes in methanol, and stained as for thick blood smears. After staining, the slide is washed gently under tap water and allowed to dry. A variation of this method is to fix in methanol for 2 minutes, apply May–Grünwald stain for 2 minutes, then add an equal volume of buffered water, pH 7.2, leave for a further 8 minutes and drain off. Approximately 50–100 fields of the stained thin smear are examined, with a ×50 or ×100 oil-immersion objective lens, before the specimen is considered to be negative. Even after a trypanosome has been detected, approximately 20 extra fields are investigated to determine if more than one species is present. The sharp extremity of the smear must be extensively explored as, because of their capillary properties, trypanosomes may be concentrated at this place (especially true for large species like *T. brucei* and *T. vivax*).

The technique described above can also be used for biopsy samples of lymph obtained from punctured lymph nodes.

Usually, both a thin and thick smear is made from the same sample. Thick smears contain more blood than thin smears and, hence, have a higher diagnostic sensitivity. Thin smears on the other hand allow...
Trypanosoma species identification. Trypanosome species can be identified by the following morphological characteristics:

Trypanosoma vivax: 20–27 µm long, undulating membrane is not obvious, free flagellum present at the anterior end, posterior end rounded, kinetoplast large and terminal.

Trypanosoma brucei is a polymorphic trypanosome species. Two distinctly different forms can be distinguished, i.e. a long slender form and a short stumpy form. Often, intermediate forms, possessing characteristics of both the slender and stumpy forms, are observed. The cytoplasm often contains basophilic granules in stained specimens.

- **Trypanosoma brucei** (long slender form): 17–30 µm long and about 2.8 µm wide, undulating membrane is conspicuous, free flagellum present at the anterior end, posterior end pointed, kinetoplast small and subterminal.

- **Trypanosoma brucei** (short stumpy form): 17–22 µm long and about 3.5 µm wide, undulating membrane is conspicuous, free flagellum absent, posterior end pointed, kinetoplast small and subterminal.

Trypanosoma congolense: 8–25 µm (small species), undulating membrane not obvious, free flagellum absent, posterior end rounded, kinetoplast is medium sized and terminal, often laterally positioned. Although T. congolense is considered to be monomorphus, a degree of morphological variation is sometimes observed. Within T. congolense, different types or subgroups exist (savannah, forest, kilifi, tsavo) that have a different pathogenicity (2). However, these types can only be distinguished using PCR.

Trypanosoma theileri: (large species), typically 60–70 µm but individual organisms can range from 19 to 120 µm (21, 25), undulating membrane is conspicuous, long free flagellum present, posterior end pointed, kinetoplast is large and positioned near the nucleus and in a marginal position. Trypanosoma theileri is normally nonpathogenic, but its presence can confuse the parasitological diagnosis. In Western Europe, T. theileri is the only trypanosome species occurring in cattle.

- **Parasite concentration techniques**

The probability of detecting trypanosomes in a sample from an infected animal depends largely on the amount of blood examined and the level of parasitaemia. The amount of blood examined with direct examination techniques is low and parasites are often very scanty in the blood of an infected animal. Both of these factors contribute to the low sensitivity of direct examination techniques. Sensitivity can be improved by increasing the volume of blood to be examined and by concentrating the trypanosomes.

a) **Microhaematocrit centrifugation technique (Woo method)**

The microhaematocrit centrifugation technique, or the Woo method (35), is widely used for the diagnosis of animal trypanosomosis. It is based on the separation of the different components of the blood sample depending on their specific gravity. The method is as follows:

i) Fresh, usually ear vein blood (about 70 µl) is collected into heparinised capillary tubes (75 × 1.5 mm).

ii) One end of the capillary tube is sealed with cristaseal or by heating, ensuring that the column of blood is not charred by the flame.

iii) The sealed capillary tubes are placed in a microhaematocrit centrifuge with the sealed ends pointing towards the outside. To ensure good balance, the tubes are loaded symmetrically.

iv) The rotary cover is screwed on and the centrifuge lid is closed.

v) The capillary tubes are centrifuged at 9000 g for 5 minutes.

vi) A tube carrier is made from a slide on which two pieces of glass 25 × 10 × 1.2 mm have been fixed, 1.5 mm apart, to form a groove.

vii) The tube is placed in the groove, a cover-slip is placed on top and the interface is flooded with water. Alternatively, examination can be done without flooding the interface with water, but in such case, the light condenser must be placed in such a way that cells become refringent.

viii) The plasma/white blood cell interface (buffy coat) is examined by slowly rotating the tube. Trypanosome movement can first be detected using the ×10 objective lens with reduced condenser aperture; the trypanosomes can be seen more clearly using the ×40 objective lens preferably with a long working distance to allow adequate depth of focus through the capillary tube.
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The microhaematocrit centrifugation technique is more sensitive than the direct examination techniques (19). In the case of T. vivax infections, the sensitivity of the Woo methods approaches 100% when the parasitaemia is >700 trypanosomes/ml blood. Sensitivity decreases to 50% when parasitaemia varies between 60 and 300 trypanosomes/ml blood. Trypanosomes become very difficult to detect when the parasitaemia is lower than 60 trypanosomes/ml blood (10). Identification of trypanosome species is difficult. As the specific gravity of T. congolense is similar to that of RBCs, parasites are often found below the buffy coat in the RBC layer. To improve the separation of RBCs and parasites, and increase the sensitivity for T. congolense, the specific gravity of RBCs can be increased by the addition of glycerol.

A modification of the Woo method is the quantitative buffy coat method (QBC) (1). The method has been used for the diagnosis of T. b. gambiense infections; it is generally too expensive for the routine large-scale use in animal trypanosomosis surveys.

b) Dark-ground/phase-contrast buffy coat technique (Murray method)

The buffy coat technique or Murray method (26) represents an improved technique for the detection of trypanosomes and is widely used. It is carried out following steps i to v above, after which the capillary tube is cut, with a diamond tipped pencil, 1 mm below the buffy coat, to include the top layer of RBCs. The buffy coat and the uppermost layer RBCs are extruded on to a clean microscope slide (check that the buffy coat is not sticking to the capillary tube; it should be visible on the slide before covering it with a cover-slip [22 × 22 mm]). Approximately 200 fields of the preparation are examined for the presence of motile trypanosomes with a dark-ground or a phase-contrast microscope with a ×40 objective lens. Trypanosome species can be identified by reference to the following criteria:

**Trypanosoma vivax:** Large, extremely active, traverses the whole field very quickly, pausing occasionally.

**Trypanosoma brucei:** Various sizes, rapid movement in confined areas; undulating membrane traps the light into ‘pockets’ moving along the body.

**Trypanosoma congolense:** Small, sluggish, adheres to RBCs by anterior end.

**Trypanosoma theileri:** More than twice the size of pathogenic trypanosomes, tends to rotate; the posterior end is clearly visible, very long and sharp.

As with the microhaematocrit centrifugation technique, the buffy coat technique is more sensitive than direct examination techniques. The sensitivity of the buffy coat method can be improved by using the buffy coat double-centrifugation technique (19). A total amount of 1500–2000 µl of blood is centrifuged, after which the buffy coat is aspirated into a microhaematocrit capillary tube and centrifuged again. The buffy coat is examined. However, collection of the buffy coat after the initial centrifugation is a delicate step and results may vary from one technician to another.

Compared with the microhaematocrit centrifugation technique, the buffy coat technique has the added advantage that preparations can be fixed and stained for more accurate identification of species and for retention as a permanent record.

Both the microhaematocrit centrifugation and buffy coat techniques give direct results and can be used for screening large numbers of animals. They require specialised equipment and an electricity supply making the test more expensive compared with the examination of the wet blood film. However, this is compensated for by increased sensitivity. Both parasite concentration techniques rely on the detection of motile, live, trypanosomes. Because trypanosomes can lose their vigour and die rather quickly once the blood sample is drawn, samples collected in capillary tubes should be cooled immediately and not be allowed to overheat in the microhaematocrit centrifuge or on the microscope stage. Samples should be examined as soon as possible after collection, preferably within a couple of hours.

The microhaematocrit centrifugation and buffy coat techniques are particularly useful in that the packed cell volume (PCV) can be assessed at the same time. To determine the PCV after centrifugation, the microhaematocrit capillary tube (containing ear vein or jugular vein blood) is placed in a haematocrit reader. The length of the packed RBC column is expressed as a percentage of the total volume of blood. Measuring the PCV is useful for determining the degree of anaemia. Anaemia can be caused by factors other than tsetse-transmitted trypanosomosis. It remains, however, one of the most important indicators of trypanosomosis in cattle. As trypanosomosis is a herd problem, the PCV-profile of a herd is influenced by the number of trypanosome-infected animals and can be used to indicate differences in disease challenge. The average PCV is also influenced by the age and level of genetic susceptibility of cattle.
c) **Anion exchange**

The miniature anion-exchange chromatography technique (m-AECT) is widely used for the diagnosis of human sleeping sickness caused by *T. b. gambiense* (23). Blood is passed through a diethyl amino-ethyl (DEAE)-cellulose column equilibrated with a phosphate buffered saline (PBS) solution of an ionic strength suited to the blood of the animal species under examination. As the RBCs are more negatively charged than the trypanosomes, they are held in the column and the trypanosomes pass through with the eluate, which is collected, centrifuged to concentrate the trypanosomes and examined under the microscope. Large volumes of blood can be examined from each animal and, therefore, the method has high sensitivity. However, the technique is cumbersome and is not suitable for the examination of a large number of animals because it is very expensive and time consuming.

**d) In-vitro cultivation**

A procedure for the *in-vitro* cultivation of *T. brucei* has been described, but success has been irregular over many years. Moreover, the method needs sophisticated equipment, produces results after a considerable delay and is certainly not suitable for large-scale use. A kit (KIVI) for *in-vitro* isolation of trypanosomes has proven to be promising in isolating and amplifying all species of *T. brucei* in humans, domestic and game animals (31). The test’s value in isolating *T. congolense* and *T. vivax* is still unknown. As it is based on the cultivation of procyclic forms of trypanosomes, species differentiation is not possible (18). It should be noted that cultivation is a highly efficient and sensitive method for the detection of tabanid-transmitted *T. theileri*, the prevalence of which is often found to be close to 100% using this technique. In the case of mixed infections, *T. theileri* easily overgrows *T. b. brucei* (33).

• **Animal inoculation**

The subinoculation of blood into rodents, usually mice or rats, is particularly useful in revealing subpatent infections. The laboratory animals are injected intraperitoneally with 0.2–5 ml (depending on the size of the rodent) of freshly collected blood. Artificial immunosuppression of recipient animals by irradiation or drug treatment will greatly increase the chances of isolating the parasite. They are bled three times a week for at least 2 months. Collected blood is examined using the wet film method.

Animal inoculation is more sensitive than direct examination of the wet blood film. Nevertheless, the method is not practical; it is expensive and diagnosis is not immediate. The method is highly sensitive in detecting *T. b. brucei* infections (it is a technique of choice for detection of the non-tsetse-transmitted animal trypanosome: *T. evansi*). However, some *T. congolense* strains are not easily transmitted and *T. vivax* rarely infects laboratory rodents. Also animal inoculation should be avoided as it raises serious animal welfare concerns.

• **Test to detect trypanosomal antigen**

Antigen-detection enzyme-linked immunosorbent assays (ELISA) for trypanosomosis have been described (27). Field evaluations of the tests have given inconsistent results (5, 15). Additional works have been done under controlled conditions, which led to the conclusion that the sensitivity and specificity of these tests are not suitable for the diagnosis of trypanosomosis (4, 5, 7, 11).

• **DNA amplification tests**

A PCR method has been developed as a tool for the diagnosis of infections with African trypanosomes in humans and animals, as well as tsetse flies. Specific repetitive nuclear DNA sequences can be amplified for *T. vivax* and three types of *T. congolense* (5, 8, 24); however current primers for *T. vivax* seem to not be able to amplify some genotypes within this species. A common primer set is available for detection of the three *T. brucei* subspecies. The primer sets available for different trypanosome subgenus, species and types are referred to as follows: Trypanozoont subgenus – TBR1 and TBR2; *T. congolense* (savannah type) – TCN1 and TCN2; *T. congolense* (forest type) – TCF1 and TCF2; *T. congolense* (Kenya Coast type) – TCK1 and TCK2; and *T. vivax* – TVW1 and TVW2. Due to the multiplicity of these taxon-specific primers in tsetse flies or cattle, a full trypanosome species identification requires that five PCR test be carried out per sample, which considerably increases the cost of diagnosis. Recently PCR restriction fragment length polymorphism (RFLP) assays and ITS1 of ribosomal DNA amplification have been developed that allow the identification of all *Trypanosoma* species as single or mixed infections using one single test (3, 8, 9, 12); however these tests are not yet suitable for routine diagnosis. Loop-mediated isothermal amplification is also under development for trypanosome diagnosis (20).

Standard monovalent PCR amplifications are carried out in a reaction mixture containing Tris/HCl, MgCl₂, KCl, each of the four deoxyribonucleotide triphosphates, primers, DNA template and Taq DNA polymerase. Samples are incubated during several cycles at varying temperatures. The PCR products are electrophoresed through
agarose. Gels are stained with ethidium bromide and visualised under UV light for the presence of specific weight products.

The procedure is extremely sensitive, but false-positive results may occur as a result of contamination of samples with other DNA. The test requires specialised equipment and highly trained personnel, so it is not suitable for use in many laboratories. False-negative results may occur when the parasitaemia is very low (<1 trypanosome/ml of blood), which occurs frequently in chronic infections; they may also occur when the specificity of the primers is too high, so that not all isolates of a particular trypanosome species are recognised. Sample collection has been simplified by adapting the test using blood or buffy coats spotted on to filter paper (12, 16). A large number of samples can be processed at one time, making it potentially suitable for large-scale surveys. However, at the moment, the cost of PCR analyses is prohibitive for the routine use of the test.

2. Serological tests

Several antibody detection techniques have been developed to detect trypanosomal antibodies for the diagnosis of animal trypanosomosis, with variable sensitivity and specificity. The methods of choice are the indirect fluorescent antibody test (IFAT) (17) and the trypanosomal antibody-detection ELISA (14, 22). The identification of major antigens of trypanosomes, and their production as recombinant molecules or synthetic peptides, should hopefully lead to the development of new tests based on the use of defined molecules. Thus, in the future, it may be possible to improve the specificity of serological tests to allow the detection of species-specific antibodies, and to reach a high level of standardisation that is currently not achieved by the use of total parasite extracts.

a) Indirect fluorescent antibody test

The original method for this test (34) has been replaced by a new technique for the preparation of trypanosomal antigens (17), which involves fixation of live trypanosomes using a mixture of 80% cold acetone and 0.25% formalin in normal saline.

- Test procedure
  i) Prepare thin smears from heavily parasitaemic blood or from a trypanosome suspension. Air-dry and fix in acetone for 5 minutes.
  ii) Mark circles of 5 mm diameter on glass slides using nail varnish.
  iii) Using a pipette, place a test serum, diluted 1/40, in each circle, ensuring that the area in each circle is completely covered.
  iv) Incubate the antigen/test serum preparation at 37°C for 30 minutes in a humid chamber.
  v) Wash the preparation three times in PBS for 5 minutes each time at 4°C, with gentle agitation. Air-dry the slides.
  vi) Apply conjugate: rabbit or goat anti-bovine IgG (for tests on bovine sera) conjugated to fluorescein isothiocyanate.
  vii) Incubate and wash as above. Rinse in distilled water. Air-dry the slides.
  viii) Mount the slides in PBS or buffered glycerol and examine for fluorescence.

b) Antibody-detection enzyme-linked immunosorbent assay

The original antibody ELISA (22) has recently been further developed for use in large-scale surveys of bovine trypanosomosis (6, 14). Recommendations have been made that allow antigen production and standardisation of the test on a local basis (6, 7, 13, 36).

The standard antigen for trypanosomosis antibody tests is derived from bloodstream-form trypanosomes. Trypanosomes are purified by DEAE anion-exchange chromatography of parasites from whole blood of infected rats. Antigens are prepared as a soluble fraction with lysis (with the addition of anti-enzyme) using seven freeze–thaw cycles and centrifugation at 10,000 g for 30 minutes. Antigens obtained from in-vitro propagated procyclic trypanosome forms can also be used (13). Soluble antigens can be stored at –80°C or –20°C for long and short periods, respectively, but they may also be lyophilised for conservation at room temperature. ELISAs using T. congolense or T. vivax precoated microtitre plates have been developed that have the advantage that a standardised denatured antigen is used that can be stored for long periods at room temperature (29).

Both the IFAT and antibody-detection ELISA have been adapted for the analysis of blood samples collected on filter paper. Blood contained in one heparinised microhaematocrit centrifuge capillary tube is extruded on to a filter paper (Whatman® No. 4). Samples are air-dried out of direct sunlight and placed in a plastic bag.
with self-indicating silica gel desiccant. The bag is sealed and should be kept as cool as possible until specimens are refrigerated or frozen.

Each ELISA-microplate is run with strong positive, weak positive and negative reference sera, which are required to comply with pre-set values for quality assurance. The absorbance of each ELISA-sample tested is expressed as a percentage (percentage positivity: PP) of the strong positive reference standard (36), or the positive and negative reference standards (6); results are, therefore, quantifiable. The cut-off value is determined using known positive and negative field or experimental samples (6, 7).

Both antibody-detection tests have high sensitivity and genus specificity. Their species specificity is generally low, but may be improved by using a standardised set of the three species-specific tests (7). They detect immune responses to current and past infections and can, therefore, only provide a presumptive diagnosis of active infection. However, persistence of antibodies after a curative treatment or a self-cure is estimated to be on the average of 3–4 months in young and adult cattle infections (7); although it might take up to 13 months before all antibodies have disappeared in some animals (32) consequently, proper sampling and knowledge of trypanocidal use will give more acute information.

Immunodiagnosis needs expensive, sophisticated equipment and expertise, which is not always available. It has to be performed in specialised laboratories and there is a substantial delay between the actual sampling and the availability of the results. Nevertheless, the antibody ELISA lends itself to a high degree of automation and standardisation. Sample collection and storage is made easy through the use of filter papers. All of these factors make the antibody ELISA a very useful test for large-scale surveys to determine the distribution of tsetse-transmitted trypanosomosis.

C. REQUIREMENTS FOR VACCINES AND DIAGNOSTIC BIOLOGICALS

No vaccines are in use at the present time. The only requirement for diagnostic biologicals in livestock is to grow parasites in rodents to avoid the presence of livestock components in IFAT and ELISA biologics.

REFERENCES


* * *

**NB:** There is an OIE Reference Laboratory for Trypanosomosis (tsetse-transmitted) (see Table in Part 3 of this *Terrestrial Manual* or consult the OIE Web site for the most up-to-date list: www.oie.int).
SECTION 2.5.

EQUIDAE

CHAPTER 2.5.1.

AFRICAN HORSE SICKNESS

SUMMARY

African horse sickness (AHS) is an infectious but noncontagious viral disease affecting all species of equidae caused by an orbivirus of the family Reoviridae and characterised by alterations in the respiratory and circulatory functions. AHS is transmitted by at least two species of Culicoides. Nine different serotypes have been described.

All serotypes of AHS occur in eastern and southern Africa. Only AHS serotype 9 and 4 have been found in West Africa from where they occasionally spread into countries surrounding the Mediterranean. Examples of outbreaks that have occurred outside Africa are: in the Middle East (1959–1963), in Spain (serotype 9, 1966, serotype 4, 1987–1990), and in Portugal (serotype 4, 1989).

Laboratory diagnosis of AHS is essential. Although the clinical signs and lesions are characteristic, they can be confused with those of other equine diseases.

As a viral disease, the laboratory diagnosis of AHS can be based on the identification of infectious virus, virus nucleic acid, viral antigens or specific antibodies. Over the past few years, a wide variety of laboratory tests have been adapted for the detection of both AHS virus (AHSV) and specific antibodies.

Identification of the agent: it is important to perform virus isolation and serotyping whenever outbreaks occur outside the enzootic regions.

AHSV can be isolated from blood collected during the early febrile stage. For virus isolation, the other tissues of choice for diagnosis are spleen, lung, and lymph nodes, collected at necropsy. Sample preparations can be inoculated in cell cultures, such as baby hamster kidney-21 (BHK-21), monkey stable (MS) or African green monkey kidney (Vero), intravenously in embryonated eggs, and intracerebrally in newborn mice. Several enzyme-linked immunosorbent assays (ELISAs) for the rapid detection of AHSV antigen in spleen tissues and supernatant from infected cells have been developed. Identification of AHSV RNA has also been achieved using a reverse-transcription polymerase chain reaction method. Virus isolates can be serotyped by a type-specific serological test such as virus neutralisation (VN) and by reverse-transcription polymerase chain reaction and sequencing.

Serological tests: Horses that survive natural infection develop antibodies against the infecting serotype of AHSV within 8–12 days post-infection. This may be demonstrated by several serological methods, such as complement fixation test, ELISA, immunoblotting and VN. The latter test is used for serotyping. Other tests that have been described are immunodiffusion and haemagglutination inhibition.

Requirements for vaccines and diagnostic biologicals: Attenuated (monovalent and polyvalent) live vaccines for use in horses, mules and donkeys, are currently commercially available. A monovalent inactivated vaccine has been produced commercially, but is no longer available. New vaccines, including a subunit vaccine, have been evaluated experimentally.
A. INTRODUCTION

African horse sickness (AHS) (Peste equina africana, Peste equine) is an infectious, noncontagious arthropod-borne disease of equidae, caused by a double-stranded RNA orbivirus belonging to the family Reoviridae. The genus Orbivirus also includes bluetongue virus and epizootic haemorrhagic disease virus, which have similar morphological and biochemical properties with distinctive pathological and antigenic properties as well as host ranges. The virion is an unenveloped particle of a size around 70 nm. The genome of AHS virus (AHSV) is composed of ten double-stranded RNA segments, which encode seven structural proteins (VP1-7), most of which have been completely sequenced for AHSV serotypes 4, 6 and 9 (29, 36, 39) and four nonstructural proteins (NS1, NS2, NS3, NS3A) (11, 19). Proteins VP2 and VP5 form the outer capsid of the virion, and proteins VP3 and VP7 are the major inner capsid proteins. Proteins VP1, VP4 and VP6 constitute minor inner capsid proteins. The NS3 proteins are the second most variable AHSV proteins (35), the most variable being the major outer capsid protein, VP2. This protein, VP2, is also the principal responsible for AHSV serotypes and, together with VP5, for virus neutralisation activity (26). Nine antigenically distinct serotypes of AHSV have been identified by virus neutralisation but some cross-reaction has been observed between 1 and 2, 3 and 7, 5 and 8, and 6 and 9, but no cross-reactions with other known orbiviruses have been observed.


The disease has both a seasonal (late summer/autumn) and a cyclical incidence with major epizootics in southern Africa during warm-phase events (1). Mortality due to AHS is related to the species of equidae affected and to the strain or serotype of the virus. At least two field vectors are involved: Culicoides imicola and C. bolitinos. Among the equidae, horses are the most susceptible to AHS with a mortality rate of 50–95%, followed by mules with mortality around 50%. In enzootic regions of Africa, donkeys are very resistant to AHS and experience only subclinical infections. In European and Asian countries, however, donkeys are moderately susceptible and have a mortality rate of 10%. Zebras are also markedly resistant with no clinical signs, except fever, and may have extended viraemia (up to 40 days) (4).

B. DIAGNOSTICS TECHNIQUES

Although some clinical signs and lesions are characteristic, for example the supraorbital swelling that is often present in horses with subacute AHS, these clinical signs combined with an appropriate history and epidemiological information may be sufficient for a tentative diagnosis. However, other signs and lesions are less specific for AHS, and other diseases such as equine encephalosis, equine infectious anaemia, equine morbillivirus pneumonia, equine viral arteritis, babesiosis and purpura haemorrhagica may be confused with one or other forms of AHS and should be excluded. These are the reasons why a laboratory diagnosis is essential to establish a correct and confirmatory diagnosis. This is the reason that laboratory confirmation is essential.

1. AHS clinical forms

There are four classical clinical forms of AHS: pulmonary, cardiac, mixed, and horse sickness fever (7).

The peracute or pulmonary form, which has a short incubation period (3–5 days), is characterised by severe dyspnoea and progressive respiratory involvement. An acute febrile reaction, lasting 1–2 days and reaching a maximum of approximately 40–41°C, may be the only sign. This is followed by various degrees of respiratory distress – respiratory rate may increase to 60 or even 75 breaths/minute. The animal may be observed to stand with its forelegs spread apart, its head extended and its nostrils fully dilated. Profuse sweating is common and spasmodic coughing may be observed terminally, with frothy fluid exuding from the nostrils. Death usually occurs within a few hours after the first clinical signs are observed, the animal having literally drowned in its own serous fluid. The pulmonary form is usually observed in completely susceptible animals, animals infected with a highly virulent strain of virus, or animals that are worked during the febrile stage of the disease. Recovery from this form is very rare, occurring in <5% of cases. This is also the form usually seen in dogs.

The incubation period of the subacute, oedematous or cardiac form varies from about 7 to 14 days, and the onset of clinical disease is marked by a febrile reaction (39–41°C) that lasts for 3–6 days. Shortly before the decline of the fever, characteristic oedematous swellings may appear. These initially involve the temporal or supraorbital fossae and the eyelids, and later extend to the lips, cheeks, tongue, intermandibular space and laryngeal region. Subcutaneous oedema sometimes extends a variable distance down the neck towards the chest and, in severe cases, may involve the chest and shoulders, but generally not the lower limbs. Terminally, petechial haemorrhages may be observed in the conjunctivae and on the ventral surface of the tongue. The animal finally becomes restless and may show signs of colic before death from cardiac failure. Difficulty in swallowing due to paralysis of the oesophagus is also seen. The mortality rate is about 50% and death usually occurs within 4–8 days after the onset of the febrile reaction. In recovering cases, swelling gradually subsides within a period of 3–8 days. This clinical form of AHS is usually associated with infection by virus strains of low
virulence or is encountered in immune animals infected by heterologous virus strains, or may be a function of biological variation in the infected animal.

The acute or mixed form represents a mixture of the pulmonary and cardiac forms and is often the most common form of AHS in horses and mules. The mortality rate is around 70% within 3–6 days after the onset of the febrile reaction. This form of the disease may manifest itself in the following ways:

- Initial pulmonary signs of a relatively mild degree are followed by marked oedematous swellings of the head and neck, with death resulting from heart failure.
- Oedematous swelling, typical of the subacute form, is followed by the sudden onset of dyspnoea and other clinical signs typical of the peracute pulmonary form.

Horse sickness fever is the mildest form and is frequently overlooked in natural outbreaks. The incubation period varies from 5 to 14 days, and is followed by a febrile reaction (39–40°C) of the remittent type, with morning remissions and afternoon exacerbations, lasting for 5–8 days. Apart from the febrile reaction, other clinical signs are rare. The conjunctivae may be slightly congested, the pulse rate may be increased, and a certain degree of anorexia, depression and oedema of supraorbital fossae may be present. There is no mortality. This form of the disease is usually observed in partially immune animals or in resistant species, such as the donkey and zebra.

There is no evidence that humans can become infected with any field strain of AHSV, either through contact with naturally or experimentally infected animals or by virus manipulation in laboratories. However, certain neurotropic vaccine strains that may cause encephalitis and meningitis in humans following transnasal infections have been described (28). Experimental and natural transmission of AHS to dogs has been reported through ingestion of infected horse meat (3). However, there is only limited and unsubstantiated evidence that dogs become infected by insect bites (34).

2. Isolation and identification of the agent

Several techniques are already available for AHS viral identification ranging from the rapid capture (indirect sandwich) enzyme-linked immunosorbent assay (ELISA), using either polyclonal antibodies (PAbS) or monoclonal antibodies (MAbS), to the polymerase chain reaction (PCR) test, including a new reverse-transcription (RT) PCR for discrimination of the nine AHSV serotypes (31), or cell culture and inoculation of newborn mice. If possible more than one test should be performed to diagnose an outbreak of AHS, especially the index case. The initial test can be a quick test such as ELISA or PCR, followed by virus isolation in tissue culture. Virus neutralisation (VN) for serotype identification should be performed as early in the outbreak as possible so that the correct vaccine can be selected. Subsequently, the ELISA may be very useful in laboratory diagnosis.

At present, there are no international standards for viruses or diagnostic reagents, and there is no standard methodology for the determination of AHSV. However, a viral panel has been evaluated, and comparative studies between different ELISAs for AHSV antigen determination have been carried out in different laboratories. The results have demonstrated a high level of correlation for antigen detection (30) using the indirect sandwich ELISAs for antigen studies (13, 18).

A very important aspect of the diagnosis is the selection of samples and their transportation to the laboratory.

a) Samples for virus isolation

Unclotted whole blood collected during the early febrile stage of the disease from sick animals, as well as small pieces (2–4 g) of spleen, lung and lymph nodes from animals that have died, are the samples of choice for diagnosis. Samples should be kept at 4°C during transportation and storage.

b) Cell culture

Successful direct isolation of AHSV has been performed on baby hamster kidney (BHK-21), monkey stable (MS) and African green monkey kidney (Vero) mammalian cell lines and on Culicoides and mosquito insect cell lines. Blood samples collected in an appropriate anticoagulant can be used undiluted as the inoculum. After 15–60 minutes of adsorption at ambient temperature or at 37°C, the cell cultures are washed and maintenance medium is added. Alternatively and more commonly, the blood is washed, lysed and diluted 1/10. This procedure removes unwanted antibody, which could neutralise free virus, and promotes release of virus associated with the red blood cell membranes. When tissue samples, such as spleen, lung, etc., are used, a 10% tissue suspension is prepared in phosphate buffered saline (PBS) or cell culture medium, containing antibiotics.

A cytopathic effect (CPE) may appear between 2 and 10 days post-infection with mammalian cells. Three blind passages should be performed before considering the samples to be negative. No CPE is observed in
insect cells but the presence of the virus can be observed 7–10 days after infected insect cells are passed onto mammalian cells.

c) Newborn mice
This method of isolation of AHSV involves the intracerebral inoculation of two families of 1–3-day-old mice. In positive cases, animals develop nervous signs between 3 and 15 days post-inoculation. The brains from sick animals may be collected, homogenised and re-inoculated intracerebrally into at least six 1–3-day-old mice. This second passage should present a shortened incubation period (2–5 days) and 100% infectivity. Virus may be typed directly from mouse brain by conventional neutralisation (VN) or by RNA extraction and sequencing.

d) Sandwich enzyme-linked immunosorbent assay
At least two serogroup-specific sandwich ELISAs have been developed and field tested for detection of AHSV antigen from both field samples and laboratory-infected tissue cultures (13, 18).

One technique (13) uses PAbs to the AHSV and the other (18) uses MAbs against one of the major proteins that is more conserved among serotypes – protein VP7. Both methods have been demonstrated to be adequate for the diagnosis of AHS, due to their high sensitivity and specificity and the availability of results in only 2–4 hours (30). The use of a chicken IgY in a double-antibody sandwich ELISA for detecting all AHSV serotypes has also been described (6).

Reagents for the ELISA may be obtained from the OIE Reference Laboratories for African horse sickness (see Table given in Part 3 of this Manual).

- The following is an example of a monoclonal antibody enzyme-linked immunosorbent assay
  i) **Solid phase:** Coat ELISA plates (e.g. high adsorption capacity Nunc Maxisorb) with a mixture of MAb 5G5 and 3D2 diluted in PBS, pH 7.2 (10 µg/ml each). Incubate overnight at 4°C.

  ii) Wash the plates five times with distilled water containing 0.05% (v/v) Tween 20 (washing solution). Gently tap the plates on to absorbent material to remove any residual wash.

  iii) Block the plates with PBS + 1% bovine serum albumin (BSA), pH 7.2, 200 µl/well, for 1 hour at 37°C.

  iv) Remove the blocking solution and gently tap the plates on to absorbent material.

  v) **Test samples:** Add the samples to be tested (twofold dilutions starting with undiluted spleen homogenates, or AHSV cell culture supernatant) diluted in PBS + 1% BSA, pH 7.2, 100 µl/well. Incubate for 1 hour at 37°C. (Spleen homogenate: homogenise approximately 2 cm³ [1 g] of spleen with 3 ml of MEM [minimal essential medium] culture medium. Centrifuge at 600 g for 10 minutes and save the supernatant.)

  vi) Wash the plates as described in step ii.

  vii) **Conjugate:** Dispense 100 µl/well of biotin-labelled 5G5 MAb diluted 1/500 in PBS + 1% BSA, pH 7.2. Incubate for 1 hour at 37°C. Wash the plates as described in step ii. Add 100 µl/well of avidin/peroxidase at optimal dilution in PBS + 1% BSA. Incubate for 45 minutes at room temperature.

  viii) Wash the plates as described in step ii.

  ix) **Substrate:** Add 200 µl/well of substrate solution (10 ml of 80.6 mM DMAB [dimethyl amino-benzaldehyde] + 10 ml of 1.56 mM MBTH [3-methyl-2-benzo-thiazolinone hydrazone hydrochloride] + 5 µl H₂O₂). Colour development is stopped by adding 50 µl of 3 N H₂SO₄ after approximately 5–10 minutes (before the negative control begins to be coloured).

  x) Read the plates at 600 nm (or 620 nm).

  xi) **Interpretation of the results:** Calculate the cut-off value as follows: C ± 0.06 = cut-off (where C is the absorbance value obtained with the negative control). Test samples giving absorbance values lower than the cut-off are regarded as negative. Test samples giving absorbance values greater than the cut-off + 0.20 are regarded as positive. Test samples giving intermediate absorbance values are doubtful and a second technique must be employed to confirm the result.

e) Polymerase chain reaction
An RT-PCR assay for the specific detection of AHSV genome has been developed. This assay (method 1) can be used to detect viral RNA in blood collected in EDTA (ethylene diamine tetra-acetic acid), homogenised equid or mouse tissue or cell culture fluids. Primers correspond to the 5’ end (nucleotides 1–21) and 3’ end (nucleotides 1160–1179) of RNA segment 7 (4, 22, 33, 40), amplifying the complete segment 7 of the genome. More recently, a new conventional technique (method 2) and a real-time RT-PCR (method 3) technique have been developed using primers from a highly conserved region of the same
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viral genome segment 7, to achieve better sensitivity and rapidity in the diagnosis of AHS (10). The three RT-PCR procedures can detect the nine virus serotypes and are described below.

- **Test procedure – method 1 (4)**

Extraction of nucleic acids from spleen samples is carried out as follows: 1 g of tissue sample is homogenised in 1 ml of denaturing solution (4 M guanidinium thiocyanate, 25 mM sodium citrate, 0.1 M 2-mercaptoethanol, 0.5% sarcosyl). After centrifugation, 1 µg of yeast RNA, 0.1 ml of 2 M sodium acetate pH 4, 1 ml of phenol and 0.2 ml of chloroform/isoamyl alcohol mixture (49/1) are added to the supernatant. The suspension is vigorously shaken and cooled on ice for 15 minutes. After centrifugation, the RNA present in the aqueous phase is phenol extracted, ethanol precipitated and resuspended in sterile water. The methods for cDNA synthesis and PCR amplification are performed using, in all cases, 37°C as renaturing temperature. The sequences of the PCR primers used are 5'-GTT-AAA-ATT-CGG-TTA-GGA-TG-3', which corresponds to the messenger RNA polarity and 5'-GTA-AGT-GTA-TTC-GGT-ATT-G-3', which is complementary to the messenger RNA polarity. The PCR procedure itself involves 40 cycles (94°C for 1 minute, 55°C for 1.5 minutes, 72°C for 2.5 minutes and 70°C for 7 minutes) and then the PCR tubes are kept at 4°C. Analysis of the PCR products is carried out by electrophoresis in 1.2% (w/v) agarose gels containing ethidium bromide. AHS-positive samples will resolve in a 1179 base-pair band.

- **Test procedure – method 2 (10)**

Viral double-stranded RNA extraction procedure can be achieved using the commercial High Pure Viral Nucleic Acid Kit (Roche Diagnostics), which is described below. A number of other RNA extraction kits are commercially available for the preparation of template suitable for RT-PCR depending on the sample submitted for analysis and may be appropriate for use. Different samples can be used in this procedure such as cell culture supernatants, EDTA/blood, serum or tissue homogenates.

The High Pure Viral Nucleic Acid Kit (Roche Diagnostics) includes the following reagents: Binding Buffer, Poly (A) carrier RNA, Proteinase K, Inhibitor Removal Buffer, Wash Buffer, and High Pure Filter Tubes and collection tubes.

For organ and tissue samples, first prepare a 1/10 homogenate of the material in PBS, then centrifuge to clarify at 12,000 × g for 5 minutes. Extract RNA from the resultant supernatant fluid. Sometimes it is recommended to process a 1/10 dilution of the supernatant in parallel.

Extraction for control samples: 1/10 tissue homogenates (same tissue as samples to be analysed): (a) a negative control: use 200 µl of a homogenate of AHSV-negative tissue; (b) a positive control: use 200 µl of a homogenate of AHSV-positive tissue. Process both controls together with the test samples.

i) Pipette 200 µl of sample into a 1.5 ml microcentrifuge tube.

ii) Add 200 µl of binding buffer supplemented with poly(A) and 50 µl of proteinase K. Mix immediately. Incubate for 10 minutes at 72°C.

iii) Briefly centrifuge the 1.5 ml microcentrifuge tube to remove drops from the inside of the lid.

iv) Mix samples with 100 µl of binding buffer. Briefly centrifuge to remove drops from the inside of the lid.

v) Place the High Pure filter tube in a collection tube and pipette the sample in the upper reservoir. Centrifuge for 1 minute at 8000 rpm (with blood samples, repeat the centrifugation step if sample remains in the filter tube).

vi) Discard the flowthrough and the collection tube, and place the filter tube into a clean collection tube.

vii) Add 500 µl of Inhibitor Removal Buffer to the upper reservoir and centrifuge for 1 minute at 8000 rpm.

viii) Discard the flowthrough and the collection tube, and place the filter tube into a clean collection tube.

ix) Add 450 µl of wash buffer to the upper reservoir and centrifuge for 1 minute at 8000 rpm.

x) Discard the flowthrough and the collection tube, and repeat the washing step.

xi) Discard the collection tube and place the filter tube into a clean collection tube. Centrifuge for 10 seconds at 13,000 rpm to remove residual wash buffer.

xii) Discard the collection tube and place the filter tube in a clean 1.5 ml microcentrifuge tube.

xiii) For the RNA elution, add 50 µl of prewarmed (70°C) RNase free sterile water to the upper reservoir. Centrifuge for 1 minute at 8000 rpm.

xiv) Use immediately or store at –20°C for future use.
Stock solutions for RT-PCR

- Nuclease-free sterile water.
- **One-step RT-PCR kit** is commercially available from Qiagen, that contains RT and PCR enzymes, PCR nucleotide mix, PCR buffer with magnesium chloride, and Q solution.
- RNase inhibitor enzyme is commercially available from several suppliers.
- Primers at a concentration of 20 pmol/µl: Primer 1 sequence 5’-GGC-TCC-AAC-ACT-CAC-AAG-ATG-T-3’ (forward primer); primer 2 sequence 5’-GGC-GGA-TTA-ATA-GGC-TGC-ATA-3’ (reverse primer).
- 10× **Loading buffer**: 0.2% xylene cyanol, 0.2% bromophenol blue, 30% glycerol.
- **TAE buffer (50×)** for agarose gel: Tris base (242 g); glacial acetic acid (57.1 ml); 0.5 M EDTA, pH 8.0 (100 ml); distilled water (to 1 litre).
- **Marker DNA**: 100 base-pair ladder is commercially available.

RT-PCR amplification assay

i) Denaturation of the viral double-stranded RNA is recommended to be performed prior to the RT-PCR step. Prepare the following mixture for each sample: primer 1, 20 pmol/µl (0.5 µl), primer 2, 20 pmol/µl (0.5 µl), 7 µl of nuclease-free sterile water, and 2 µl of extracted sample template.

ii) Place the tubes in a thermal cycler or heating block, and incubate at 95°C for 5 minutes. Briefly centrifuge the tubes to remove drops from the inside of the lid, and immediately, place the tubes on ice.

iii) In a sterile 1.5 ml microcentrifuge tube prepare the RT-PCR reaction mixture described below for each sample. Prepare the reaction mixture in bulk for the number of samples to be assayed allowing for one extra sample.

iv) Nuclease-free sterile water (3.25 µl), PCR Buffer 5× with magnesium chloride (5 µl), dNTP mix 10 mM (0.5 µl), Q solution 5× (5 µl), RNase inhibitor 20 U/µl (0.25 µl), enzymes mix (1 µl).

v) Add 15 µl of the RT-PCR reaction mix to each PCR tube containing the denaturated RNA template.

vi) Include a positive reaction control (2 µl of AHSV RNA) and a negative reaction control (2 µl of distilled water) for each RT-PCR run.

vii) Place all the tubes in an automated thermal cycler and run the following programme:

- One cycle at 55°C for 30 minutes.
- One cycle at 95°C for 15 minutes.
- 40 cycles at 94°C for 30 seconds, 58°C for 30 seconds and 72°C for 30 seconds.
- One cycle at 72°C for 7 minutes.
- Hold at 4°C.

viii) At the end of the programme, remove PCR tubes and add 2.5 µl of 10× loading buffer to each tube.

ix) Load all the samples in a 3% agarose gel in TAE buffer containing ethidium bromide at a final concentration of 0.5 µg/ml. Add marker DNA to one lane on each side of the gel.

x) Run the gel at a constant voltage of 150–200 volts for about 30 minutes.

xi) **Reading the results:** Examine the gel over a UV light source. In a positive sample, a discrete band will be present that should co-migrate with the PCR product of the positive control. Calculate the size of the PCR products in the test samples and the positive control by reference to the standard markers. The PCR product of the positive control has a size of 102 base pairs. No bands should be seen in the negative control.

Test procedure – method 3: real-time RT-PCR (10)

Follow the sample preparation and viral RNA extraction procedure as described for the conventional RT-PCR method 2 described above.

Stock solutions for RT-PCR

- Nuclease-free sterile water.
- **QuantiTect probe RT-PCR kit** is commercially available from Qiagen, which contains RT enzymes (RT mix) and PCR enzymes, PCR nucleotide mix, PCR buffer with magnesium chloride (master mix).
– RNase inhibitor enzyme is commercially available from several suppliers.
– Use the same primers than in the conventional RT-PCR method 2 described above. Primers at a concentration of 20 pmol/µl: Primer 1 sequence 5'-GGC-TCC-AAC-ACT-CAC-AAG-ATG-T-3' (forward primer); primer 2 sequence 5'-GGC-GGA-TTA-ATA-GGC-TGC-ATA-3' (reverse primer).
– TaqMan-MGB\textsuperscript{®} probe at a concentration of 10 pmol/µl: (5'-[6-carboxy-fluorescein (FAM)]-TGG-CAC-GCC-TTA-GCG-GC-[minor groove binder molecule (MGB)]-3').

**RT-PCR amplification assay**

i) Denaturation of the viral double-stranded RNA is recommended to be performed prior to the RT-PCR step. Prepare the following mixture for each sample: primer 1 20 pmol/µl (0.75 µl), primer 2 20 pmol/µl (0.75 µl), 6.5 µl of nuclease-free sterile water, and 2 µl of extracted sample template.

ii) Place the tubes in a thermal cycler or heating block, and incubate at 95°C for 5 minutes. Briefly centrifuge the tubes to remove drops from the inside of the lid, and immediately, place the tubes on ice.

iii) In a sterile 1.5 ml microcentrifuge tube, prepare the RT-PCR reaction mixture described below for each sample. Prepare the reaction mixture in bulk for the number of samples to be assayed allowing for one extra sample.

iv) Nuclease-free sterile water (1.375 µl), TaqMan-MGB\textsuperscript{®} probe 10 pmol/µl (0.625 µl) Master mix 2× (12.5 µl), RNase inhibitor 20 U/µl (0.25 µl), RT mix (0.25 µl).

v) Add 15 µl of the RT-PCR reaction mix to each optical PCR tube or to each well of an optical reaction plate, containing the denaturated RNA template.

vi) Include a positive reaction control (2 µl of AHSV RNA) and a negative reaction control (2 µl of distilled water) for each RT-PCR run.

vii) Place all the tubes in an automated real-time thermal cycler and run the following program:

- One cycle at 55°C for 30 minutes.
- One cycle at 95°C for 15 minutes.
- 45 cycles at 94°C for 15 seconds, 60°C for 1 minute. Make the fluorescence measurement at the end of each cycle in the appropriate channel.

viii) *Reading the results:* Assign a threshold cycle (Ct) value to each PCR reaction from a scan of all amplification plots (a plot of the fluorescence signal versus cycle number). Negative test samples, uninfected negative or extraction blank controls should have a Ct value >45.0. Positive test samples and controls should have a Ct value <45.0 (strongly positive samples have a Ct value <30.0).

**AHSV serotyping**

Until recently VN test has been the method of choice for serotyping as well as the ‘gold’ standard test for identifying AHSV’s isolated from the field using type specific antisera (37). This technique takes five or more days before results are obtained. The recent development of a type-specific RT-PCR for identification and differentiation of the nine AHSV serotypes provided a method of confirming the identity of AHSV in tissue samples within a few hours (27, 31). Nine pairs of primers were designed in the virus genome segment 2 for each specific serotype. The results obtained show a perfect agreement between the RT-PCR and the VN test.

Typing of nine AHS serotypes has also been carried out with probes developed from a set of cloned full-length VP2 genes and can be an alternative to amplification of genome segment 2 (17).

3. Serological tests

OIE International Reference sera are available (consult the OIE web site [www.oie.int] for the address). These sera were developed to standardise the ELISA, which is an OIE prescribed test. In addition, a panel of reference antiserum has been evaluated and comparative studies between different ELISAs using MAbs and PAbs, and involving several laboratories have been carried out. The results have demonstrated a high level of correlation using indirect or competitive ELISAs for antibody detection (12, 20, 30). More recently, a panel of sera has been generated and is now being used for the annual quality assurance of three antibody detection ELISAs as used by the national laboratories in Europe (9, 12, 20).

Indirect and competitive ELISAs using either soluble AHSV antigen or a recombinant protein VP7 (12, 20) have proved to be good methods for the detection of anti-AHSV group-reactive antibodies, especially for large-scale investigations (30). Both of these tests have been recognised by the European Commission (9). The competitive
ELISA can also be used for testing wildlife as species-specific anti-globulin is not required with this method. An immunoblotting test has been adapted for anti-AHS antibody determination (20). It is especially suitable for small numbers of sera. An indirect ELISA is also available that uses the AHSV serotype 4 nonstructural protein NS3 as antigen. The assay can be used to differentiate between animals infected or vaccinated with the live vaccine from those vaccinated with an inactivated vaccine made with purified virions (21). However, it should be noted that no such inactivated vaccine is at present on the market. The complement fixation (CF) test has been widely used, but some sera are anti-complementary, particularly donkey and zebra.

a) Indirect enzyme-linked immunosorbent assay (a prescribed test for international trade)

The recombinant VP7 protein has been used as antigen for AHSV antibody determination with a high degree of sensitivity and specificity (20, 38). Other advantages of this antigen are its stability and its lack of infectivity (23).

- **Test procedure**
  
  i) **Solid phase:** Coat ELISA plates (e.g. high adsorption capacity Nunc Maxisorb) with recombinant AHSV-4 VP7 diluted in carbonate/bicarbonate buffer, pH 9.6. Incubate overnight at 4°C.
  
  ii) Wash the plates five times with distilled water containing 0.01% (v/v) Tween 20 (washing solution). Gently tap the plates on to absorbent material to remove any residual wash.
  
  iii) Block the plates with PBS, pH 7.2 + 5% (w/v) skimmed milk, 200 µl/well, for 1 hour at 37°C.
  
  iv) Remove the blocking solution and gently tap the plates on to absorbent material.
  
  v) **Test samples:** Serum samples to be tested, and positive and negative control sera, are diluted 1/25 in PBS + 5% (w/v) skimmed milk + 0.05% (v/v) Tween 20, 100 µl per well. Incubate for 1 hour at 37°C. For titration, add twofold dilution series from 1/25 (100 µl/well), one serum per plate column, and do the same with positive and negative controls. Incubate for 1 hour at 37°C.
  
  vi) Wash the plates as described in step ii.
  
  vii) **Conjugate:** Dispense 100 µl/well of horseradish peroxidase conjugated anti-horse gamma-globulin diluted in PBS + 5% milk + 0.05% Tween 20, pH 7.2. Incubate for 1 hour at 37°C.
  
  viii) Wash the plates as described in step ii.
  
  ix) **Substrate:** Add 200 µl/well of substrate solution (10 ml DMAB + 10 ml of MBTH + 5 µl H₂O₂). Colour development is stopped by adding 50 µl of 3 N H₂SO₄ after approximately 5–10 minutes (before the negative control begins to be coloured). Other substrates such as ABTS (2,2'-azino-di-[3-ethyl-benzothiazoline]-6-sulphonic acid), TMB (tetramethyl benzidine), or OPD (orthophenyldiamine) can also be used.
  
  x) Read the plates at 600 nm (or 620 nm).
  
  xi) **Interpretation of results:** Calculate the cut-off value by adding 0.6 to the value of the negative control. (0.06 is the standard deviation derived with a group of 30 negative sera) Test samples giving absorbance values lower than the cut-off are regarded as negative. Test samples giving absorbance values greater than the cut-off + 0.15 are regarded as positive. Test samples giving intermediate absorbance values are doubtful and a second technique must be employed to confirm the result.

b) Immunoblotting

The binding of antibodies to viral proteins separated by electrophoresis and transferred to nitrocellulose paper has been used for the determination of anti-AHSV antibodies (20).

- **Test procedure**
  
  Semipurified proteins of AHSV are separated by SDS/PAGE (sodium dodecyl sulphate/polyacrylamide gel electrophoresis) in 15% (w/v) acrylamide-N,N'-diallyltartar-diamide (DATD) gels. Separated proteins are transferred to a nitrocellulose membrane filter at a constant current of 280 mA for 6 hours at 4°C. Immunoblotting of a nitrocellulose membrane cut into strips is carried out using the sera at 1/20 dilution, peroxidase-conjugated rabbit-anti-horse immunoglobulin at 1/500 dilution and an incubation time of 1 hour at 37°C. The bands recognised by the sera are developed by the 4-chloro-naphthol technique.

  **Interpretation of results:** The comparison between the band pattern of a positive control and a negative control serum permits the identification of the specific viral bands. The appearance of two or more of these specific bands in a problem serum allows it to be classified as positive anti-AHS serum.

c) NS3 enzyme-linked immunosorbent assay

An indirect ELISA to distinguish between infected horses and horses vaccinated with an inactivated purified AHSV serotype 4 vaccine, using a recombinant NS3 protein as antigen, has been described (21). The
results obtained indicate that recombinant NS3 can differentiate between infected animals and those vaccinated animals vaccinated with an inactivated vaccine, implying that this recombinant could be an important diagnostic reagent that could allow the transportation of vaccinated horses. To ensure the reliability of results it is essential that the selected AHSV-inactivated vaccine be a purified exclude with certainty any trace of NS3 that, if present, would stimulate the production of anti-NS3 antibodies vaccine. This is to in vaccinated horses, thus mimicking the response to a natural infection. This type of ELISA would also be useful for distinguishing between infected horses and horses vaccinated with a subunit vaccine in which NS3 protein is not present. However, it should be noted that no inactivated vaccines against AHS are at present on the market.

d) Complement fixation (a prescribed test for international trade)

The CF test has been used extensively, but due to the anti-complementary effect of some sera, as well as the good results obtained with the ELISA, its use is decreasing. The CF test is frequently used for the demonstration of group-specific antibodies against AHSV. A sucrose/acetone mouse-brain extract is commonly used as antigen.

- **Reagents**
  i) Veronal buffered saline containing 1% gelatin (VBSG).
  ii) Serum samples, free from erythrocytes, must be heat inactivated: horse serum at 56°C, zebra serum at 60°C and donkey serum at 62°C, for 30 minutes.
  iii) The antigen is a sucrose/acetone extract of AHSV-infected mouse brain. The control antigen is uninfected mouse brain, extracted in the same way. In the absence of an international standard serum, the antigen should be titrated against a locally prepared positive control serum. In the test, four to eight units are used.
  iv) The complement is a normal guinea-pig serum.
  v) The haemolysin is a hyperimmune rabbit serum against sheep red blood cells (SRBCs).
  vi) The SRBCs are obtained by aseptic puncture of the jugular vein and preserved in Alsever’s solution\(^1\) or sodium citrate.
  vii) The haemolytic system (HS) is prepared by diluting the haemolysin to contain two haemolytic doses and using this to sensitise washed SRBCs. The SRBCs are standardised to a 3% concentration.
  viii) **Control sera:** A positive control serum is obtained locally and validated. Serum from a healthy antibody-negative horse is used as the negative control serum.

- **Test procedure**
  i) Sera, complement and antigen are reacted in 96-well round-bottom microtitre plates, or in tubes if the macro-technique is used, at 4°C for 18 hours.
  ii) Sensitised SRBCs (3%) are added to all wells on the microtitre plate.
  iii) The test plate is incubated for 30 minutes at 37°C.
  iv) Plates are then centrifuged at 200 \(g\), and the wells are scored for the presence of haemolysis.
  v) The following controls are used: (a) serum and complement; (b) serum and SRBCs; (c) CF antigen and control antigen each with 4 CH\(_{50}\) (50% complement haemolytic units); 2 CH\(_{50}\), and 1 CH\(_{50}\) of complement; (d) CF antigen and SRBCs; (e) control antigen and SRBCs; (f) complement dilutions of 4 CH\(_{50}\), 2 CH\(_{50}\), and 1 CH\(_{50}\); and (g) SRBCs.
  vi) Results are read using 50% haemolysis as the end point. The inverse of the highest dilution of serum specifically fixing complement with the CF antigen is called the titre.
  vii) A titre of 1/10 or more is positive, under 1/10 is negative.

e) Virus neutralisation (VN)

Serotype-specific antibody can be detected using the VN test (14, 15). The VN test may have additional value in epidemiological surveillance and transmission studies, mainly in endemic areas where multiple serotypes are likely to be present (27).

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\(^{1}\) 20.5 g dextrose (114 mM), 7.9 g sodium citrate 2\(\text{H}_2\text{O}\) (27 mM), 4.2 g NaCl (71 mM), \(\text{H}_2\text{O}\) to 1 litre. Adjust to pH with 1 M citric acid.
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- **VN Test procedure**
  i) Stock virus is diluted to yield 100 TCID$_{50}$ (50% tissue culture infective dose), with a range of 30–300 TCID$_{50}$, per 25 µl, and 25 µl is added to each of four microtitre wells containing 25 µl serum dilutions. For screening, a final serum dilution of 1/10 is used. Doubling dilutions are used for titrations.
  ii) Serum/virus mixtures are incubated for 60 minutes at 37°C prior to the addition of 0.1 ml of Vero cell suspension (200,000 cells/ml) to each test well.
  iii) A back titration of virus stock is prepared for each test using four wells per tenfold dilution, 25 µl per well. Test plates are incubated at 37°C, 5% CO$_2$, 95% humidity for 4–5 days, until the back titration indicates that the stock virus contains 30–100 TCID$_{50}$.
  iv) The plates are then fixed and stained in a solution of 0.15% (w/v) crystal violet in 2% (v/v) glutaraldehyde and rinsed. Alternatively, they may be fixed with 70% ethanol and stained with 1% basic fuschsin.
  v) The 50% end-point titre of the serum is calculated by the Spearman–Kärber method and expressed as the negative log$_{10}$.

C. REQUIREMENTS FOR VACCINES AND DIAGNOSTIC BIOLOGICALS

Polyvalent or monovalent live attenuated AHS vaccines based on the selection in Vero cell culture of genetically stable macroplaques are commercially available from Onderstepoort Biological Products, Onderstepoort, South Africa (8). An inactivated monovalent (serotype 4) AH SV vaccine based on virus purification and inactivation with formalin has been produced commercially, but is not available at the present time (5, 16). Requirements for both attenuated and inactivated vaccines are summarised below.

Guidelines for the production of veterinary vaccines are given in Chapter 1.1.8 Principles of veterinary vaccine production. The guidelines given here and in Chapter 1.1.8 are intended to be general in nature and may be supplemented by national and regional requirements.

C1. Attenuated African horse sickness vaccine

1. **Seed management**
   a) **Characteristics of the seed**
      The seed virus is prepared by selection in Vero cells of genetically stable large plaques from low passage levels of AHSV. The plaque mutants are then further multiplied by three passages in Vero cells. A large quantity of this antigen is lyophilised and stored at −20°C as seed stock antigen.
   b) **Method of culture**
      The seed virus is grown in roller cultures of Vero cells.
   c) **Validation as a vaccine**
      The seed virus must be shown to be free of contaminating viruses, bacteria and mycoplasmas by the appropriate techniques. The serotype identity of the seed virus is confirmed.

2. **Method of manufacture**
At the onset of a production run, working antigens are produced from the seed stock antigen in either BHK-21 or Vero cell cultures. The working antigens are tested for sterility, purity and identity and should contain at least 1 × 10$^6$ plaque-forming units (PFU)/ml of infectious virus.

Roller bottle cultures of Vero or BHK-21 cells are grown using gamma-irradiated bovine serum in the growth medium. Once the cultures are confluent, the medium is poured off and the cells are seeded with the working antigens. After 1 hour, maintenance medium is added to the cultures. Incubation is continued at 37°C for 2–3 days. When the CPE is advanced, both cells and supernatant medium are harvested. The products from the same serotype are pooled and stored at 4°C.
3. **In-process control**

The pooled harvests of the individual serotypes are tested for sterility and assayed for infectivity by plaque titration on Vero cell cultures. The minimum acceptable titre is $1 \times 10^6$ PFU/ml.

Finally, two quadrivalent vaccines are constituted by mixing equal volumes of serotypes 1, 3, 4, 5 and 2, 6, 7, 8 respectively. Subsequently, AHSV serotype 5 was withdrawn from this vaccine. A monovalent type can also be prepared. After addition of suitable stabiliser, the vaccine is distributed in 1.0 ml volumes into glass vials and freeze-dried.

4. **Batch control**

a) **Sterility**

Following lyophilisation, five bottles of vaccine are selected at random and tested for sterility by internationally accepted methods. Tests for sterility and freedom from contamination of biological products are given in Chapter 1.1.9.

b) **Safety**

Innocuity of a vaccine is determined by the inoculation of reconstituted vaccine into mice (0.25 ml intraperitoneally), guinea-pig (1.0 ml intraperitoneally), and a horse (5.0 ml subcutaneously). All the animals are observed daily for 14 days. The rectal temperature of the horse is taken twice daily for 14 days and should never exceed 39°C.

c) **Potency**

Potency is largely based on virus concentration in the vaccine.

The minimum immunising dose for each serotype is about $1 \times 10^3$ PFU/dose. The infectivity titre of the final product is assayed by plaque titration in Vero cell cultures and should contain at least $1 \times 10^5$ PFU/dose. The horse used for safety testing is also used for determining the immunogenicity of a vaccine.

Serum samples are collected on the day of vaccination and 21 days later, and are tested for neutralising antibodies against each serotype by the plaque-reduction test using twofold serum dilutions and about 100 PFU of virus. The horse should develop a neutralising antibody titre of at least 20 against at least three of the four serotypes in the quadrivalent vaccine.

d) **Duration of immunity**

Duration of immunity is not assessed with every batch of vaccine, but immunity is known to persist for at least 4 years. However, in the light of possible interference between the individual serotypes in each quadrivalent vaccine, annual revaccination is advocated in enzootic regions. Vaccination with monovalent vaccine stimulates a practically lifelong immunity.

e) **Stability**

In the lyophilised state, the vaccine is known to retain its potency for many years when stored at 4–8°C. However, an expiration date of 2 years is normally given.

C2. **Inactivated African horse sickness vaccine**

1. **Seed management**

a) **Characteristics of the seed**

The seed virus is the attenuated vaccine strain (AHSV serotype 4) used widely in the field in Africa and southern Europe (seed stocks are available from the OIE Reference Laboratory [Onderstepoort]). The virus was passaged ten times in the brains of newborn mice for attenuation, and then passaged a further ten times in roller tube cultures of BHK-21 cells. This material was plaque-purified three times in Vero cell cultures by selection of a large plaque (4–6 mm) at terminal dilutions. The final plaque material was passaged once in the brain of newborn mice and four times in Vero cell cultures. This material was lyophilised and constitutes the master seed virus.

b) **Method of culture**

The attenuated virus is propagated by passage in BHK-21 cells in roller bottles.
c) Validation as a vaccine

The seed virus must be shown to be free of contaminating viruses, bacteria and mycoplasmas by the appropriate techniques. The serotype identification is confirmed by a double-sandwich ELISA using MAbs.

2. Method of manufacture

Virus is harvested when characteristic CPE is fully developed. Cultivation thereafter is carried out in cell suspensions with serum-free Stoker’s medium (MEM) at 37°C. Virus growth is checked by cell viability (CPE), and when optimum conditions are reached, the fermentor culture temperature is lowered to 4°C. The virus suspension is aseptically harvested.

The filtered suspension of attenuated virus is inactivated with formaldehyde at a final concentration of 1/1,000. The suspension is transferred to another tank and kept at 4°C under mild agitation for at least 10 days to ensure that all virus is inactivated. Inactivation controls are also performed.

After inactivation, the antigen suspension is concentrated by ultrafiltration, and then purified by selective precipitation by a complex of ethylene oxide and bivalent cations according to a patented process.

3. In-process control

Control of virus identity by double-sandwich ELISA, titration of virus before inactivation in Vero cell lines, sucrose gradient analysis to estimate the viral particle concentration, and sterility tests are performed.

4. Batch control

a) Sterility

Tests for sterility and freedom from contamination of biological products are given in Chapter 1.1.9.

b) Safety

AHSV-susceptible horses are inoculated intramuscularly or subcutaneously with a single or double dose of vaccine. Temperatures are recorded daily for 14 days, and horses are observed for signs of abnormality. Five guinea-pigs receive a horse vaccine dose by the intramuscular route. Sera collected at day 21 are tested for antibodies by VN.

c) Potency

The potency control is performed by challenge of vaccinated horses. Some animals are vaccinated with one dose, and others with two doses, of vaccine. The challenge is made by the intravenous route at 77 days after the first vaccine inoculation. Where two vaccinations are used, the second dose is given 21 days after the first dose.

Serum samples are collected on the day of vaccination and 21 days later, and are tested for neutralising antibodies and virus isolation.

d) Duration of immunity

No challenges have been performed on horses; only serological studies have been carried out. If the level of antibodies after two injections (recommended field protocol) and at 12 months post-vaccination is equivalent to the levels conferred by a single vaccination at 28 days post-vaccination, the horses are considered to be protected. With this standard, the duration of immunity is 1 year, starting 7–10 days after the first injection. One booster every year is sufficient to confer a protective level in the following years. The protocol for vaccination recommended by the manufacturer is two vaccinations (given 21 days apart) and one booster given at 365 days after the first vaccination.

e) Stability

Due to the fact that the inactivated vaccine is a relatively new product, not much data are available on the duration of its stability. Results have been obtained that demonstrate vaccine stability in excess of 3 years.

C3. African horse sickness subunit vaccine

AHSV serotype 4 outer capsid protein VP2 and VP5 plus inner capsid protein VP7, derived from single and dual recombinant baculovirus expression vectors have been used in different combination to immunise horses (24). However, this vaccine is not commercially available.
An experimental vaccine using crude cell extracts containing the three structural proteins was sufficient to obtain a complete protective immune response in horses challenged with AHS virulence virus \((10^6 \text{ TCID}_{50})\). Viraemia was not detectable in vaccinated horses (24). Further analysis of partially protective crude lysate revealed that only soluble VP2 was capable of inducing neutralising antibodies. A definition of neutralising sites of the VP2 virus protein of AHSV serotypes 3, 4 and 9 has been determined (2, 25, 36) and very recently, the full protection of horses immunised with a soluble recombinant VP2 protein (AHSV serotype 5) administrated with saponin to a lethal AHSV challenge has been reported (32). Although further experiments need to be performed to estimate the duration of the immunity induced by these proteins, the data indicate the effectiveness of this candidate vaccine.

REFERENCES


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NB: There are OIE Reference Laboratories for African horse sickness (see Table in Part 3 of this Terrestrial Manual or consult the OIE Web site for the most up-to-date list: www.oie.int).
CHAPTER 2.5.2.
CONTAGIOUS EQUINE METRITIS

SUMMARY

Contagious equine metritis is an inflammation of the endometrium of mares caused by Taylorella equigenitalis, which usually results in temporary infertility. It is a nonsystemic infection, the effects of which are restricted to the reproductive tract of the mare.

If present, the chief clinical signs are a slight to copious mucopurulent vaginal discharge and a variable cervicitis and vaginitis. Recovery is uneventful, but prolonged asymptomatic carriage is established in a proportion of infected mares. Taylorella equigenitalis is most frequently transmitted by sexual contact with carrier stallions, which are always asymptomatic and in which the principal sites of T. equigenitalis colonisation are the urogenital membranes (urethral fossa, urethral sinus, urethra and penile sheath). Inadequate hygiene during the cleansing or examination of the genitalia of horses can also be responsible for the transmission of infection. The sites of persistence of T. equigenitalis in the mare are urogenital membranes, principally in the clitoral sinuses and fossa and very infrequently in the uterus. Foals born of carrier mares may also become carriers. The organism can infect equid species other than horses, e.g. donkeys.

Washing with disinfectants combined with local and systemic antibiotic treatment can eliminate T. equigenitalis. Vaccination has been found to be ineffective. The principal means of control is through preventing transmission by establishing that stallions and mares are free from T. equigenitalis before breeding commences. Determination of the carrier state depends on the detection of T. equigenitalis on urogenital swabs of mares and stallions and its accurate identification. Serum antibody to T. equigenitalis can be detected in mares for 3–7 weeks after infection and can also be demonstrated in the occasional carrier mare, but never in the stallion. Serology is of value in detecting recent, but not chronic, infection in the mare, but the emphasis for control of the disease should be on the detection of carriers by culture.

Identification of the agent: Swabs must be transported to the laboratory with precautions to avoid loss of viability. The swab should be fully submerged in Amies charcoal medium and transported to the testing laboratory, preferably in temperature-controlled conditions, for plating out within 48 hours of collection. Growth of T. equigenitalis is likely to take at least 72 hours and may take up to 14 days, but usually does not take longer than 6 days at 37°C on medium enriched with heated blood and an atmosphere of 5–10% CO₂. An incubation time of at least 7 days is advisable before certifying cultures negative for T. equigenitalis. After 72 hours under appropriate culture conditions colonies may be small – up to 2–3 mm in diameter – watery to opaque and yellowish grey, and are smooth with an entire edge. Taylorella equigenitalis is a Gram-negative, small, coccoid rod that is sometimes pleomorphic and exhibits bipolar staining. It produces catalase and phosphatase, and is strongly oxidase positive. It is otherwise unreactive biochemically, and identification is finally dependent on antigenic characterisation of an isolate using specific antibodies. The fastidious nature of T. equigenitalis makes it difficult to isolate and test-breeding of stallions for detection of the carrier state has been used as a valuable adjunct to cultural examination.

Recently another species of Taylorella, T. asinigenitalis, has been isolated from male donkeys and horse stallions in the United States of America and from stallions in Europe. This bacterium has not been associated with naturally occurring disease; it resides in the genital tract of male donkeys and can be passed to other donkeys and horses during mating.
Antibody to whole killed T. equigenitalis and a latex agglutination kit employing these antibodies may be used. Specificity for the organism and evidence of its failure to react with other oxidase positive/catalase positive, Gram-negative bacteria that might be cultured from the urogenital tract of horses is essential. Monoclonal antibodies have been developed that can be used successfully to identify T. equigenitalis and distinguish it from strains of T. asinigenitalis.

**Serological tests:** No serological test described to date will, by itself, reliably detect infection for diagnosis and control purposes. However, serological tests can be used as an adjunct to culture for T. equigenitalis in screening mares recently bred to a carrier stallion, but must not be used as a substitute for culture.

**Requirements for vaccines and diagnostic biologicals:** Effective vaccines that protect against contagious equine metritis or prevent colonisation by T. equigenitalis are not yet available.

### A. INTRODUCTION

Contagious equine metritis was first described in the United Kingdom (UK) in 1977 (12), after which it was diagnosed in a number of countries world-wide. It first presented as disease outbreaks characterised by a mucopurulent vaginal discharge originating from inflammation of the endometrium and cervix, resulting in temporary infertility. The fastidious nature and slow growth of the causative bacterium, *Taylorella equigenitalis*, caused difficulties in initial attempts at culture (22), but the disease was reproduced by experimental clitoral challenge with isolated laboratory-grown bacteria (21, 23, 27). Using appropriate culture conditions, *T. equigenitalis* can be isolated from infective vaginal discharge. Mares may encounter more than one episode of the disease in a short period of time (32). Serum antibody persists for 3–7 weeks after infection, but often it is not detectable for up to 15–21 days after recovery from acute infection in the mares (14). Most mares recover uneventfully, but some may become carriers of *T. equigenitalis* for many months (21). Colonisation by *T. equigenitalis* is most consistently demonstrated by culture of swabs taken from the recesses of the clitoral fossa and sinuses, but it may be recovered from the cervix and endometrium in pure culture (21). Carriage does not always affect conception (33), and in such cases pregnancy may proceed so that foals are born, become infected by passage down the birth canal, and thereby also become long-term and subclinical carriers (30). Many primary cases of infection with *T. equigenitalis* in the mare are subclinical, and a frequent indicator of infection is a mare returning in oestrus prematurely after being bred to a putative carrier stallion.

Carrier mares and stallions act as reservoirs of *T. equigenitalis*, but stallions, because they mate with numerous mares, play a much more important role in transmission of the bacterium. The urogenital membranes of stallions become contaminated at coitus, leading to a carrier state that may persist for many months or years (25). Unhygienic examination of mares and unsanitary washing of the stallion’s penis may also spread the organism. Other sites of the horse’s body are not known to harbour *T. equigenitalis*. Most carrier mares are clitoral carriers of *T. equigenitalis*. Long-term persistence of the organism in the uterus, though uncommon, can occur. To detect such carriers of *T. equigenitalis*, cervical or endometrial swab samples should be taken routinely in addition to sampling the clitoral area of all mares. *Taylorella equigenitalis* can cause abortion in the mare but this is a rare occurrence.

### B. DIAGNOSTIC TECHNIQUES

Prior infection and vaccination are not fully protective (15), and failure of antibody to persist has meant that control of infection has relied entirely on prevention of transmission through the detection of *T. equigenitalis* on swabs of urogenital membranes. In spite of difficulties in culturing *T. equigenitalis*, screening mares and stallions prior to and while on the stud farm has successfully eliminated the disease from thoroughbred horses in countries using a voluntary code of practice. These have been based on the widely adopted UK’s Horserace Betting Levy Board’s Code of Practice (16), which is reviewed annually and updated as necessary; the key recommendations of the Code are summarised below.

At the start of the breeding season, swabs are taken from all stallions, including those in their first breeding season, from the urethra, urethral fossa and sinuses, pre-sheath and pre-ejaculatory fluid, on two occasions no fewer than 7 days apart. Mares are classified according to the degree of risk that they represent, and the frequency of sampling is adjusted accordingly. High-risk mares are defined as: (a) those from which *T. equigenitalis* has been isolated (the high risk status will remain until three sets of negative swabs have been taken at three different oestrous periods in each of 2 years); (b) mares that have visited any premises on which *T. equigenitalis* has been isolated within the previous 12 months; (c) mares arriving from Canada, France, Germany, Ireland, Italy, the UK and the USA that have been mated during the last breeding season with stallions resident outside these countries; d) all mares that have been in countries other than Canada, France, Germany,
Ireland, Italy, the UK and the USA within the last 12 months. ‘Low risk’ mares are any mares not defined as ‘high risk’.

High risk stallions are defined as: (a) stallions that have not previously been used for breeding purposes; (b) stallions from which *T. equigenitalis* has been isolated (the ‘high risk’ status will remain until treatment has been undertaken and required swab results are negative); (c) stallions that have been, within the last 12 months, on any premises on which *T. equigenitalis* has been isolated; (d) stallions that have mated a mare which has not been swabbed negative in accordance with the Code of Practice. ‘Low risk’ stallions are any stallions not defined as ‘high risk’. There is a potentially serious problem with contagious equine metritis in this group of horses, especially those belonging to non-thoroughbred breeds.

Results of laboratory tests for *T. equigenitalis* should be entered on an officially approved certificate, which is sent to the veterinarians and stallion stud farm managers who supervise the breeding. The certificate should record the animal’s name, the sites and date of swabbing, the name of the veterinarian taking the swabs, identity of the testing laboratory, the date the swabs were received and cultured by the laboratory, and whether the swabs were negative or positive, or whether the culture was overgrown by other bacteria to an extent that the laboratory could not be confident that small numbers of *T. equigenitalis* would be detected and, therefore, requiring another set of swabs to be collected.

Difficulties with the culture of *T. equigenitalis* caused by its fastidious nature necessitate the use of a quality control system that should be approved before a laboratory is permitted to undertake official testing for contagious equine metritis and to issue certificates of the test results. The task of quality control should be undertaken by an experienced, reliable, and impartial microbiology laboratory, authorised for the purpose, which is not involved in routine testing of contagious equine metritis swabs. At 6-month intervals, swabs inoculated into mixed cultures that are designed to test a laboratory’s ability to recover and identify *T. equigenitalis* in the face of contaminants, as well as procedures for reporting results, should be sent to laboratories wishing to be approved for testing for this bacterium. A list of those laboratories satisfactorily passing the quality control should be published in a veterinary journal that is widely read by the national veterinarians. The veterinarians and stallion stud farm managers who supervise the breeding mares and stallions should accept only certificates provided by laboratories currently approved for that purpose.

Any mares with abnormal vaginal exudate, or returning to oestrus prematurely, should be investigated and managed as though infected with *T. equigenitalis* until results of laboratory testing prove otherwise. Other causes of outbreaks of endometritis include *Pseudomonas aeruginosa*, *Streptococcus zooepidemicus* and certain capsule types of *Klebsiella pneumoniae*. Swabs should be examined for these bacteria, and an attempt should be made to culture and identify *K. pneumoniae* and *P. aeruginosa* so as to establish a differential diagnosis.

If carriers of *T. equigenitalis* are detected, the organism can be eliminated by treatment with systemic antibiotics combined with disinfectant washing of exposed genital membranes (1). Particular attention should be paid to cleansing of the recesses of the clitoral fossa and sinuses of mares, where colonisation by *T. equigenitalis* is frequently found in carrier animals. A course of treatment may take several weeks and may need to be repeated before intensive swabbing consistently fails to recover *T. equigenitalis* in stallions and mares (13). A significant number of carrier mares can be refractive to several courses of treatment. These may require surgery and ablation of the clitoral sinuses for permanent elimination of the carrier state in such animals.

Control measures for countries regarded as free from *T. equigenitalis* infection should be based on the screening of animals prior to importation and/or during a post-importation quarantine period using swabbing and testing regimes broadly based on those described above for breeding populations.

1. Identification of the agent (the prescribed test for international trade)

Various bacteria may be present on the urogenital membranes of horses as harmless commensals and may interfere with the culture of *T. equigenitalis*. Some may initially be present in small numbers, but multiply on the swab before it is cultured. Overgrowth of these organisms on the culture plates may obscure the presence of *T. equigenitalis*. Swabs must be placed in a transport medium with activated charcoal, such as Amies medium, to absorb inhibitory by-products of bacterial metabolism (28). Over time, numbers of *T. equigenitalis* decline on swabs with time, and this effect is more pronounced at higher temperatures (24). Swabs must be kept cool during transportation and should arrive at the laboratory no later than 24–48 hours after they were taken. Negative culture results from swabs plated out more than 48 hours after they were taken are unreliable. Antibiotic treatment for whatever cause should cease at least 7 days before swabbing. The presence of antibiotics may sublethally damage *T. equigenitalis*, which nonetheless persists on the urogenital membranes but cannot be grown on laboratory media.

Each swab must be inoculated on to 5% (v/v) heated blood, (‘chocolate’), agar plates, produced by heating the liquid medium, containing blood, at 70–80°C for 12 minutes. When cooled to 45–50°C, trimethoprim (1 µg/ml), clindamycin (5 µg/ml), and amphotericin B (5 µg/ml), is added to the medium, Timoney *et al.* (31). Thymidine,
which will inactivate trimethoprim, is present in bacteriological media containing peptone, so it is important to add 5% lysed horse blood at this stage. Lysed horse blood contains thymidine phosphorylase, which will inactivate thymidine, thus allowing the trimethoprim to exert its selective effect. This is the preferred medium for isolating *T. equigenitalis*; this medium has been used successfully to isolate equally well both biotypes of this pathogen and to eliminate the growth of many commensal bacteria. As inhibitors may prevent the isolation of some strains of *T. equigenitalis*, swabs should also be inoculated on to plates of 5% ‘chocolate’ blood agar with a rich peptone agar base containing additional cysteine (0.83 mM), sodium sulphite (1.59 mM) and a fungicide (5 µg/ml amphotericin B). *Taylorella equigenitalis* will grow on blood agar, but it can tolerate less than ideal conditions better when grown on ‘chocolate’ blood agar as described above. Some manufacturers’ produce a peptone agar base that is quality controlled for its ability to support the growth of *T. equigenitalis*. The quality of the commercial agar should be confirmed by the testing laboratory. An important feature of all good *T. equigenitalis* media is the absence of fermentable carbohydrates. These do not enhance the growth of *T. equigenitalis*, but their fermentation by other bacteria inhibits *T. equigenitalis* growth (4, 15). A third medium containing streptomycin sulphate (200 µg/ml) is sometimes used as some isolates of *T. equigenitalis* are resistant to this concentration of antibiotic, which serves to reduce the extent of growth of other bacteria that might otherwise obscure the presence of small numbers of *T. equigenitalis* (28). However, a streptomycin-sensitive biotype is now the most common strain isolated and will not be detected on this medium; consequently, it should only be used in conjunction with medium without streptomycin. Growth by other bacteria, for example *Proteus mirabilis*, however, may be so extensive that the laboratory should record that they cannot issue a negative result. In this event, further swabs should be requested in the hope that the problem will not recur.

All culture media prepared should be subjected to quality control and must support growth of the suspect organism from a small inoculum, before their use on suspect samples. The reference strain of *T. equigenitalis* must also be cultured in parallel with the test samples to ensure that the culture conditions are optimal for isolation of this organism.

The fastidious nature of *T. equigenitalis* makes it difficult to isolate. Test breeding of stallions has been used to increase the sensitivity of detection of the carrier state and it serves as a valuable adjunct to cultural examination. The numbers of *Taylorella* mechanically carried by stallions can be very low and may be missed by culturing swabs, but can be detected after multiplication in the mare that has been test bred. The use of test breeding as a diagnostic tool can be especially important in countries that are considered free from contagious equine metritis. Occasionally, the urogenital membranes of stallions or mares will be persistently colonised by another bacterium that interferes with diagnosis, and it will prove necessary to attempt to eliminate this by washing and antibiotic treatment. Swabbing for *T. equigenitalis* shall not recommence until at least 7 days after treatment has stopped. The use of Timoney’s medium (31), described above, should overcome this difficulty in most cases. It is important to emphasise that alongside each day’s tests, additional plates should be inoculated with a culture of *T. equigenitalis* to check that each batch of medium will support growth.

Plates must be incubated at 35–37°C in 5–10% (v/v) CO₂ in air or by use of a candle jar. At least 72 hours is normally required before colonies of *T. equigenitalis* become visible, after which time daily inspection is needed. Visual detection of colonies may take up to 14 days (35). A standard incubation time of at least 7 days is advisable before certifying cultures negative for *T. equigenitalis*. Plates should be examined for contaminants after the first 24 hours’ incubation. Colonies of *T. equigenitalis* may be up to 2–3 mm in diameter, smooth with an entire edge, glossy and yellowish grey. Laboratories should be aware that certain countries require the prolonged incubation period as a standard procedure and should therefore ascertain the particular import requirements and/or indicate the incubation period on which their cultural findings are based.

*Taylorella equigenitalis* is a Gram-negative, nonmotile, bacillus or cocco-bacillus that is often pleomorphic (up to 6 µm long) and may exhibit bipolar staining. It is catalase positive, phosphatase positive, and strongly oxidase positive (see ref. 5 for methods for examining catalase, phosphatase and oxidase activities). It is otherwise inert in tests for biochemical activity. If a slow-growing organism is isolated that fits the description for cellular morphology and that is strongly oxidase positive, it should be tested for reactivity with *T.-equigenitalis*-specific antiserum. A variety of serotyping tests have been developed ranging in complexity from slide agglutination to direct or indirect immunofluorescence. Each method has its advantages and disadvantages. The disadvantage of the slide agglutination test is that occasionally autoagglutination of isolates occurs: culturing in bottled CO₂ in air, as opposed to in a candle jar, may reduce autoagglutination (29). It has been suggested that immunofluorescence can be used to identify autoagglutinating isolates, some workers have reported cross-reaction with *Mannheimia haemolytica* but this is very rare. If a cross-reaction is suspected, it may be necessary to repeat the test using

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1 For example, Mast Diagnostics, Mast House, Derby Road, Bootle, Merseyside L20 1EA, United Kingdom (UK), and Lab M, Tomley House, Wash Lane, Bury BL9 6AU, UK.
adsorbed antisera (29). The immunofluorescence test can be improved by the use of monoclonal antibodies, which are now available².

Antiserum is produced by vaccinating rabbits with killed *T. equigenitalis*. A number of different immunisation regimes can be employed, ranging from those used for producing *Escherichia-coli*-typing antisera (26), to immunisation together with an adjuvant, such as Freund’s incomplete. Monoclonal antibodies are available commercially that provide a highly specific means of identifying *T. equigenitalis*. A standard strain, such as NCTC 11184³, should be used for immunisation. However, the most important consideration is the specificity of the antiserum produced. It should agglutinate *T. equigenitalis*, but fail to agglutinate other bacteria that might be cultured from horse urogenital membranes, even if rarely. In particular, it should not agglutinate any oxidase-positive and Gram-negative rods, such as *Mannheimia haemolytica*, *Actinobacillus equuli*, *Bordetella bronchiseptica* (to which *T. equigenitalis* is closely related, see ref. 9), and *Pseudomonas aeruginosa*. Recently another species of *Taylorella*, *T. asinigenitalis*, has been isolated from male donkeys in the United States of America (17) and from a horse stallion in Europe (6). This newly described bacterium, which has not been associated with naturally occurring disease, resides in the genital tract of male donkeys and can be passed to donkeys and horses during mating. Moreover it has similar, though not identical, colonial appearance and cultural characteristics and gives identical biochemical test results to those used to confirm the identity of *T. equigenitalis*. There is even serological cross-reactivity between the two organisms. At the National Veterinary Services Laboratories (NVSL) in Ames, Iowa, USA⁴, and at the Veterinary Laboratories Agency (VLA), Bury St Edmunds, United Kingdom⁴, differentiation of *T. asinigenitalis* from *T. equigenitalis* is possible using the polymerase chain reaction (PCR).

A latex agglutination kit is available commercially for the antigenic identification of *T. equigenitalis*. It is based on polyclonal antibodies produced using methods similar to those described above. This is widely used by routine testing laboratories for the confirmation of the identity of colonies growing on selective medium that give a biochemical reaction consistent with *T. equigenitalis*. As *T. equigenitalis* is antigenically relatively distinct, and small amounts of cross-reactive antibody are easily absorbed during production of the reagent, the test has proved to be highly specific and sensitive. It should be emphasised that it will not necessarily distinguish strains of *T. equigenitalis* from *T. asinigenitalis*.

A PCR method has been used for detecting *T. equigenitalis* and was compared with culture methods in the Netherlands and Japan (2, 7, 10). In these studies, a much higher rate of detection by PCR was found than by culture, even among horses imported from a source without previous evidence of *T. equigenitalis* infection or clinical disease. The authors proposed that carriage is more widespread than previously believed, and that recently discovered genetic variation among strains (8, 18) may relate to differences in pathogenicity. The PCR has also been used in the UK (11). It was highly specific and was able to detect very small numbers of *T. equigenitalis* in the presence of very large numbers of bacteria comprising the background flora harvested from culture plates inoculated with samples of the equine urogenital tract. Recently in Japan, the field application of the PCR in the eradication of contagious equine metritis was evaluated. It was demonstrated that the PCR was more sensitive than culture for the detection of *T. equigenitalis* from genital swabs of horses in the field (2, 3, 19, 20). In 2004–2005, a real-time PCR was developed in the UK for use directly on genital swabs and compared with culture (34). There was no significant difference in the performance of the direct PCR and culture, but the PCR had the added advantage of speed of result and also differentiated *T. equigenitalis* from *T. asinigenitalis*. This promising technique needs to be more fully and widely evaluated, especially for detection of the carrier state in the stallion.

2. Serological tests

No serological test described to date will, by itself, reliably detect infection for diagnosis and control. However, the complement fixation test has been used successfully as an adjunct to culture for *T. equigenitalis* in screening mares between 21 and 45 days after being bred to suspect a carrier stallion.

### C. REQUIREMENTS FOR VACCINES AND DIAGNOSTIC BIOLOGICALS

Effective vaccines that protect against contagious equine metritis or prevent colonisation by *T. equigenitalis* are not yet available.

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² Institut Pourquier, 326 rue de la Galera, Parc Euromedicine, 34090 Montpellier, France. E-mail: info@institut-pourquier.fr

³ Obtainable from the National Collection of Type Cultures, Colindale, London, UK.

⁴ For further information contact the NVSL at NVSL, P.O. Box 844, Ames, Iowa 50010, USA. E-mail: NVSL_Concerns@aphis.usda.gov or the VLA at VLA, Rougham Hill, Bury St Edmunds, IP33 2RX, UK. E-mail: bury-st-edmunds@vla.defra.gsi.gov.uk
REFERENCES


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**NB:** There are OIE Reference Laboratories for Contagious equine metritis (see Table in Part 3 of this *Terrestrial Manual* or consult the OIE Web site for the most up-to-date list: www.oie.int).
CHAPTER 2.5.3.

DOURINE

SUMMARY

Dourine is a chronic or acute contagious disease of breeding solipeds that is transmitted directly from animal to animal during coitus. The causal organism is Trypanosoma equiperdum (Doflein, 1901).

Dourine is the only trypanosomosis that is not transmitted by an invertebrate vector. Trypanosoma equiperdum differs from other trypanosomes in that it is primarily a tissue parasite that rarely invades the blood. There is no known natural reservoir of the parasite other than infected equids. It is present in the genital secretions of both infected males and females. The incubation period, severity, and duration of the disease vary considerably; it is often fatal, but spontaneous recoveries do occur but latent carriers do exist. Subclinical infections occur, and donkeys and mules are more resistant than horses and may remain inapparent carriers. Infection is not always transmitted by an infected animal at every copulation. Rats can be infected experimentally, and can be used to maintain strains of the parasite indefinitely. Trypanosoma equiperdum strains are best stored in liquid nitrogen.

The clinical signs are marked by periodic exacerbation and relapse, ending in death, sometimes after paraplegia or, possibly, recovery. Fever, local oedema of the genitalia and mammary glands, cutaneous eruptions, incoordination, facial paralysis, ocular lesions, anaemia, and emaciation may all be observed. Oedematous cutaneous plaques, 5–8 cm in diameter and 1 cm thick, are pathognomonic.

Identification of the agent: Definitive diagnosis depends on the recognition of clinical signs and identification of the parasite. As this is rarely possible, diagnosis is usually based on clinical signs, together with serological evidence from complement fixation (CF) tests.

Serological tests: Humoral antibodies are present in infected animals whether or not they display clinical signs. The CF test is used to confirm infection in clinical cases or in latent carriers. Noninfected animals, especially donkeys, often yield unclear results. The indirect fluorescent antibody test can be used to confirm infection or resolve inconclusive CF test results. Enzyme-linked immunosorbent assays are also used.

Requirements for vaccines and diagnostic biologicals: There are no vaccines available. The only effective control is through the slaughter of infected animals. Good hygiene is essential during assisted matings because infection may be transmitted through contaminated fomites.

A. INTRODUCTION

Dourine is a chronic or acute contagious disease of breeding solipeds that is transmitted directly from animal to animal during coitus. The causal organism is Trypanosoma equiperdum (Doflein, 1901). Dourine is also known under other names: mal de coït, el dourin, morbo coitale maligno, Beschäleuchte, slapsiekte, sluchnaya bolyezn, and covering disease (1, 8).

Although the disease has been known since ancient times, its nature was established only in 1896 when Rouget discovered trypanosomes in infected Algerian horses. Dourine is the only trypanosomosis that is not transmitted by an invertebrate vector. Trypanosoma equiperdum differs from other trypanosomes in that it is primarily a tissue parasite that is rarely detected in the blood. There is no known natural reservoir of the parasite other than infected equids.
Infection is transmitted during copulation, more commonly from stallion to mare, but also from mare to stallion, due to the presence of the parasite in the seminal fluid and mucous exudate of the penis and sheath of the infected male, and in the vaginal mucus of the infected female. Initially, parasites are found free on the surface of the mucosa or between the epithelial cells of a newly infected animal. Invasion of the tissues takes place, and oedematous patches appear in the genital tract. Parasites then may pass into the blood, where they are carried to other parts of the body. In typical cases, this metastatic invasion gives rise to characteristic cutaneous plaques.

The incubation period, severity and duration of the disease vary considerably. In South Africa, the disease is typically chronic, usually mild, and may persist for several years (6). In other areas, such as northern Africa and South America, the disease tends to be more acute, often lasting only 1–2 months or, exceptionally, 1 week. Although dourine is a fatal disease with an average mortality of 50% (especially in stallions), spontaneous recovery can occur. Subclinical infections are recognised. Donkeys and mules are more resistant than horses.

As trypanosomes are not continually present in the genital tract throughout the course of the disease, transmission of the infection does not necessarily take place at every copulation involving an infected animal. Transmission of infection from mare to foal can occur via the mucosa, such as the conjunctiva. Mares' milk has been shown to be infectious. Animals other than equids can be infected experimentally. Rat-adapted strains can be maintained indefinitely; infected rat blood can be satisfactorily cryopreserved. Antigens for serological tests are commonly produced from infected laboratory rats.

The disease is marked by stages of exacerbation, tolerance or relapse, which vary in duration and which may occur once or several times before death or recovery. The signs most frequently noted are: pyrexia, tumefaction and local oedema of the genitalia and mammary glands, oedematous cutaneous eruptions, knuckling of the joints, incoordination, facial paralysis, ocular lesions, anaemia, and emaciation. A pathognomonic sign is the oedematous plaque consisting of an elevated lesion in the skin, up to 5–8 cm in diameter and 1 cm thick. The plaques usually appear over the ribs, although they may occur anywhere on the body, and usually persist for between 3 and 7 days. They are not a constant feature.

Generally, the oedema disappears and returns at irregular intervals. During each recess, an increasing extent of permanently thickened and indurated tissue can be seen. The vaginal mucosa may show raised and thickened semitransparent patches. Folds of swollen membrane may protrude through the vulva. It is not uncommon to find oedema of the mammary glands and adjacent tissues. Depigmentation of the genital area, perineum, and udder may occur. In the stallion, the first clinical sign is a variable swelling involving the glans penis and prepuce. The oedema extends posteriorly to the scrotum, inguinal lymph nodes, and perineum, with an anterior extension along the inferior abdomen. In stallions of heavy breeds, the oedema may extend over the whole floor of the abdomen.

Pyrexia is intermittent; nervous signs include incoordination, mainly of the hind limbs, lips, nostrils, ears, and throat. Facial paralysis is usually unilateral. In fatal cases, the disease is usually slow and progressive, with increasing anaemia and emaciation, although the appetite remains good almost throughout.

At post-mortem examination, gelatinous exudates are present under the skin. In the stallion, the scrotum, sheath, and testicular tunica are thickened and infiltrated. In some cases the testes are embedded in a tough mass of sclerotic tissue and may be unrecognisable. In the mare, the vulva, vaginal mucosa, uterus, bladder, and mammary glands may be thickened with gelatinous infiltration. The lymph nodes, particularly in the abdominal cavity, are hypertrophied, softened and, in some cases, haemorrhagic. The spinal cord of animals with paraplegia is often soft, pulpy and discoloured, particularly in the lumbar and sacral regions.

B. DIAGNOSTIC TECHNIQUES

1. Identification of the agent

A definitive diagnosis depends on the recognition of the clinical signs and the demonstration of the parasite. This is rarely possible because: (a) although the clinical signs and gross lesions in the developed disease may be pathognomonic, they cannot always be identified with certainty, especially in the early stages or in latent cases; they can be confused with other conditions, such as coital exanthema (moreover, in some countries [e.g. in South America], T. evansi infections give rise to similar clinical signs); (b) the trypanosomes are only sparsely present and are extremely difficult to find, even in oedematous areas; and (c) the trypanosomes are only fleetingly present in the blood, and in small numbers that defy detection. For unknown reasons, no parasite strain of T. equiperdum has been isolated in any country of the world since 1982 and most of the strains currently available in national veterinary diagnostic laboratories are related to T. evansi (3).
In practice, diagnosis is based on clinical evidence supported by serology. Recently, other approaches have been studied and reported on (3).

In infected animals, trypanosomes are present, in low numbers only, in lymph and oedematous fluids of the external genitalia, in the vaginal mucus, and fluid contents of plaques. They are usually undetectable in the blood, but may be found in the urethral or vaginal mucus collected from preputial or vaginal washings or scrapings 4–5 days after infection. Later, parasites may be found in the fluid contents of oedemas and plaques, especially shortly after their eruption. The skin of the area over the plaque should be washed, shaved and dried, and the fluid contents aspirated by syringe. Blood vessels should be avoided. The fresh aspirate is examined microscopically for motile trypanosomes. These are present for a few days only, so that lesions should be examined at intervals. The parasite is rarely found in thick blood films, but is sometimes detectable after centrifuging blood and examining the recentrifuged plasma.

As dourine is the only trypanosome to affect horses in temperate climates, the observation of trypanosomes in thick blood films is sufficient for a positive diagnosis. In countries where nagana or surra occur, it is difficult to distinguish *T. equiperdum* microscopically (morphology, motility) from other members of the subgenus Trypanozoon (*T. evansi*, *T. brucei*). In particular, *T. equiperdum* and *T. evansi* cannot be differentiated on the basis of morphological criteria. Both are monomorphic, slender trypomastigotes with a free flagellum, although pleomorphic, stumpy, proteonuclear forms are recognised. However, in kinetoplastic strains, the presence of maxi-circles in *T. equiperdum* and the absence in *T. evansi* provides a possible differentiation (14).

Typical strains of the parasite range in length from 15.6 to 31.3 µm.

2. **Serological tests**

Humoral antibodies are present in infected animals, whether they display clinical signs or not. The complement fixation (CF) test (12) is used to confirm clinical evidence and to detect latent infections. Uninfected equids, particularly donkeys and mules, often give inconsistent or nonspecific reactions because of the anticomplementary effects of their sera. In the case of anticomplementary sera, the indirect fluorescent antibody (IFA) test is of advantage. There is no internationally adopted protocol. Cross-reactions are possible due to the presence in some countries of other trypanosomes, for example, *T. cruzi* and *T. evansi*. Enzyme-linked immunosorbent assays (ELISAs) are also used. *Trypanosoma equiperdum* is closely related to other Old World trypanosomes, including *T. brucei* and *T. evansi*. Members of this genus all share conserved cytoskeletal elements that provoke a strong and cross-reactive serological response. All diagnostic antigens and antisera (monoclonal and polyclonal) currently available for use in serodiagnostic testing contain these conserved elements or antibodies to them, and therefore none of the serological procedures described below is specific for dourine. The diagnosis of dourine must include history, clinical, and pathological findings as well as serology. Significant improvements in dourine serodiagnosis will require development of more trypanosome-specific subunit antigens and antibodies to them.

a) **Complement fixation test (the prescribed test for international trade)**

Standard or microplate techniques may be used (7). Guinea-pig serum (available commercially) is used as a source of complement. Other reagents are sheep red blood cells (RBCs) washed in veronal buffer, and rabbit haemolytic serum (i.e. rabbit anti-sheep RBC) (commercial) as well as known negative and positive control sera.

- **Antigen production**
  i) A rat is inoculated with *T. equiperdum* cryopreserved stock. The rat must be free from *T. lewisi*, which could be achieved by injection with neoarsphenamine, but is better accomplished by using specific-pathogen free rats. Adult rats receive 0.5–1.0 ml of rapidly thawed frozen stablate, intramuscularly or intraperitoneally. At maximum parasitaemia, blood is collected into an anticoagulant, such as heparin, which will serve as a stock culture for the inoculation of additional rats.
  ii) Twenty large rats are inoculated intramuscularly or intraperitoneally with 0.5–1.0 ml of this stock culture. All rats are to have a heavy infection concurrently. If necessary, the dose is adjusted and additional rats are inoculated to reach maximum parasitaemia at the desired time of 72–96 hours. Rats usually die within 3–5 days; prior to this, blood is taken from the tail for thick smears and examined microscopically. When parasitaemia is maximal, the rat is killed and blood is collected in Alsevers or acid–citrate–dextrose (ACD) saline solution. If parasitaemia is not synchronous, blood can be collected and held in Alsevers or ACD saline at 4°C until blood has been collected from all the rats.
  iii) The blood is filtered through muslin gauze and centrifuged at 800 g for 4 minutes. The RBCs are mostly deposited while the trypanosomes remain in suspension.
iv) The supernatant fluid is transferred to a fresh tube; the upper layer of RBCs is mixed with trypanosomes to a second tube, and the next layer to a third. Alsevers or ACD saline is added to tubes 2 and 3 to prevent clotting of cells. All tubes are mixed and centrifuged at 1500 g for 5 minutes.

v) The supernatant fluid is discarded and the upper white layer of trypanosomes is transferred from all tubes into a clean tube. The next pink layer is transferred into a second tube, and the lower layer to a third tube.

vi) Physiological saline is added and mixed and the tubes are centrifuged again at 1500 g for 5 minutes to separate the trypanosomes. The washing step is repeated until all the trypanosomes are collected as a pure white mass. Ten rats should produce 3–5 g of antigen. This purification procedure can also be carried out using a column of DEAE (diethylaminoethyl) cellulose in a solution of phosphate buffered saline (PBS) containing glucose, pH 8.0 (11).

vii) The concentrated trypanosomes are diluted with two volumes of veronal buffer and 5% polyvinylpyrrolidone as a cryopreservative. Before use in CF tests, the antigen must be dispensed to a fine suspension with a hand-held or motorised ground glass homogeniser chilled in ice (15). This antigen may be divided into aliquots, frozen and lyophilised.

The antigen is standardised by titration against a 1/5 dilution of a standard low-titre antiserum.

Sera: Positive and negative sera should be inactivated at 58°C for 30 minutes before being used in the tests. Mule and donkey sera are normally inactivated at 62°C for 30 minutes. The USDA complement fixation protocol calls for inactivation of sera for 35 minutes (13). Dilutions of sera that are positive in the screening test are titrated against two units of antigen. Test sera are screened at a dilution of 1/5. Sera showing more than 50% complement fixation at this dilution are usually deemed to be positive.

Anticomplementary sera: If the anticomplementary control shows only a trace, this may be ignored. For all other anticomplementary sera, the activity must be titrated. A duplicate series of dilutions is made and the sample is restested using *T. equiperdum* antigen in the first row and veronal buffer only in the second. The second row gives the titre of the anticomplementary reaction. Provided the first row shows an end-point that is at least three dilutions greater than the second, the anticomplementary effect may be ignored and the sample rated as positive. If the results are any closer, a fresh sample of serum must be requested. Dilution of the serum 1/2 and heat inactivation at 60–63°C for 30 minutes may result in reduction or removal of the anticomplementary effect.

Buffers and reagents: 0.15 M veronal buffered saline, pH 7.4, is used for diluting reagents and for washing sheep RBCs. Antigen is pretested by checkerboard titration, and two units are used in the test. Guinea-pig complement (C) is tested for its haemolytic activity, and diluted to provide two units for the test. Sheep RBCs in Alsever’s or ACD saline solution are washed three times. A 3% solution is used for the haemolytic system. The USDA protocol calls for 2% solution in the microtitration procedure with confirmation in a tube test with 3% RBC (13). Titrated rabbit-anti-sheep RBCs – the rabbit haemolytic serum – is taken at double the concentration of its haemolytic titre (two units). All test sera, including positive and negative control sera, are inactivated at a 1/5 dilution before testing.

- **Primary dilutions**
  
i) 100 µl of test serum is diluted with 400 µl of veronal buffer (1/5).
  
ii) 100 µl of both positive and negative control sera is diluted with 400 µl of veronal buffer (1/5).
  
iii) The solutions are incubated in a water bath at 58°C for 30 minutes to inactivate complement and destroy anticomplementary factors.

- **Screening test procedure**
  
i) 25 µl of inactivated test serum is placed in each of three wells.
  
ii) 25 µl of inactivated control serum is placed in each of three wells.
  
iii) 25 µl of *T. equiperdum* antigen diluted to contain two units is placed in the first well only for each serum.
  
iv) 25 µl of complement diluted to contain two units is added to the first two wells only for each serum.
  
v) 25 µl veronal buffer, pH 7.4, is added to the second well for each serum (anticomplementary well).
  
vi) 50 µl veronal buffer, pH 7.4, is added to the third well for each serum (lysis activity well).
  
vii) The complement control is prepared.
  
viii) The plate is shaken on a microshaker sufficiently to mix the reagents.
  
ix) The plate is incubated for 1 hour in a water bath, incubator or in a humid chamber at 37°C.
x) The haemolytic system is prepared. After the first 50 minutes of incubation, the sheep RBCs are sensitised by mixing equal volumes of rabbit haemolytic serum, diluted to contain two units per 50 µl, and a 3% suspension of washed RBCs; the solution is mixed well and incubated for 10 minutes at 37°C.

xi) After incubation, 50 µl of haemolytic system is added to each well.

xii) The plate is shaken on a microshaker sufficiently to mix the reagents.

xiii) The plate is incubated for 30 minutes at 37°C. To aid in reading the results, the plates can be centrifuged after incubation.

xiv) **Reading the results:** the plate is viewed from above with a light source beneath it. The fixation in every well is assessed by estimating the proportion of cells not lysed. The degree of fixation is expressed as 0, 1+, 2+, 3+, 4+ (0%, 25%, 50%, 75% or 100% cells not lysed). Reactions are interpreted as follows: 4+, 3+, 2+ = positive, 1+ = suspicious, trace = negative, complete haemolysis = negative.

xv) **End-point titration:** All sera with positive reactions at 1/5 are serially double diluted and tested according to the above procedure for end-point titration.

**b) Indirect fluorescent antibody test**

An IFA test for dourine can also be used (12) as a confirmatory test or to resolve inconclusive results obtained by the CF test. The test is performed as follows:

**Antigen:** (For method, see preparation of CF test antigen in Section B.2.a) Blood is collected into heparinised vacutainers or into a solution of acid–citrate–dextrose from an animal in which the number of trypanosomes is still increasing (about ten parasites per 10× microscope field should be present).

i) The blood is centrifuged for 10 minutes at 800 g.

ii) One to two volumes of PBS is added to the packed RBCs, the mixture is agitated, and smears are made that evenly cover the whole slide.

iii) The smears are air-dried and then wrapped in bundles of four, with paper separating each slide. The bundles of slides are wrapped in aluminium foil, sealed in an airtight container over silica gel, and stored at –20°C or –76°C.

iv) Slides stored at –20°C should retain their activity for about 1 year, at –76°C they should remain usable for longer.

**Acid–citrate–dextrose solution:** Use 15 ml per 100 ml of blood.

**Conjugate:** Fluorescein-labelled sheep anti-horse immunoglobulins.

• **Test procedure**

i) The antigen slides are allowed to reach room temperature in a desiccator. An alternative method is to remove slides directly from the freezer and fix them in acetone for 15 minutes.

ii) The slides are marked out.

iii) Separate spots of test sera diluted in PBS are applied, and the slides are incubated in a humid chamber in a water bath at 37°C for 30 minutes.

iv) The slides are washed in PBS, pH 7.2, three times for 5 minutes each, and air-dried.

v) Fluorescein-labelled conjugate is added at the correct dilution. Individual batches of antigen and conjugate should be titrated against each other using control sera to optimise the conjugate dilution. The slides are incubated in a humid chamber in a water bath at 37°C for 30 minutes.

vi) The slides are washed in PBS, three times for 5 minutes each, and air-dried. An alternative method, to reduce background fluorescence, is to counter-stain, using Evans Blue (0.01% in distilled water) for 1 minute, rinse in PBS and then air-dry

vii) The slides are mounted in glycerol/PBS (50/50) or immersion oil (commercially available, non-fluorescing grade).

viii) The slides are then examined under UV illumination. Incident light illumination is used with barrier filter K 530 and exciter filter BG 12. Slides may be stored at 4°C for 4–5 days. Sera diluted at 1/80 and above showing strong fluorescence of the parasites are usually considered to be positive. Estimating the intensity of fluorescence demands experience on the part of the observer.

Standard positive and negative control sera should be included in each batch of tests, and due consideration should be given to the pattern of fluorescence in these controls when assessing the results of test sera.
c) **Enzyme-linked immunosorbent assay**

The ELISA has been developed and compared with other serological tests for dourine (14, 16).

**Carbonate buffer, pH 9.6, for antigen coating on to microtitre plates:** Na$_2$CO$_3$ (1.59 g); NaHCO$_3$ (2.93 g); and distilled water (1 litre).

**Blocking buffer:** Carbonate buffer + 3% fetal calf serum (FCS).

**PBS, pH 7.4, with Tween 20 (PBST) for washing:** KH$_2$PO$_4$ (0.2 g); Na$_2$HPO$_4$ \( \times \) 12 H$_2$O (2.94 g); NaCl (8.0 g); KCl (0.2 g in 1 litre distilled water), and Tween 20 (0.5 ml).

**Sample and conjugate buffer:** PBST + 6% FCS.

**Citric phosphate buffer:** Citric acid monohydrate (4.2 g in 200 ml distilled water); Na$_2$HPO$_4$ \( \times \) 12 H$_2$O (in 200 ml distilled water). Both components are mixed at equal volumes.

**Substrate indicator system:** ABTS (2,2'-Azino-bis-[3-ethylbenzothiazoline-6-sulphonic acid]) (40 mg) is dissolved in citric phosphate buffer (100 ml), and stored at 4°C in the dark. Just before use, 100 µl of 1/40 H$_2$O$_2$ is added to 10 ml of ABTS.

**Conjugate:** Rabbit anti-horse IgG (H+L) PO or IgY anti-horse Ig-PO.

**Antigen:** Lyophilised *T. equiperdum* antigen (0.5 ml) is reconstituted with coating buffer (5 ml), sonicated twice for 10 seconds each at 12 µm peak to peak, and centrifuged at 10,000 g for 4 minutes. The supernatant is further diluted to a pretested working dilution (e.g. 1/500).

**Test procedure**

i) Wells in columns 2, 4, 6, etc., are charged with 50 µl of antigen, columns 1, 3, 5, etc., are charged with the same amount of carbonate buffer. The plate is incubated for 40 minutes at 37°C in a humid chamber, washed in tap water, and 50 µl of blocking buffer is added to each well. The plate is incubated for 20 minutes, washed in tap water followed by three wash cycles with PBST, with soaking times of 3 minutes/cycle.

ii) 50 µl of test samples and equine control sera prediluted 1/100 in sample/conjugate buffer is added in parallel to wells with and without antigen. The plate is incubated for 30 minutes, washed in tap water, followed by three wash cycles with PBST.

iii) Properly diluted conjugate in sample/conjugate buffer is added in volumes of 50 µl to all wells. The plate is incubated for 30 minutes with subsequent washing as above.

iv) 100 µl of substrate indicator system is added to all wells.

v) The reaction is stopped after 15 minutes at room temperature by the addition of 25 µl of 37 mM NaCN. Alternatively, commercially available detergents can be used after pretesting. The results are read photometrically at a wavelength of 405 nm.

vi) **Calculation of results:** absorbance (with antigen) minus absorbance (without antigen) = net extinction. A reaction exceeding a net extinction of 0.3 is regarded as a positive result.

A competitive ELISA has also been described for detecting antibody against *Trypanosoma equiperdum* (10).

d) **Other serological tests**

Other serological tests have been used, including radioimmunoassay, counter immunoelectrophoresis and agar gel immunodiffusion (AGID) tests (2, 5). The AGID has been used to confirm positive tests and to test anticomplementary sera. A seven-well pattern in 0.8% agarose in Tris buffer is used, with the CF test antigen in the centre well and positive control sera and unknown sera in alternate peripheral wells. A method has been published for diagnosing equine piroplasmosis, glanders and dourine at the same time, using immunoblotting (9). A card agglutination test has been developed that compares favourably with the CF test (4).

C. **REQUIREMENTS FOR VACCINES AND DIAGNOSTIC BIOLOGICALS**

There are no biological products available. Control of the disease depends on compulsory notification and slaughter of infected animals. Good hygiene at assisted matings is also essential.
REFERENCES


16. **WORKING PROTOCOLS, BGVV: ELISA ON DOURINE** (1995). Bundesinstitut f. gesundheitlichen Verbraucherschutz u. Veterinärmedizin (BgVV). P.O. Box 33 00 13, D-14191 Berlin, Germany.

* * *

**NB:** There is an OIE Reference Laboratory for Dourine (see Table in Part 3 of this *Terrestrial Manual* or consult the OIE Web site for the most up-to-date list: www.oie.int).
CHAPTER 2.5.4.

EPIZOOTIC LYMPHANGITIS

SUMMARY

Epizootic lymphangitis is a contagious, chronic disease of horses and other Equidae characterised clinically by a spreading, suppurative, ulcerating pyogranulomatous dermatitis and lymphangitis. This is seen particularly in the neck, legs and chest. It can also present as an ulcerating conjunctivitis, or rarely a multifocal pneumonia. Transmission is by contact of infected material with traumatised skin, by biting flies, ticks or inhalation. The causative agent, Histoplasma capsulatum var. farciminosum, is a thermally dimorphic, fungal soil saprophyte. Differential diagnoses include glanders (farcy), caused by Burkholderia mallei, ulcerative lymphangitis due to Corynebacterium pseudotuberculosis, sporotrichosis caused by Sporothrix schenckii, and the skin lesions of histoplasmosis caused by H. capsulatum var. capsulatum. Amphotericin B injection with local wound drainage and inorganic iodides are used to treat early cases.

Identification of the agent: Identification of the agent is made by its appearance in smears of the exudate or in histological sections of the lesion material. The yeast form of the organism is present in large numbers in well established lesions, and appears as pleomorphic ovoid to globose structures, approximately 2–5 µm in diameter, located both extracellularly and intracellularly in macrophages and giant cells. Organisms are usually surrounded by a ‘halo’ when stained with Gram stain, haematoxylin and eosin, Periodic acid–Schiff reaction or Gomori methenamine–silver stain. The mycelial form of the organism grows slowly under aerobic conditions at 25–30°C on a variety of media, including Mycobiotic agar, enriched Sabouraud’s dextrose agar, brain–heart infusion agar, and pleuropneumonia-like organism nutrient agar. Conversion to the yeast phase at 37°C must be demonstrated.

Serological and other tests: Antibodies to H. capsulatum var. farciminosum develop at or before the onset of clinical signs. Assays reported for detection of antibody include fluorescent antibody, enzyme-linked immunosorbent assay, and passive haemagglutination tests. In addition, a skin hypersensitivity test has been described.

Requirements for vaccines and diagnostic biologicals: Killed and live vaccines have been used on a limited scale in endemic areas, but they are not readily available.

A. INTRODUCTION

Epizootic lymphangitis is a contagious, chronic disease of horses, mules and donkeys. The disease is characterised clinically by a suppurative, ulcerating, and spreading pyogranulomatous, multifocal dermatitis and lymphangitis. It is seen most commonly in the extremities, chest wall and the neck, but it can also be present as an ulcerating conjunctivitis of the palpebral conjunctiva, or rarely as a multifocal pneumonia. The organism may also invade open lesions including ruptured strangles abscesses and castration wounds. It has also been called pseudofarcy or pseudoglanders. Another synonym is equine histoplasmosis, which may be a more accurate name for the disease, as not all clinical cases present obvious lymphangitis. The form that the disease takes seems to depend primarily on the route of entry (17). The traumatised skin is either infected directly by infected pus, nasal or ocular excretions or indirectly by soil or contaminated harnesses, grooming equipment, feeding and watering utensils, wound dressings or flies. It is also believed that ticks may play a role in the transmission of this agent (4). The conjunctival form of the disease is believed to be spread by flies of the Musca or Stomoxys genera (17). The pulmonary form of the disease is infrequent and is presumed to occur after inhalation of the organism. The incubation period is from about 3 weeks to 2 months (3). In all cases, the lesions are nodular and granulomatous in character, and the organism, once established, spreads locally by invasion and then via the lymphatics. There is often thickening, or ‘cording’, of lymphatics, with the formation of pyogranulomatous nodules. Regional lymph nodes may by enlarged and inflamed. Lesions usually heal spontaneously after 2–
3 months, resulting in stellate scar formation. However, extensive lesions with high mortality rates can occur in areas where there is poor veterinary care and nutrition (3).

The causative agent, *Histoplasma capsulatum* var. *farciminosum*, is a thermally dimorphic fungus. The mycelial form is present in soil; the yeast form is usually found in lesions. *Histoplasma farciminosum* was formerly described as an independent species, but this assessment has been changed and it is now considered to be a variety of *H. capsulatum* due to the close morphological similarities of both the mycelial and yeast forms (19). Antigenically, *H. capsulatum* var. *farciminosum* and *H. capsulatum* var. *capsulatum* are indistinguishable, however the latter is the cause of disseminated histoplasmosis, is endemic in North America and has a wide host range (16). DNA sequences of four protein-coding genes have been analysed to elucidate the evolutionary relationships of *H. capsulatum* varieties. This indicated that *H. capsulatum* var. *farciminosum* is deeply buried in the branch of *Sam Hcc* group A, (H60 to -64, -67, -71, -74 and -76), looking as if it were an isolate of South American *H. capsulatum* var. *capsulatum* (14).

The cutaneous form of the disease may be confused with farcy (the skin form of glanders), which is caused by *Burkholderia mallei*, ulcerative lymphangitis, which is caused by *Corynebacterium pseudotuberculosis*, indolent ulcers acused by *Rhodococcus equi*, sporotrichosis caused by *Sporothrix schenckii*, and histoplasmosis caused by *H. capsulatum* var. *capsulatum*, cryptococcosis, strangles, sarcois and cutaneous lymphosarcomas (13, 15).

The disease is more common in the tropics and subtropics and is endemic in north, east and north-east Africa, and some parts of Asia, including some countries bordering the Mediterranean Sea, India, Pakistan and Japan. The disease is common in Ethiopia, especially in cart horses, affecting an average of 18.8% of horses in warm, humid areas between 1500 and 2300 metres above sea level (3, 4). Reports from other parts of the world are sporadic and all cases must be verified by laboratory testing. The prevalence of the disease increases with assembling of animals; it was much more common, historically, when large numbers of horses were stabled together for cavalry and other transportation needs. Mainly, it is horses, mules, and donkeys that are affected by the disease, although infection may occur in camels, cattle and dogs (19). Experimentally, other animals are refractory to infection subsequent to inoculation, with the exception of certain laboratory animal species such as mice, guinea-pigs and rabbits (12, 17). Infection in humans has also been reported (2, 6, 11).

The disease is eradicated by the humane slaughter of infected horses, disinfection of infected premises and restricting the movement of equids from infected premises. In endemic areas where eradication is not possible, inorganic iodides can be used for therapy in early cases (1). Localised nodules can also be lanced, the pus drained and the nodules packed with a 7% tincture of iodine. If affordable, Amphotericin B can be used.

As the clinical signs of epizootic lymphangitis can be confused with those of other diseases in the field, definitive diagnosis rests on laboratory confirmation.

**B. DIAGNOSTIC TECHNIQUES**

1. **Identification of the agent**

Material should be collected directly from unruptured nodules. For microbiological isolation, the material should be placed in a liquid nutrient medium with antibacterials and kept refrigerated until culturing, which should be attempted as soon as possible. For direct examination, swabs of lesion material can be smeared on glass slides and fixed immediately. For histopathology, sections of lesion material, including both viable and nonviable tissue, should be placed in 10% neutral buffered formalin. Confirmation of the disease is dependent on the demonstration of *H. capsulatum* var. *farciminosum*.

a) **Direct microscopic examination**

- **Gram-stained smears**

Smears can be stained directly with Gram’s stain and examined for the typical yeast form of the organism, which will appear as Gram-positive, pleomorphic, ovoid to globose structures, approximately 2–5 µm in diameter (2). They may occur singly or in groups, and may be found either extracellularly or within macrophages. A halo around the organisms (unstained capsule) is frequently observed.

- **Histopathology**

In haematoxylin and eosin (H&E)-stained histological sections, the appearance of the lesion is quite characteristic and consists of pyogranulomatous inflammation with fibroplasia. Langhans giant cells are common. The presence of numerous organisms, both extracellularly and intracellularly within macrophages or multinucleated giant cells in tissue sections stained with H&E, Periodic acid–Schiff reaction and Gomori methenamine–silver stain are observed (16). There is some indication that the number of organisms increases with chronicity. The organisms are pleomorphic, often described as slightly lemon-shaped.
basophilic masses, varying from 2 to 5 µm in diameter, that are surrounded by a ‘halo’ when stained with H&E or Gram’s stain (1).

- Electron microscopy

Electron microscopy has been applied to skin biopsy samples of 1.5–2.0 mm immediately prefixed in phosphate buffered 2% glutaraldehyde solution at 4°C and post-fixed in 1% osmium tetroxide. Ultra-thin sections were cut and stained with uranyl acetate and lead citrate. Examination demonstrated the fine internal structure of the organism, *H. capsulatum* var. *farciminosum*, including the cell envelope, plasma membrane, cell wall, capsule and inner cell structures (1).

**b) Culture**

The mycelial form of *H. capsulatum* var. *farciminosum* grows slowly on laboratory media (2–8 weeks at 26°C). Media that can be used include Mycobiotic agar (2), Sabouraud’s dextrose agar agar enriched with 2.5% glycerol, brain–heart infusion agar supplemented with 10% horse blood, and pleuropneumonia-like organism (PPLO) nutrient agar enriched with 2% dextrose and 2.5% glycerol, pH 7.8 (11, 16). The addition of antibiotics to the media is recommended: cycloheximide (0.5 g/litre) and chloramphenicol (0.5 g/litre). Broad-spectrum antibacterial activity is obtained if gentamicin (50 mg/litre) and penicillin G (6 × 10⁶ units/litre) are used instead of chloramphenicol. Colonies appear in 2–8 weeks as dry, grey-white, granular, wrinkled mycelia. The colonies become brown with aging. Aerial forms occur, but are rare. The mycelial form produces a variety of conidia, including chlamydospores, arthroconidia and some blastoconidia. However, the large round double-walled macroconidia that are often observed in *H. capsulatum* var. *capsulatum* are lacking.

As a confirmatory test the yeast form of *H. capsulatum* var. *farciminosum* can be induced by subculturing some of the mycelium into brain–heart infusion agar containing 5% horse blood or by using Pine’s medium alone at 35–37°C in 5 % CO₂. Yeast colonies are flat, raised, wrinkled, white to greyish brown, and pasty in consistency (16). However, complete conversion to the yeast phase may only be achieved after four to five repeated serial transfers on to fresh media every 8 days.

**c) Animal inoculation**

Experimental transmission of *H. capsulatum* var. *farciminosum* has been attempted in mice, guinea-pigs and rabbits. Immunosuppressed mice are highly susceptible to experimental infection and can be used for diagnostic purposes (1).

**2. Serological tests**

There are published reports of various tests to detect antibodies as well as a skin hypersensitivity test for detection of cell-mediated immunity. Antibodies usually develop at or just after the onset of clinical signs.

**a) Fluorescent antibody tests**

- Indirect fluorescent antibody test

The following non-quantitative procedure is as described by Fawi (7).

i) Slides containing the organisms are made by smearing the lesion contents on to a glass slide or by emulsifying the cultured yeast phase of the organism in a saline solution and creating a thin film on a glass slide.

ii) The slides are heat-fixed by passing the slide through a flame.

iii) The slides are then washed in phosphate buffered saline (PBS) for 1 minute.

iv) Undiluted test sera are placed on the slides, which are then incubated for 30 minutes at 37°C.

v) The slides are washed in PBS three times for 10 minutes each.

vi) Fluorescein isothiocyanate (FITC)-conjugated anti-horse antibody at an appropriate dilution is flooded over the slides, which are then incubated for 30 minutes at 37°C.

vii) Washing in PBS is repeated three times for 10 minutes each.

viii) The slides are examined using fluorescence microscopy.

- Direct fluorescent antibody test

The following procedure is as described by Gabal et al. (8).
i) The globulin fraction of the test serum is precipitated, and then re-suspended to its original serum volume in saline. The serum is then conjugated to FITC.

ii) Small colony particles of the cultured mycelial form of the organism are suspended in 1–2 drops of saline on a glass slide. With a second slide, the colony particles are crushed and the solution is dragged across the slide to create a thin film.

iii) The smears are heat-fixed.

iv) The slides are washed in PBS for 1 minute.

v) The slides are incubated with dilutions of conjugated serum for 60 minutes at 37°C.

vi) The slides are washed in PBS three times for 5 minutes each.

vii) The slides are examined using fluorescence microscopy.

b) Indirect Enzyme-linked immunosorbent assay

The following procedure is as described by Gabal & Mohammed (10).

i) The mycelial form of the organism is produced on Sabouraud’s dextrose agar in tubes, and incubated for 4 weeks at 26°C. Three colonies are ground in 50 ml of sterile PBS. The suspension is diluted 1/100 and the 96-well microtitre plates are coated with 100 µl/well.

ii) The plates are incubated at 4°C overnight.

iii) The plates are washed with PBS containing Tween 20 (0.5 ml/litre) (PBS-T) three times for 3 minutes each.

iv) The plates are incubated with 5% bovine serum albumin, 100 µl/well, at 23–25°C for 30 minutes, with shaking.

v) The plates are washed with PBS-T three times for 3 minutes each.

vi) The sera are serially diluted using twofold dilution in duplicate in PBS-T, starting with a 1/50 dilution and incubated for 30 minutes at 23–25°C.

vii) The plates are washed with PBS-T three times for 3 minutes each.

viii) Peroxidase-labelled goat anti-horse IgG is diluted 1/800 and used at 100 µl/well, with incubation for 30 minutes at 23–25°C, with shaking.

ix) The plates are washed with PBS-T three times for 3 minutes each.

x) Finally, 100 µl/well of hydrogen peroxide and ABTS (2,2'-Azino-di-[3-ethyl-benzthiazoline]-6-sulphonic acid) in a citric acid buffer, pH 4, is added.

xi) The plates are read at 60 minutes in a spectrophotometer at wavelength 405 nm.

xii) The absorbance values are obtained twice from each serum dilution and the standard deviation and average percentage of the absorbance values of the different serum samples are considered in the interpretation of the results.

c) Passive haemagglutination test

The following procedure is as described by Gabal & Khalifa (9).

i) The organism is propagated for 8 weeks on Sabouraud’s dextrose agar. Five colonies are scraped, ground, suspended in 200 ml of saline, and sonicated for 20 minutes. The remaining mycelial elements are filtered out, and the filtrate is diluted 1/160.

ii) Normal sheep red blood cells (RBCs) are washed, treated with tannic acid, washed, and re-suspended as a 1% cell suspension.

iii) Different dilutions of the antigen preparation are mixed with the tanned RBCs and incubated in a water bath at 37°C for 1 hour. The RBCs are collected by centrifugation, washed three times in buffered saline and re-suspended to make a 1% cell suspension.

iv) Test sera are inactivated by heating at 56°C for 30 minutes and then absorbed with an equal volume of washed RBCs.

v) Dilutions of serum (0.5 ml) are placed in test tubes with 0.05 ml of antigen-coated tanned RBCs.

vi) Agglutination is recorded at 2 and 12 hours.
vii) Agglutination is detected when the RBCs form a uniform mat on the bottom of the tube. A negative test is indicated by the formation of a ‘button’ of RBCs at the bottom of the tube.

d) Skin hypersensitivity tests

Two skin hypersensitivity tests for the diagnosis of epizootic lymphangitis have been described. The first test was described by Gabal & Khalifa and adapted by Armeni et al. (5, 9).

i) A pure culture of *H. farciminosum* is propagated for 8 weeks on Sabouraud’s dextrose agar containing 2.5% glycerol. Five colonies are scraped, ground, suspended in 200 ml of saline, undergo five freeze–thaw cycles and are sonicated at an amplitude of 40° for 20 minutes. The remaining mycelial elements are removed by centrifugation at 1006 g at 4°C for 11 minutes. Sterility of the preparation is verified by incubating an aliquot on Sabouraud’s dextrose agar at 26°C for 4 weeks.

ii) Animals are inoculated intradermally with 0.1 ml containing 0.2 mg/ml protein in the neck.

iii) The inoculation site is examined for the presence of a local indurated and elevated area at 24–48 hours post-injection. An increase in skin thickness of > 4 mm is considered to be positive.

Alternatively, a ‘histofarcin’ test has been described by Soliman et al. (18).

i) The mycelial form of the organism is grown on polystyrene discs floating on 250 ml of PPLO media containing 2% glucose and 2.5% glycerine at 23–25°C for 4 months.

ii) The fungus-free culture filtrate is mixed with acetone (2/1) and held at 4°C for 48 hours.

iii) The supernatant is decanted and the acetone is allowed to evaporate.

iv) Precipitate is suspended to 1/10 original volume in distilled water.

v) Animals are inoculated intradermally with 0.1 ml of antigen in the neck.

vi) The inoculation site is examined for the presence of a local indurated and elevated area at 24, 48 and 72 hours post-injection.

C. REQUIREMENTS FOR VACCINES AND DIAGNOSTIC BIOLOGICALS

Control of the disease is usually through elimination of the infection. This is achieved by culling infected horses and application of strict hygiene practices to prevent spread of the organism. There are published reports on the use of killed (2) and live attenuated vaccines (20) in areas where epizootic lymphangitis is endemic, apparently with relatively good results.

The antigens used for skin hypersensitivity testing are described in the previous section.

REFERENCES


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CHAPTER 2.5.5.

EQUINE ENCEPHALOMYELITIS
(Eastern and Western)

SUMMARY

Eastern and Western equine encephalomyelitis viruses belong to the genus Alphavirus of the family Togaviridae. Alternate infection of birds and mosquitoes maintain these viruses in nature. The disease occurs sporadically in horses and humans from mid-summer to late autumn. Horses and humans are tangential dead-end hosts. The disease in horses is characterised by fever, anorexia, and severe depression. In severe cases, the disease in horses progresses to hyperexcitability, ataxia, severe mental depression, recumbency, convulsions, and death. Eastern equine encephalomyelitis (EEE) virus infection in horses is often fatal, while Western equine encephalomyelitis (WEE) virus can cause a subclinical or mild disease with less than 30% mortality. EEE and WEE have been reported to cause disease in poultry, game birds and ratites. Sporadic cases of EEE have been reported in cows, sheep, pigs, deer, and dogs.

Identification of the agent: A presumptive diagnosis of EEE or WEE can be made when susceptible horses display the characteristic somnolence and other signs of neurological disease in areas where haematophagous insects are active. There are no characteristic gross lesions. Histopathological lesions can provide a presumptive diagnosis. EEE virus can usually be isolated from the brain and sometimes other tissues of dead horses, however WEE virus is rarely isolated. EEE and WEE viruses can be isolated from field specimens by inoculating newborn mice, embryonating chicken eggs, cell cultures, or newly hatched chickens. The virus is identified by complement fixation (CF), immunofluorescence, or plaque reduction neutralisation (PRN) tests. EEE and WEE viral RNA may also be detected by reverse-transcription polymerase chain reaction methods.

Serological tests: Antibody can be identified by PRN, haemagglutination inhibition (HI), CF tests, or IgM capture enzyme-linked immunosorbent assay.

Requirements for vaccines and diagnostic biologicals: EEE and WEE vaccines are safe and immunogenic. They are produced in cell culture and inactivated with formalin.

A. INTRODUCTION

Eastern equine encephalomyelitis (EEE) and Western equine encephalomyelitis (WEE) viruses are members of the genus Alphavirus of the family Togaviridae. The natural ecology for virus maintenance occurs via alternate infection of birds and ornithophilic mosquitoes. Clinical disease may be observed in humans and horses, both of which are dead-end hosts for these agents. EEE has been diagnosed in Quebec and Ontario in Canada, central and eastern regions of the United States of America (USA), the Caribbean Islands, Mexico, and Central and South America. Disease caused by the WEE virus has been reported in the western USA and Canada, Mexico, and Central and South America (13, 16, 24). Highlands J virus, antigenically related to WEE virus, has been isolated in eastern USA. Although Highlands J virus is generally believed not to cause disease in mammals, it has been isolated from the brain of a horse dying of encephalitis in Florida (7).

Even though the mortality is lower for WEE, the clinical signs of EEE and WEE can be identical. The disease caused by either virus is also known as sleeping sickness. Following an incubation period of 5–14 days, clinical signs include fever, anorexia, and depression. A presumptive diagnosis of EEE or WEE virus infection in unvaccinated horses can be made if the characteristic somnolence is observed during the summer in temperate climates or the wet season in tropical and subtropical climates, when the mosquito vector is plentiful. However, a number of other diseases, such as West Nile virus and Venezuelan equine encephalomyelitis (chapters 2.1.20 and 2.5.14, respectively), produce similar clinical signs and the diagnosis must be confirmed by the described
EEE virus causes severe disease in humans with a mortality rate of 30–70% and a high frequency of permanent sequelae in patients who survive. WEE is usually mild in adult humans, but can be a severe disease in children. The fatality rate is between 3 and 14%. Severe infection and death caused by EEE and WEE viruses have been reported in laboratory workers; therefore, any work with these viruses must be performed at containment level 3 (see Chapter 1.1.2 Biosafety and biosecurity in the veterinary microbiology laboratory and animal facilities). It is recommended that personnel be immunised against EEE and WEE viruses (22). Precautions should also be taken to prevent human infection when performing post-mortem examinations on horses suspected of being infected with the equine encephalomyelitis viruses.

**B. DIAGNOSTIC TECHNIQUES**

1. **Identification of the agent**

The most definitive method for diagnosis of EEE or WEE is the isolation of the viruses. EEE virus can usually be isolated from the brains of horses, unless more than 5 days have elapsed between the appearance of clinical signs and the death of the horse. EEE virus can frequently be isolated from brain tissue even in the presence of a high serum antibody titre. WEE virus is rarely isolated from tissues of infected horses. Brain is the tissue of choice for virus isolation, but the virus has been isolated from other tissues, such as the liver and spleen. It is recommended that a complete set of tissues be collected in duplicate, one set for virus isolation and the other set in formalin for histopathological examination. Specimens for virus isolation should be sent refrigerated if they are to be received in the laboratory within 48 hours of collection; otherwise, they should be frozen and sent with dry ice. A complete set of tissues will allow the performance of diagnostic techniques for other diseases. For isolation, a 10% suspension of tissue is prepared in phosphate buffered saline (PBS), pH 7.8, containing bovine serum albumin (BSA) (fraction V; 0.75%), penicillin (100 units/ml), and streptomycin (100 µg/ml). The suspension is clarified by centrifugation at 1500 g for 30 minutes.

EEE and WEE viruses can be isolated in a number of cell culture systems. The most commonly used cell cultures are primary chicken or duck embryo fibroblasts, continuous cell lines of African green monkey kidney (Vero), rabbit kidney (RK-13), or baby hamster kidney (BHK-21). Isolation is usually attempted in 25 cm² cell culture flasks. Confluent cells are inoculated with 1.0 ml of tissue suspension. Following a 1–2-hour absorption period, maintenance medium is added. Cultures are incubated for 6–8 days, and one blind passage is made. EEE and WEE viruses will produce a cytopathic change in cell culture. Cultures that appear to be infected are frozen. The fluid from the thawed cultures is used for virus identification.

The newborn mouse is also considered to be a sensitive host system. Inoculate intracranially one or two litters of 1–4-day-old mice with 0.02 ml of inoculum using a 26-gauge 3/8 inch (9.3 mm) needle attached to a 1 ml tuberculin syringe. The inoculation site is just lateral to the midline into the midportion of one lateral hemisphere. Mice are observed for 10 days. Mice that die within 24 hours of inoculation are discarded. From 2 to 10 days postinoculation, dead mice are collected daily and frozen at −70°C. Mouse brains are harvested for virus identification by aspiration using a 20-gauge 1 inch (2.5 cm) needle attached to a 1 ml tuberculin syringe. A second passage is made only if virus cannot be identified from mice that die following inoculation.

The chicken embryo is considered to be less sensitive than newborn mice when used for primary isolation of EEE and WEE viruses. Tissue suspensions can be inoculated by the yolk-sac route into 6–8-day-old embryonating chicken eggs. There are no diagnostic signs or lesions in the embryos infected with these viruses. Inoculated embryos should be incubated for 7 days, but deaths usually occur between 2 and 4 days post-inoculation. Usually only one passage is made unless there are dead embryos from which virus cannot be isolated. Newly hatched chickens are susceptible and have been used for virus isolation. If this method is used, precautions must be taken to prevent aerosol exposure of laboratory personnel, as infected birds can shed highly infectious virus.

EEE or WEE viruses can be identified in infected mouse or chicken brains, cell culture fluid, or amnionic-allantoic fluid by complement fixation. A 10% brain suspension is prepared in veronal (barbitone) buffer; egg and cell culture fluids are used undiluted or diluted 1/10 in veronal buffer. The fluid or suspension is centrifuged at 9000 g.
for 30 minutes, and the supernatant fluid is tested against hyperimmune serum or mouse ascitic fluid prepared against EEE and WEE viruses using a standard CF procedure (21). The CF test requires the overnight incubation at 4°C of serum-antigen with 7 units of complement. Virus can be identified in cell culture by direct immunofluorescent staining. The less commonly used method of virus identification is the neutralisation test, as outlined below.

Reverse-transcription polymerase chain reaction (RT-PCR) methods to detect EEE, WEE and VEE viral nucleic acid in mosquitoes and vertebrate tissues have been described, although few have been extensively validated for mammalian samples (8, 9, 12, 23). A multiplex PCR method was developed to expedite differential diagnosis in cases of suspected EEE or West Nile arboviral encephalomyelitis in horses (6). The assay has enhanced speed and sensitivity compared to cell culture virus isolation and has been used effectively in the USA during several recent arbovirus seasons. Recently, a combination of an RT-PCR with an enzyme-linked immunosorbent assay (ELISA: RT-PCR-ELISA) was reported as a method to identify alpha-viruses that are pathogenic to humans (25).

Antigen-capture ELISA has been developed for EEE surveillance in mosquitoes. This can be used in countries that do not have facilities for virus isolation or PCR (2). Immunohistochemical procedures for diagnosis of EEE have also been described (15).

2. Serological tests

Serological confirmation of EEE or WEE virus infection requires a four-fold or greater increase or decrease in antibody titre in paired serum samples collected 10–14 days apart. Most horses infected with EEE or WEE virus have a high antibody titre when clinical disease is observed. Consequently, a presumptive diagnosis can be made if an unvaccinated horse with appropriate clinical signs has antibody against only EEE or WEE virus. The detection of IgM antibody by the ELISA can also provide a presumptive diagnosis of acute infection (17). The plaque reduction neutralisation (PRN) test or, preferably, a combination of PRN and haemagglutination inhibition (HI) tests is the procedure most commonly used for the detection of antibody against EEE and WEE viruses. There may be cross-reactions between antibody against EEE and WEE virus in the CF and HI tests. CF antibody against both EEE and WEE viruses appears later and does not persist; consequently, it is less useful for the serological diagnosis of disease.

a) Complement fixation

The CF test is frequently used for the demonstration of antibodies, although the antibodies detected by the CF test may not persist for as long as those detected by the HI or PRN tests. A sucrose/acetone mouse brain extract is commonly used as antigen. The positive antigen, or control antigen, is mouse brain from uninoculated mice similarly extracted and diluted.

Sera are diluted 1/4 in veronal buffered saline containing 1% gelatin (VBSG), and inactivated at 56°C for 30 minutes. Titrations of positive sera may be performed using additional twofold dilutions. The CF antigens and control antigen (normal mouse brain) are diluted in VBSG to their optimal amount of fixation as determined by titration against the positive sera; guinea-pig complement is diluted in VBSG to contain 5 complement haemolytic units-50% (CH<sub>50</sub>). Sera, antigen, and complement are reacted in 96-well round-bottom microtitre plates at 4°C for 18 hours. The sheep red blood cells (SRBCs) are standardised to 2.8% concentration. Haemolysin is titrated to determine the optimal dilution for the lot of complement used. Haemolysin is used to sensitise 2.8% SRBCs and the sensitised cells are added to all wells on the microtitre plate. The test is incubated for 30 minutes at 37°C. The plates are then centrifuged (200 g), and the wells are scored for the presence of haemolysis. The following controls are used: (a) serum and control serum each with 5 CH<sub>50</sub> and 2.5 CH<sub>50</sub> of complement; (b) CF antigen and control antigen each with 5 CH<sub>50</sub> and 2.5 CH<sub>50</sub> of complement; (c) complement dilutions of 5 CH<sub>50</sub>, 2.5 CH<sub>50</sub>, and 1.25 CH<sub>50</sub>; and (d) cell control wells with only SRBCs and VBSG diluent. These controls test for anticomplementary serum and anticomplementary antigen, activity of complement used in the test, and integrity of the SRBC indicator system in the absence of complement, respectively.

To avoid anticomplementary effects, sera should be separated from the blood as soon as possible. Positive and negative control sera should be used in the test.

b) Haemagglutination inhibition

The antigen for the HI test is the same as described above for the CF test. The antigen is diluted so that the amount used in each haemagglutinating unit (HAU) is from four to eight times that which agglutinates 50% of the RBCs in the test system. The haemagglutination titre and optimum pH for each antigen are determined with goose RBCs diluted in pH solutions ranging from pH 5.8 to pH 6.6, at 0.2 intervals.
Sera are diluted 1/10 in borate saline, pH 9.0, and then inactivated at 56°C for 30 minutes. Kaolin treatment is used to remove nonspecific serum inhibitors. Alternatively, nonspecific inhibitors may be removed by acetone treatment of serum diluted 1/10 in PBS followed by reconstitution in borate saline. Sera should be absorbed before use by incubation with a 0.05 ml volume of washed packed goose RBCs for 20 minutes at 4°C.

Following heat inactivation, kaolin treatment and absorption, twofold dilutions of the treated serum are prepared in borate saline, pH 9.0 with 0.4% bovalbumin. Serum dilutions (0.025 ml/well) are prepared in a 96-well round-bottom microtitre plate in twofold dilutions in borate saline, pH 9.0, with 0.4% bovalbumin. Antigen (0.025 ml/well) is added to the serum. Plates are incubated at 4°C overnight. RBCs are derived from normal white male geese¹ and washed three times in dextrose/gelatin/veronal (DGV), and a 7.0% suspension is prepared in DGV. The 7.0% suspension is then diluted 1/24 in the appropriate pH solution, and 0.05 ml per well is added immediately to the plates. Plates are incubated for 30 minutes at 37°C. Positive and negative control sera are incorporated into each test. A test is considered to be valid only if the control sera give the expected results. Titres of 1/10 and 1/20 are suspect, and titres of 1/40 and above are positive.

c) Enzyme-linked immunosorbent assay

The ELISA is performed by coating flat-bottomed plates with anti-equine IgM capture antibody (17). The antibody is diluted according to the manufacture’s recommendations in 0.5 M carbonate buffer, pH 9.6, and 50 µl is added to each well. The plates are incubated at 37°C for 1 hour, and then at 4°C overnight. Prior to use, the coated plates are washed three times with 200–300 µl/well of 0.01 M PBS containing 0.05% Tween 20. After the second wash, 200 µl/well of PBS/Tween/5% nonfat dried milk is added and the plates are incubated at room temperature for 1 hour. Following incubation, the plates are washed again three times with PBS/Tween. Test and control sera are diluted 1/400 in 0.01 M PBS, pH 7.2, containing 0.05% Tween 20, and 50 µl is added to each well. The plates are incubated at 37°C for 90 minutes and then washed three times. Next, 50 µl of viral antigen is added to all wells. (The dilution of the antigen will depend on the source and should be empirically determined.) The plates are incubated overnight at 4°C, and washed three times. Then, 50 µl of horseradish-peroxidase-conjugated monoclonal antibody (Mab) to encephalitis virus² is added. The plates are incubated for 90 minutes at 37°C and then washed six times. Finally, 50 µl of freshly prepared ABTS (2,2’-Azino-bis-[3-ethylbenzo-thiazoline-6-sulphonic acid]) substrate + hydrogen peroxidase is added, and the plates are incubated at room temperature for 15–40 minutes The absorbance of the test serum is measured at 405 nm. A test sample is considered to be positive if the absorbance of the test sample in wells containing virus antigen is at least twice the absorbance of negative control serum in wells containing virus antigen and at least twice the absorbance of the sample tested in parallel in wells containing normal antigen.

d) Plaque reduction neutralisation

The PRN test is very specific and can be used to differentiate between EEE and WEE virus infections. The PRN test is performed in duck embryo fibroblast, Vero, or BHK-21 cell cultures in 25 cm² flasks or six-well plates. Volumes listed are for flasks; the volume should be halved for wells in six-well plates. The sera can be screened at a 1/10 and 1/100 final dilution. Endpoints can be established using the PRN or HI test. Serum used in the PRN assay is tested against 100 plaque-forming units (PFU) of virus (50 PFU for six-well plates). The virus/serum mixture is incubated at 37°C for 75 minutes before inoculation on to confluent cell culture monolayers in 25 cm² flasks. The inoculum is adsorbed for 1 hour, followed by the addition of 6 ml of overlay medium. The overlay medium consists of two solutions that are prepared separately. Solution I contains 2 × Earle’s Basic Salts Solution without phenol red, 4% fetal bovine serum, 100 µg/ml gentamicin, 200 µg/ml nystatin, 0.45% solution of sodium bicarbonate, and 0.002% neutral red. When duck embryo fibroblasts are used, Solution I also contains 6.6% yeast extract lactalbumin hydrolysate. Solution II consists of 2% Noble agar that is sterilised and maintained at 47°C. Equal volumes of solutions I and II are adjusted to 47°C and mixed together just before use. The test is incubated for 48–72 hours, and endpoints are based on a 90% reduction in the number of plaques compared with the virus control flasks, which should have about 100 plaques.

C. REQUIREMENTS FOR VACCINES AND DIAGNOSTIC BIOLOGICALS

Inactivated vaccines against EEE and WEE viruses are available commercially. Attenuated EEE and WEE virus vaccines have not proven satisfactory. The vaccines licensed for use in the USA are prepared using the following

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¹ RBCs from adult domestic white male geese are preferred, but RBCs from other male geese can be used. If cells from female geese are used, there may be more test variability. It has been reported that rooster RBCs cause a decrease in the sensitivity of the test.

² Available from: Centers for Disease Control and Prevention, Biological Reference Reagents, 1600 Clifton Road NE, Mail Stop C21, Atlanta, Georgia 30333, United States of America.
combinations: EEE and WEE; EEE, WEE, and Venezuelan equine encephalomyelitis (VEE); and EEE and VEE. In addition, tetanus toxoid, inactivated influenza virus, and inactivated West Nile virus have been combined with EEE and WEE or EEE, WEE, and VEE. Early vaccines were produced from virus propagated in embryonating chicken eggs and inactivated with formalin. Current vaccines are prepared from virus propagated in cell culture, and inactivated with formalin (10) or monoethylamine.

1. Seed management
   a) Characteristics of the seed
      Standard strains of EEE and WEE viruses that were isolated over 20 years ago have been used for vaccine production and have been proven to produce a protective immunity. Strains of EEE virus that differ antigenically and in molecular structure have been identified from different geographical regions. However, the North American and Caribbean isolates appear to be similar (26). Strains of WEE virus isolated from different countries have been found to be similar both by MAb testing and RNA oligonucleotide fingerprinting analysis (16). A recent well-characterised isolate from the country where the vaccine is to be used would be advantageous. Viruses that are selected must be immunogenic and replicate to high titres in cell culture.
   b) Method of culture
      Primary chicken embryo fibroblasts and Vero cells have been used for propagation of viruses used for vaccine production. The fibroblasts should be prepared from specific pathogen free embryos. Other susceptible cell lines could also be used.
   c) Validation as a vaccine
      If a cell line is used, the master cell stock is tested to confirm the identity of the cell line, species of origin, and freedom from extraneous agents. If primary cell cultures are used, a monolayer from each batch of each subculture should be tested for extraneous agents including bacteria, fungi, mycoplasma, and viruses. The master seed virus should also be tested to ensure freedom from bacteria, fungi, mycoplasma, and extraneous viruses (see Chapter 1.1.9 Tests for sterility and freedom from contamination of biological materials for a description of the method).

      The vaccines are administered by the intramuscular (in most cases) or intradermal route in the cervical region in two doses given 2–4 weeks apart. Annual revaccination is recommended. All foals vaccinated before 1 year of age should be revaccinated before the next vector season.

2. Method of manufacture
   Details of the manufacture of vaccines currently on the market are not available. Consequently, the information provided here is intended only as background reference material on the vaccines and not as a method of manufacture. The virus and cell culture system should be selected so that a high virus titre, $\geq 10^6$ TCID$_{50}$ (50% tissue culture infective dose) per ml, is obtained in under 48 hours. Virus for vaccine production can be prepared from the supernatant fluid from infected cell cultures. The fluid is harvested when 70–100% of the monolayers have the characteristic cytopathic changes. The virus titre is determined by titration in cell culture or mice. The fluid is clarified by low speed centrifugation and filtered through gauze. The virus is inactivated by adding formalin to a final concentration of 1/2000 (0.05%) and holding at 37°C for 24 hours. Residual formaldehyde is neutralised by sodium bisulphite (10). The residual free formalin content in the inactivated vaccine should not exceed 0.2% formaldehyde.

3. In-process control
   Cultures should be examined daily for virus-induced cytopathic effect. The harvested virus should be tested for microbial contamination. The efficacy of the inactivation process should be checked by testing for viable virus.

4. Batch control
   a) Sterility
      Tests for sterility and freedom from contamination of biological materials may be found in Chapter 1.1.9.
   b) Safety
      The inactivated vaccine is safety tested by inoculating subcutaneously at least ten 6–12-hour-old chickens with 0.5 ml of the vaccine. The chickens are observed each day for 10 days for unfavourable reactions that are attributable to the vaccine (20). Safety testing can also be carried out by inoculating intracerebrally at
least eight 1–4-day-old mice with 0.02 ml of the vaccine, and observing for 7 days. It is critical that safety tests be conducted on each lot of vaccine to insure that there is no residual virulent virus present.

c) Potency
Potency testing is performed by inoculating each of ten guinea pigs with either EEE or WEE virus, using one-half the horse dose on two occasions, 14–21 days apart, by the route recommended for the horse. Serum samples from each vaccinate and each control are tested 14–21 days after the second dose using the PRN test. The EEE titres should be ≥1/40, and the WEE titres should be ≥1/40 (20), using Vero cells. If duck embryo fibroblasts are used in the PRN test, the titres will be lower. An alternative potency test is to use intracerebral challenge, 14–21 days after the second vaccination. Each guinea pig is inoculated with 0.1 ml of virus containing 100 LD₅₀ (50% lethal dose). Simultaneous titration is carried out. In order for the vaccine to be approved, 80% of the guinea pigs must survive both viruses.

d) Duration of immunity
Comprehensive studies on duration of immunity are not available. An annual revaccination is recommended. Foals that are vaccinated before 1 year of age should be revaccinated before the next vector season.

e) Stability
The lyophilised vaccine is stable and immunogenic for 3 years if kept refrigerated at 2–7°C. After 3 years, vaccine should be discarded. The vaccines should be used immediately after reconstitution.

f) Preservatives
The preservatives used are thimerosal at a 1/10,000 dilution and antibiotics (neomycin, polymyxin amphotericin B and gentamicin).

g) Precautions (hazards)
Severe infection and death caused by EEE and WEE viruses have been reported in laboratory workers; therefore, any work with these viruses must be carried out at least in a containment level 3 laboratory (see Chapter 1.1.2) using biological safety cabinets, and it is recommended that personnel be immunised against EEE and WEE viruses (22).

Pregnant mares and foals under 2 weeks old should not be vaccinated.

5. Tests on the final product
a) Safety
See Section C.4.b.

b) Potency
See Section C.4.c.

REFERENCES


Chapter 2.5.5. — Equine encephalomyelitis (Eastern and Western)


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**NB:** There is an OIE Reference Laboratory for Equine encephalomyelitis (Eastern and Western) (see Table in Part 3 of this *Terrestrial Manual* or consult the OIE Web site for the most up-to-date list: [www.oie.int](http://www.oie.int)).
CHAPTER 2.5.6.

EQUINE INFECTIOUS ANAEMIA

SUMMARY

Equine infectious anaemia (EIA) is a persistent viral infection of equids. The causative agent, EIA virus (EIAV) is a lentivirus in the family Retroviridae, subfamily Orthoretrovirinae. Other members of the lentivirus genus include: bovine immunodeficiency virus; caprine arthritis encephalitis virus; feline immunodeficiency virus; human immunodeficiency virus 1; human immunodeficiency virus 2; and maedi/visna virus. EIA can be diagnosed on the basis of clinical signs, pathological lesions, serology and molecular methods. Infected horses remain viraemic carriers for life and, with very rare exceptions, yield a positive serological test result. Antibody response usually persists and antibody-positive animals, older than 6–8 months, are identified as virus carriers (below 6–8 months of age, serological reactions can be due to maternal antibodies; status can be confirmed by molecular techniques). Infected equids are potential virus reservoirs. Biting flies are mechanical vectors for the virus in nature.

Identification of the agent: Virus from a horse can be isolated by inoculating suspect blood into a susceptible horse or on to leukocyte cultures prepared from susceptible horses. Recognition of infection in horses that have been inoculated experimentally may be made on the basis of clinical signs, haematological changes and a positive antibody response determined by an immunodiffusion test or enzyme-linked immunosorbent assay (ELISA) or by molecular techniques. Successful virus isolation in horse leukocyte cultures is confirmed by the detection of specific EIA antigen, by immunofluorescence assay, polymerase chain reaction, reverse-transcriptase assay, or by the inoculation of culture fluids into susceptible horses. Virus isolation is rarely attempted because of the time, difficulty and expense involved.

Serological tests: Agar gel immunodiffusion (AGID) tests and ELISAs are simple, reliable tests for the demonstration of EIAV infection. When ELISAs are positive they should be confirmed using the AGID test. EIA antigens can be prepared from infected tissue cultures or by using recombinant DNA technology.

Requirements for vaccines and diagnostic biologicals: There are no biological products currently available.

A. INTRODUCTION

Equine infectious anaemia (EIA) occurs world-wide. The infection, formerly known as swamp fever, is limited to equids. The disease is characterised by recurrent febrile episodes, thromboctopenia, anaemia, rapid loss of weight and oedema of the lower parts of the body. If death does not result from one of the acute clinical attacks, a chronic stage develops and the infection tends to become inapparent. The incubation period is normally 1–3 weeks, but may be as long as 3 months. In acute cases, lymph nodes, spleen and liver are hyperaemic and enlarged. Histologically these organs are infiltrated with nests of immature lymphocytes and plasma cells. Kupffer cells in the liver often contain haemosiderin or erythrocytes. The enlarged spleen may be felt on rectal examination. Differential diagnoses include equine viral arteritis (Chapter 2.5.10), and other causes of oedema, fever, or anemia.

Once a horse is infected with EIA virus (EIAV), its blood remains infectious for the remainder of its life. This means that the horse is a viraemic carrier and can potentially transmit the infection to other horses (4). Transmission occurs by transfer of blood from an infected horse. In nature, spread of the virus is most likely via interrupted feeding of bloodsucking horseflies on a clinically ill horse and then on susceptible horses. Transmission can also occur by the iatrogenic transfer of blood through the use of contaminated needles. In utero infection of the fetus may occur (9). The virus titre is higher in horses with clinical signs and the risk of transmission is higher from these animals than the carrier animals with a lower virus titre.
B. DIAGNOSTIC TECHNIQUES

Agar gel immunodiffusion (AGID) tests (7) and enzyme-linked immunosorbent assays (ELISAs) (17) are accurate, reliable tests for the detection of EIA in horses, except for animals in the early stages of infection and foals of infected dams. In other rare circumstances, misleading results may occur when the level of virus circulating in the blood during an acute episode of the disease is sufficient to bind available antibody, and if initial antibody levels never rise high enough to be detectable (18). Although the ELISA will detect antibodies somewhat earlier and at lower concentrations than the AGID test, positive ELISAs are confirmed using the AGID test. This is because false-positive results have been noted with ELISAs. The AGID test also has the advantage of distinguishing between EIA and non-EIA antigen–antibody reactions by lines of identity.

The EIAV is a lentivirus in the family Retroviridae, subfamily Orthoretrovirinae. Other members of the lentivirus genus include: bovine immunodeficiency virus; caprine arthritis encephalitis virus; feline immunodeficiency virus; human immunodeficiency virus 1; human immunodeficiency virus 2; and maedi/visna virus. Nucleic acid sequence comparisons have demonstrated a marked relatedness among these viruses.

1. Identification of the agent

a) Virus isolation and identification

Virus isolation is usually not necessary to make a diagnosis.

Isolation of the virus from suspect horses may be made by inoculating their blood on to leukocyte cultures prepared from horses free of infection. Virus production in cultures can be confirmed by detection of specific EIA antigen by ELISA (16), by immunofluorescence assay (20), by molecular tests or by subinoculation into susceptible horses. Virus isolation is rarely attempted because of the difficulty of growing horse leukocyte cultures.

When the exact status of infection of a horse cannot be ascertained, the inoculation of a susceptible horse with suspect blood should be employed. In this case a horse that has previously been tested for antibody and shown to be negative is given an immediate blood transfusion from the suspect horse, and its antibody status and clinical condition are monitored for at least 45 days. Usually, 1–25 ml of whole blood given intravenously is sufficient to demonstrate infection, but in rare cases it may be necessary to use a larger volume of blood (250 ml) or washed leukocytes from such a volume (5).

b) Polymerase chain reaction

A nested polymerase chain reaction (PCR) assay to detect EIA proviral DNA from the peripheral blood of horses has been described (13). The nested PCR method is based on primer sequences from the gag region of the proviral genome. It has proven to be a sensitive technique to detect field strains of EAV in white blood cells of EIA infected horses; the lower limit of detection is typically around 10 genomic copies of the target DNA (13, 14). A real-time reverse-transcriptase PCR assay has also been described (8). To confirm the results of these very sensitive assays, it is recommended that duplicate samples of each diagnostic specimen be processed. Because of the risk of cross contamination, it is also important that proper procedures are followed. Methods to insure the validity of PCR testing are discussed in detail in Chapter 1.1.5 Validation and quality control of polymerase chain reaction methods used for the diagnosis of infectious diseases.

The following are some of the circumstances where the PCR assay maybe used for the detection of EIAV infection in horses:

- Conflicting results on serologic tests;
- Suspected infection but negative or questionable serologic results;
- Complementary test to serology for the confirmation of positive results;
- Confirmation of early infection, before serum antibodies to EIAV develop;
- Ensuring that horses that are to be used for antiserum or vaccine production or as blood donors are free of EIAV;
- Confirmation of the status of a foal from an infected mare.
2. Serological tests

Due to the persistence of EIAV in infected equids, detection of serum antibody to EIAV confirms the diagnosis of EIAV infection.

a) Agar gel immunodiffusion test (the prescribed test for international trade)

Precipitating antibody is rapidly produced as a result of EIA infection, and can be detected by the AGID test. Specific reactions are indicated by precipitin lines between the EIA antigen and the test serum and confirmed by their identity with the reaction between the antigen and the positive standard serum. Horses in the first 2–3 weeks after infection will usually give negative serological reactions. In rare cases the post-infection time prior to the appearance of detectable antibody may extend up to 60 days.

Reagents for AGID are available commercially from several companies. Alternately, AGID antigen and reference serum may be prepared as described below.

• Preparation of antigen

Specific EIA antigen may be prepared from the spleen of acutely infected horses (6), from infected equine tissue culture (11), from a persistently infected canine thymus cell line (3), or from proteins expressed in bacteria or baculovirus using the recombinant DNA technique (2, 10). Preparation from infected cultures or from recombinant DNA techniques gives a more uniform result than the use of spleen cells and allows for better standardisation of reagents.

To obtain a satisfactory antigen from spleen, a horse must be infected with a highly virulent strain of EIAV. The resulting incubation period should be 5–7 days, and the spleen should be collected 9 days after inoculation, when the virus titre is at its peak and before any detectable amount of precipitating antibody is produced. Undiluted spleen pulp is used in the immunodiffusion test as antigen (6). Extraction of antigen from the spleen with a saline solution and concentration with ammonium sulphate does not give as satisfactory an antigen as selection of a spleen with a very high titre of EIA antigen.

Alternatively, equine fetal kidney or dermal cells or canine thymus cells are infected with a strain of EIAV adapted to grow in tissue culture (American Type Culture Collection). Virus is collected from cultures by precipitation with 8% polyethylene glycol or by pelleting by ultracentrifugation. The diagnostic antigen, p26, is released from the virus by treatment with detergent or ether (11). EIAV core proteins, expressed in bacteria or baculovirus, are commercially available and find practical use as high quality antigens for serological diagnosis.

The p26 is an internal structural protein of the virus that is coded for by the *gag* gene. The p26 is more antigenically stable among EIAV strains than the virion glycoproteins gp45 and gp90 (12). There is evidence of strain variation in the p26 amino acid sequence; however there is no evidence to indicate that this variation influences any of the serological diagnostic tests (21).

• Preparation of standard antiserum

A known positive antiserum may be collected from a horse previously infected with EIAV. This serum should yield a single dense precipitation line that is specific for EIA, as demonstrated by a reaction of identity with a known standard serum. It is essential to balance the antigen and antibody concentrations in order to ensure the optimal sensitivity of the test. Reagent concentrations should be adjusted to form a narrow precipitation line approximately equidistant between the two wells containing antigen and serum.

• Test procedure (1, 6, 15)

i) Immunodiffusion reactions are carried out in a layer of agar in Petri dishes. For Petri dishes that are 100 mm in diameter, 15–17 ml of 1% Noble agar in 0.145 M borate buffer (9 g H₃BO₃, plus 2 g NaOH per litre), pH 8.6 (± 0.2) is used. Six wells are punched out of the agar surrounding a centre well of the same diameter. The wells are 5.3 mm in diameter and 2.4 mm apart. Each well must contain the same volume of reagent.

ii) The antigen is placed in the central well and the standard antiserum is placed in alternate exterior wells. Serum samples for testing are placed in the remaining three wells.

iii) The dishes are maintained at room temperature in a humid environment.

iv) After 24–48 hours the precipitation reactions are examined over a narrow beam of intense, oblique light and against a black background. The reference lines should be clearly visible at 24 hours, and at that time any test sera that are strongly positive may also have formed lines of identity with those between the standard reagents. A weakly positive reaction may take 48 hours to form and is indicated by a slight bending of the standard serum precipitation line between the antigen well and the test
serum well. Sera with high precipitating antibody titres may form broader precipitin bands that tend to be diffuse. Such reactions can be confirmed as specific for EIA by dilution at 1/2 or 1/4 prior to retesting; these then give a more distinct line of identity. Sera devoid of EIA antibody will not form precipitation lines and will have no effect on the reaction lines of the standard reagents.

v) Interpretation of the results: Horses that are in the early stages of an infection may not give a positive serological reaction in an AGID test. Such animals should be bled again after 3–4 weeks. In order to make a diagnosis in a young foal, it may be necessary to determine the antibody status of the dam. If the mare passes EIA antibody to the foal through colostrum, then a period of 6 months or longer after birth must be allowed for the maternal antibody to wane. Sequential testing of the foal at monthly intervals may be useful to observe the decline in maternal antibody. To conclude that the foal is not infected, a negative result must be obtained (following an initial positive result) at least 2 months after separating the foal from contact with the EIA positive mare or any other positive horse. Alternatively PCR could be performed on the blood of the foal to determine the presence/absence of EIA provirus.

b) Enzyme-linked immunosorbent assay

There are four ELISAs that are approved by the United States Department of Agriculture for the diagnosis of equine infectious anaemia and are available internationally; a competitive ELISA and three non-competitive ELISAs. The competitive ELISA and two non-competitive ELISAs detect antibody produced against the p26 core protein antigen. The third non-competitive ELISA incorporates both p26 core protein and gp45 (viral transmembrane protein) antigens. Typical ELISA protocols are used in all tests. If commercial ELISA materials are not available, a non-competitive ELISA using p26 antigen purified from cell culture material may be employed (16).

A positive test result by ELISA should be retested using the AGID test to confirm the diagnosis because some false-positive results have been noted with the ELISA. The results can also be confirmed by the immunoblot technique. A standard antiserum for immunodiffusion, which contains the minimum amount of antibody that should be detected by laboratories, is available from the OIE Reference Laboratories (see Table given in Part 3 of this Terrestrial Manual). Uniform methods for EIA control have been published (19).

C. REQUIREMENTS FOR VACCINES AND DIAGNOSTIC BIOLOGICALS

No biological products are available currently.

REFERENCES


* * *

NB: There are OIE Reference Laboratories for Equine infectious anaemia (see Table in Part 3 of this Terrestrial Manual or consult the OIE Web site for the most up-to-date list: www.oie.int).
CHAPTER 2.5.7.

EQUINE INFLUENZA

SUMMARY

Equine influenza is an acute respiratory infection of horses, donkeys and mules caused by two distinct subtypes (H7N7, formerly equi-1, and H3N8, formerly equi-2) of influenza A virus within the genus Influenzavirus A of the family Orthomyxoviridae. Viruses of the H7N7 subtype have not been isolated since the late 1970s. Infection of zebras, dogs and humans has also been reported. In fully susceptible equidae, clinical signs include pyrexia and a harsh dry cough followed by a mucopurulent nasal discharge; neurological signs have been described as a rare event. In partially immune vaccinated animals, one or more of these signs may be absent. Characteristically, influenza spreads rapidly in a susceptible population. While normally confined to equidae, the H3N8 subtype has crossed the species barrier to dogs. Infection normally produces mild fever and coughing; occasionally fatal pneumonia develops. While equine influenza has not been shown to cause disease in humans, serological evidence of infection has been described. Diagnosis of influenza virus infections is based on virus isolation, antigen and/or genome detection from horses with acute respiratory illness, or on the demonstration of a serological response to infection. Ideally, both methods are used. The disease is endemic in most countries with substantial equine populations. In recent years infection has been introduced into Australia and re-introduced into South Africa and Japan; to date New Zealand and Iceland are reported to be free. Extensive infection of dogs has been reported in North America as a result of cross species transmission and subsequent within species transmission.

Identification of the agent: Embryonated hens’ eggs and/or cell cultures can be used for virus isolation from nasopharyngeal swabs or nasal and tracheal washes. Viral growth is monitored by haemagglutination (HA) or, in cell cultures, by haemadsorption (HAD) using chicken or guinea-pig red blood cells. Isolates can be characterised by haemagglutination inhibition (HI) using strain-specific antisera. Isolates should always be sent immediately to an International Reference Laboratory (OIE or World Health Organization). Samples that yield negative results should be repassaged; up to five passages may be necessary to isolate viruses from vaccinated horses. Infection may also be demonstrated by detection of viral antigen in respiratory secretions using an enzyme-linked immunosorbent assay.

Serological tests: Diagnosis of influenza virus infections is usually only accomplished by tests on paired sera; the first sample should be taken as soon as possible after the onset of clinical signs and the second approximately 2 weeks later. Antibody levels are determined by HI or single radial haemolysis (SRH).

Requirements for vaccines and diagnostic biologicals: Spread of infection and severity of disease may be reduced by the use of potent inactivated equine influenza vaccines containing epidemiologically relevant virus strains. Inactivated equine influenza vaccines contain whole viruses or their subunits. However, vaccinated infected horses can still shed the virus. The vaccine viruses are propagated in embryonated hens’ eggs or tissue culture, concentrated, and purified before inactivation with agents such as formalin or beta-propiolactone. Vaccines provide protection by inducing humoral antibody to the haemagglutinin protein. Responses are generally short-lived and multiple doses are required to maintain protective levels of antibody. An adjuvant is usually required to stimulate durable protective levels of antibody. Live attenuated virus and viral vectored vaccines have recently been licensed in some countries.

Influenza vaccines are widely available and are routinely used in competition horses in Europe, the Americas, and Asia. In some countries, vaccination is mandatory for sport horses that are competing under rules of equestrian organisations. Vaccines are not widely or routinely used on
Chapter 2.5.7. — Equine influenza

the Indian subcontinent, China, Australia or New Zealand, the latter region, having remained free from infection.

Following a primary course of three doses at intervals of around 0, 1 and 6 months, an annual booster is the usual minimum requirement. In some regions, repeated vaccinations are given every 3–6 months. Foals born to well vaccinated dams should not commence their vaccination programme until 6–9 months of age when all maternal immunity has waned; earlier vaccination may induce tolerance and a long-term poor response to vaccination. Foals with no maternal antibody may be vaccinated from an early age, particularly in high risk situations.

Immunity stimulated by inactivated and a nonreplicating vectored vaccine is dependent primarily, on the induction of antibody to the haemagglutinin (HA) and vaccine induced protection correlates well with levels of circulating antibody to the HA. By contrast immunity induced by infection or vaccination with a live attenuated vaccine is not dependent on the presence of circulating antibody and mucosal and cellular responses are likely to contribute.

Vaccine breakdown has been attributed to inadequate vaccine potency, inappropriate vaccination schedules, and outdated vaccine viruses that have become irrelevant as a result of antigenic drift. An in-vitro potency test (single radial diffusion) can be used for in-process testing of the antigenic content of inactivated products before addition of an adjuvant. In process testing of live and vectored vaccines relies on titration of infectious virus A surveillance programme is underway to monitor antigenic drift among equine influenza viruses and to provide information on strain selection for vaccines.

A. INTRODUCTION

Equine influenza is caused by two subtypes: H7N7 (formerly subtype 1) and H3N8 (formerly subtype 2) of influenza A viruses (genus Influenzavirus A of the family Orthomyxoviridae); however there have been very few reports of H7N7 subtype virus infections in the last 20 years (15, 29). Although these are not genuine human pathogens, humans can become infected with equine influenza virus subtypes. Such infections are unusual and subclinical, but may represent a potential biohazard to laboratory personnel (1).

In fully susceptible equidae, clinical signs include pyrexia, and a harsh dry cough; pneumonia in young foals and donkeys and encephalitis in horses have been described as rare events (7, 10). Clinical signs associated with infection in dogs also include fever and a cough; occasionally infection results in suppurative bronchopneumonia and peracute death (4). Characteristically, influenza spreads rapidly in a susceptible population, but in partially immune vaccinated animals, one or more of these signs may be absent and spread of the disease within a vaccinated population may be limited (6). This makes clinical diagnosis of equine influenza more difficult as other viral disease, such as equine herpesvirus, may clinically resemble a mild form of influenza. Horses infected with equine influenza virus become susceptible to secondary bacterial infection and may develop mucopurulent nasal discharge, which can lead to diagnosis of bacterial disease with the underlying cause being overlooked. Vaccination does not produce sterile immunity; vaccinated horses may shed virus and contribute to the spread of EI. Appropriate risk management strategies to deal with this possibility should be developed.

B. DIAGNOSTIC TECHNIQUES

Laboratory diagnosis of influenza virus infections is based on virus isolation from horses with acute respiratory illness, or on the demonstration of a serological response to infection. Ideally, both methods are used. Infection may also be demonstrated by detection of viral antigen in respiratory secretions using an enzyme-linked immunosorbent assay (ELISA) or viral genome using polymerase chain reaction (PCR) assays. All influenza viruses are highly contagious for susceptible hosts, including embryonated hens’ eggs and cell cultures. Care must therefore be taken during the handling of infected eggs or cultures to avoid accidental cross-contamination (28). Standard strains should not be propagated in the diagnostic laboratory, at least never at the same time or in the same place where diagnostic samples are being processed. All working areas must be efficiently disinfected before and after virus manipulations, which should preferably be conducted within biohazard containment.

It is important to obtain samples as soon as possible after the onset of clinical signs, preferably within 3–5 days. These samples include nasopharyngeal swabs and nasal or tracheal washings, the latter taken by endoscopy. Swabs may consist of absorbent cotton wool sponge/gauze on wire, and should be long enough to be passed via the ventral meatus into the nasopharynx. Swabs should be transferred to a tube containing transport medium immediately after use. This medium consists of phosphate buffered saline (PBS) containing 40% glycerol, or PBS containing 2% tryptose phosphate broth, 2% antibiotic solution (penicillin [10,000 units], streptomycin
[10,000 units] in sterile distilled water [100 ml], and 2% fungizone (250 mg/ml stock). If the samples are to be inoculated within 1–2 days they may be held at 4°C, but, if kept for longer, they should be stored at −70°C or below. Preferably, samples should also be transported on ice.

Only one sample is processed at a time. The liquid is expelled from the swab by squeezing with forceps, which is then disposed of suitably. Further antibiotics may be added if samples appear to be heavily contaminated with bacteria. The remainder of the fluid is stored at −70°C. Samples treated with antibiotics are allowed to stand on ice for 30–60 minutes and are then centrifuged at 1500 g for 15 minutes to remove bacteria and debris; the supernatant fluids are used for inoculation. Filtration of samples is not advised as influenza virus may adsorb on to the filter and be lost from the sample.

1. Identification of the agent

Isolation of infectious virus may be carried out in embryonated hens' eggs or cell cultures and EI nucleic acid can be identified by PCR. Traditionally, eggs have been preferred for isolation of equine influenza. Comparison of H3N8 viruses isolated in eggs and Madin–Darby canine kidney (MDCK) cells indicated that MDCK cells are capable of selecting variant viruses that are not representative of the predominant virus in clinical specimens (11). However, in recent years some viruses have been successfully isolated in MDCK cells but not in eggs and selection of variants has occurred as a result of culture in eggs (24), therefore isolation should be attempted using both substrates. PCR techniques have been described for the identification of equine influenza virus from clinical specimens and for molecular epidemiology (8, 13, 23).

In situations where laboratory facilities for virus isolation are unavailable, influenza virus antigen in nasal secretions may be detected directly by an antigen-capture ELISA for the H3N8 virus using a monoclonal antibody (MAb) against the nucleoprotein (3, 14). Commercial self-contained kits for detecting human influenza are available and have been shown to detect equine influenza antigen (2). This approach provides a rapid result on which management decisions may be based. It should not be used in preference to virus isolation, as it is essential that new viruses be isolated and sent to reference laboratories for characterisation as part of the surveillance programme to monitor antigenic drift and emergence of new viruses and to provide isolates for inclusion in updated vaccines. Positive ELISA results are useful in the selection of samples if resources are limited or for the selection of specimens to be sent to a reference laboratory for virus isolation attempts.

a) Virus isolation in embryonated hens' eggs

Fertile eggs are set in a humid incubator at 37–38°C and turned twice daily; after 10–11 days, they are examined by candling and live embryonated eggs are selected for use. The area above the air sac is cleansed with alcohol and a small hole is made through the shell. Several eggs/sample are inoculated (0.1 ml) in the amniotic cavity with no additional dilution of the sample (sample may also be diluted). The syringe is withdrawn approximately 1 cm and a further 0.1 ml is inoculated into the allantoic cavity. Alternatively, the sample may be inoculated into the allantoic cavity alone through a second hole drilled just below the line of the air sac. The hole(s) is/are sealed with wax or Sellotape, and the eggs are incubated at 34–35°C for 3 days. The embryos that die within 24 hours following inoculation should be discarded. The eggs that contain embryos that die more that 24 hours after inoculation or contain live embryos after 3 days are examined for the presence of EI virus.

The eggs are transferred to 4°C for 4 hours or overnight to kill the embryos and to reduce bleeding at harvest. The shells are disinfected, and the amniotic and/or allantoic fluid is harvested by pipette, each harvest being kept separate. These are tested for haemagglutination (HA) activity by mixing in equal volumes (0.025 ml) with chicken red blood cells (RBCs) (0.5% [v/v] packed cells in PBS) in V- or U-bottomed microtitre plates or 0.4% guinea-pig RBCs (0.4% [v/v] packed cells in PBS) in V- or U-bottomed plates. If chicken RBCs are used, the plates may be read by tilting to 70° so that non-agglutinated cells ‘stream’ to the bottom of the well. Non-agglutinated guinea-pig cells appear as a button at the bottom of the well and may take longer to settle. If there is no HA activity, aliquots of each harvest are pooled and passed into further eggs. All HA positive samples are divided into aliquots and stored at −70°C; one aliquot is titrated for HA immediately. If the HA titre is 1/16 or more, the isolate is characterised immediately.

If titres are low, positive samples should be passaged. Care should be taken to avoid generation of defective interfering particles by prediluting the inoculum 1/10, 1/100, 1/1,000. Positive samples arising from the highest dilution should be selected as stocks for storage. It may be necessary to undertake as many as five passages to isolate the virus, particularly from vaccinated horses. If virus has not been recovered by the fifth passage, further passages are unlikely to be successful.

b) Virus isolation in cell cultures

Cultures of the MDCK cell line (MDCK, ATCC CCL34) may be used to isolate equine influenza viruses. The cells are grown to confluence in tubes and then infected in triplicate with 0.25–0.5 ml of each sample, processed as described above. Prior to inoculation, the cell monolayer is washed at least once with tissue culture medium containing trypsin (2 µg/ml) without serum. The cultures are maintained with serum-free
medium containing 0.5–2 µg/ml trypsin (treated with TPCK [L-1-tosylamine-2-phenylethyl chloromethyl ketone] to remove chymotrypsin, available pretreated, e.g. from Sigma), and examined daily for evidence of cytopathic effects (CPE). If positive, or after 7 days in any case, the supernatant fluids are tested for HA. Fluids with titres of ≥1/16 are characterised immediately. Negative fluids and those with titres <1/16 are repassaged up to five passages.

Alternatively, the cells are screened for evidence of haemadsorption (HAD). This procedure detects expression of viral antigens at the cell surface. The medium is removed from the cultures and the tubes are washed with PBS. One or two drops of a 50% suspension of chicken or guinea-pig RBCs are added, the tubes are rotated carefully, and kept at room temperature (23°C ±2°C) for 30 minutes. Unbound RBCs are washed off with PBS, and the cultures are examined microscopically for evidence of HAD.

c) Haemagglutinin typing

The HA subtype of new isolates of equine influenza viruses is best determined by haemagglutination inhibition (HI; Section B.2.a) using H7N7- and H3N8-specific antisera. Isolates may first be treated with Tween 80/ether, which destroys viral infectivity and reduces the risk of cross-contamination. In the case of H3N8 viruses particularly, this treatment enhances the HA activity (12). However, treatment with Tween 80/ether may increase the variability of the results obtained. Standard antigens must be titrated in parallel with tests to identify viruses and should include H7N7 strains (e.g. A/eq/Prague/56, A/eq/Newmarket/77) and H3N8 strains (e.g. A/eq/Newmarket/2/93, and A/eq/Kentucky/94). Virus strains may be obtained from OIE Reference Laboratories (see Table given in Part 3 of this *Terrestrial Manual*). Additionally, recent isolates from the same geographical area should be included if available. The standard antigens should be treated with Tween 80/ether to avoid cross-contamination. Test antigens and standard antigens are always back-titrated to confirm their antigen content.

New isolates of equine influenza viruses may be further characterised by HI using strain-specific antisera. The species in which antibodies are raised will influence the cross-reactivity of the antiserum, with ferrets providing the most strain-specific antibody (17).

All isolates should be sent immediately to an International Reference Laboratory designated by OIE or the World Health Organization (WHO) for inclusion in the strain surveillance programme to monitor antigenic drift and emergence of new viruses.

d) Neuraminidase typing

Typing of neuraminidase requires specific antisera and no routine technique is available. Typing can be done using specific PCR primers.

e) Polymerase chain reaction

PCR assays are increasingly used for the detection of equine influenza genome in nasal secretions. Real-time Light Cycler reverse transcription PCR technology has been shown to be more sensitive for the detection of positive samples than virus culture in eggs or detection of nucleoprotein using ELISA technology (26). Although genetic sequence of isolates can also be derived from PCR assays it remains essential to isolate infectious virus in order to examine antigenic properties of new isolates and antigenic drift.

2. Serological tests

Infections are detected by performing serological tests on paired sera to show a rise in antibody. These tests should be carried out whether virus isolation has been attempted or not. Two simple methods exist, HI and single radial haemolysis (SRH), each equally efficient and widely used. The complement fixation (CF) test can also be applied, but is not in general use. Both of the paired serum samples should be tested together at the same time to minimise variability. The standard antigens are described above (Section B.1.c). If available, isolates from recent cases should be included. Freeze-dried post-infection equine antisera to A/eq/Newmarket/77 (H7N7), A/eq/Newmarket/1/93 (‘American-like’ H3N8) and A/eq/Newmarket/2/93 (‘European-like’ H3N8) and an influenza-negative equine serum, are available from the OIE Reference Laboratory, Newmarket (see Table given in Part 3 of this *Terrestrial Manual*). These sera have been assigned SRH values through an international collaborative study and can be used as primary reference sera for this assay.

a) Haemagglutination inhibition test

The antigen is first treated with Tween 80/ether in order to increase the sensitivity of the test, particularly for H3N8 viruses. The test is best done in microtitre plates using the appropriate dilution equipment. A macrotest may be used, for which antigen is diluted to a final HA titre of 1/8 per well and the volumes for PBS, sera and antigen are 0.5 ml. Sera are pretreated to remove nonspecific haemagglutinins, and
inactivated at 56°C for 30 minutes. Pretreatments include the use of one of the following: (a) kaolin and RBCs absorption, (b) potassium periodate, or (c) \textit{Vibrio cholerae} receptor-destroying enzyme. Potassium periodate or \textit{V. cholerae} receptor-destroying enzyme is the treatment of choice. The treated sera are diluted in PBS, a standard dose of antigen is added (HA titre of 1/4 per well for microtitration assay), and these are kept at room temperature (23°C ± 2°C) for 30 minutes. After gentle mixing, RBCs are added and the test is read 30 minutes later. The HI titres are read as the highest dilution of serum giving complete inhibition of agglutination. Either chicken RBCs (1% [v/v] packed cells) in V-bottomed microtitre plates or guinea-pig RBCs (0.5% [v/v] packed cells) in V- or U-bottomed plates may be used. If chicken RBCs are used, the plates may read by tilting to 70° so that non-agglutinated cells ‘stream’ to the bottom of the well. Non-agglutinated guinea-pig cells appear as a ‘button’ in the bottom of the well and may take longer to settle. Titre increases of fourfold or more between paired sera indicate recent infection (28).

- **Tween 80/ether treatment of the virus**
  i) To 39.5 ml of infective allantoic fluid, add 0.5 ml of a 10% (v/v) suspension of Tween 80 in PBS to give a 0.125% (v/v) concentration of Tween 80.
  ii) After mixing gently at room temperature for 5 minutes, add 20 ml of diethyl ether to give a final concentration of 33.3% by volume, and mix the suspension well at 4°C for 15 minutes.
  iii) After allowing the layers to separate by standing, remove the aqueous layer containing the disrupted virus particles to a glass bottle with a loose lid and allow the excess ether to evaporate off overnight (12).
  iv) Store treated virus in aliquots at –70°C.

- **Titration of haemagglutination**
  i) Add 25 µl of PBS to all wells in a row of a microtitre plate.
  ii) Add 25 µl of virus to first well (dilution = 1/2) and titrate through, leaving the last well as a control.
  iii) Add an extra 25 µl of PBS to all wells.
  iv) Add 50 µl of RBCs to all wells. Leave at 22°C (±2°C) for 30 minutes. The HA titre is taken as the last virus dilution giving partial HA.

- **Potassium periodate pretreatment of sera**
  i) Mix one volume (150 µl) of serum with two volumes (300 µl) of freshly prepared 0.016 M potassium periodate (0.38 g in 100 ml PBS), and leave at 22°C (±2°C) for 15 minutes.
  ii) Add a further one volume of 3% glycerol in PBS to neutralise any excess periodate solution, mix and leave at room temperature (23°C ± 2°C) for 15 minutes.
  iii) Inactivate in a 56°C water bath for 30 minutes.

- **Test procedure**
  i) Dispense 25 µl of PBS to all wells of a microtitre plate.
  ii) Add serum (25 µl) to the first well of a row of 12, and titrate through, leaving the last well as a control.
  iii) Dilute the antigen to give a dose of 4 HA units (4 × minimum agglutinating dose, i.e. titre/4).
  iv) Add 25 µl to each well, and incubate at 22°C (±2°C) for 30 minutes.
  v) Add 50 µl of RBCs to each well. Leave at 22°C (±2°C) for 30 minutes.
  vi) The plates may be read by tilting to 70° so that non-agglutinated cells ‘stream’ to the bottom of the well. No agglutination is recorded as a positive result.

b) **Single radial haemolysis**

In this test, viral antigens are coupled to fixed RBCs that are suspended in agarose containing guinea-pig complement (C'). Wells are punched in the agarose and filled with test sera. Influenza antibodies and C' lyse the antigen-coated RBCs, resulting in a clear, haemolytic zone around the well; the size of this zone is directly proportional to the level of strain-specific antibody in the serum sample (16, 25, 27).

Special immunodiffusion plates (MP Biomedical) may be used for the assay, but simple Petri dishes are also suitable. Sheep RBCs collected into Alsever’s solution are washed three times. The C’ can be obtained commercially, or normal guinea-pig serum can be used. The antigens are allantoic fluids or purified preparations; the strains used are the same as for the HI tests. The viruses are coupled to RBCs by
potassium periodate or by chromic chloride. The coupled antigen/RBCs preparations are mixed with C', together with a 1% solution of agarose (low melting grade) in PBS. Care must be taken to ensure that the temperature is not allowed to rise above 42°C at any time. The mixture is poured into plates and left to set. Wells of 3 mm in diameter and 12 mm apart are punched in the solidified agarose, at least 6 mm from the edge of the plates. Such plates may be stored at 4°C for 12 weeks. Plates are prepared for each antigen.

Sera are inactivated at 56°C for 30 minutes, but no further treatment is necessary. Paired sera should be assayed on the same plate. As a minimum, a subtype-specific antiserum should be included as a control serum in one well on each plate. All sera are tested in a control plate containing all components except virus to check for nonspecific lysis. Alternatively, an unrelated virus, such as A/PR/8/34 (H1N1), may be used in the control plate. Sera that show haemolytic activity for sheep RBCs must be pre-absorbed with sheep RBCs. Zones of lysis should be clear and not hazy or translucent. All clear zones should be measured and the area of haemolysis calculated.

- Preparation of reagents
  i) Saline/HEPES: 0.85% NaCl (4.25 g/500 ml); 0.05 M HEPES (N-2-hydroxyethylpiperazine, N-2-ethanesulphonic acid; 5.95 g/500 ml); and 0.02% sodium azide. Make to pH 6.5 with NaOH.
  ii) Saline/HEPES/BSA: as saline/HEPES with 0.2% (w/v) bovine serum albumin (BSA).
  iii) CrCl₃ stock solution (2.25 M) 6 g/10 ml: Make fresh 1/400 dilution in 0.85% NaCl for each assay.
  iv) PBS (London)/PBS ‘A’: NaCl (10.00 g); KCl (0.25 g); Na₂PO₄ (1.45 g); KH₂PO₄ (0.25 g); and Na azide (0.20 g). Make up to 1 litre with distilled water.
  v) Agarose in PBS: Place flask containing PBS ‘A’ on a stirrer. Slowly add 10 g agarose to the stirring solution. Liquefy in a pressure cooker. Dispense into glass bottles for storage at 22°C (±2°C).
  vi) Virus antigen: Allantoic fluid containing infectious virus is harvested and stored at –70°C. A short titration curve determines the optimum ratio of virus antigen to RBCs to be used when preparing sensitised sheep RBCs. The H7N7 influenza strains always produce clear zones; the H3N8 strains sometimes produce hazy zones, in which case it is necessary to concentrate the virus by centrifugation.
  vii) Sheep blood: Collect blood into an equal volume of Alsever’s solution and store at 4°C. It may be necessary to test bleed several sheep, as characteristics of RBCs from individual sheep vary. Keep the blood for 2 days before use, then it may be usable for up to 3 weeks, providing sterility is maintained.
  viii) Complement: Use commercially available guinea-pig complement or collect serum from young guinea-pigs of 300–350 g body weight and store in small volumes at –70°C. For use, thaw in cold water and hold at 4°C prior to mixing.
  ix) Treatment of sera: Use undiluted sera heat inactivated at 56°C for 30 minutes. Avoid repeated freeze–thaw cycles.

- Test procedure
  i) Wash sheep RBCs at least three times in saline/HEPES.
  ii) Prepare an appropriate volume of 8% RBCs (v/v packed cells) in saline/HEPES, having first calculated the number of plates required and allowing 1 ml per 6 × 11 cm immunoplate and 1–2 ml extra.
  iii) Add a predetermined volume₁ of virus antigen to the 8% RBCs solution. Hold the mixture at 4°C for 10 minutes. Haemagglutination may be observed.
  iv) SLOWLY add CrCl₃ (1/400 in 0.85% NaCl) at half the total volume of virus/RBCs suspension. Hold at 22°C (±2°C) for 5 minutes with occasional mixing.
  v) Sediment the sensitised RBCs by centrifugation at 1500 g for 5 minutes.
  vi) Gently resuspend in saline/HEPES/BSA and centrifuge at 1500 g for 5 minutes.
  vii) Resuspend RBCs to an 8% suspension in PBS ‘A’.

During the sensitisation process, melt the agarose in a pressure cooker. Shortly before use, pipette 7.8 ml volumes to Universal bottles and retain at 42°C. Check that the agar has cooled to 42°C before use.

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₁ Prepare three plates by adding 0.6, 1.2 or 1.8 ml of virus antigen to 2 ml RBCs. Add 1.3, 1.6 and 1.9 ml CrCl₃ respectively and resuspend to 2 ml in PBS ‘A’. Optimum volume of virus antigen is that which results in the largest and clearest zones with appropriate reference serum.
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• Preparation of plates
  i) Add 0.9 ml of virus-sensitised sheep RBCs to 7.8 ml of agarose (42°C). Mix quickly, but gently.
  ii) Add 0.3 ml of undiluted guinea-pig serum. Mix again and pour into immunoplates on a levelling table. Allow to set and air dry without a lid for 5 minutes.
  iii) Place lids on plates and store at 4°C in a humid box until used.
  iv) Prepare control plates with unsensitised cells or cells sensitised with an unrelated virus. Batches of prepared plates can be stored for several weeks.
  v) Punch 3 mm holes in the set gels to a prepared template, allow for 16 test sera and a positive control serum. On antigen control plates, prepare five rows of eight wells.
  vi) Pipette 10 µl of heat-inactivated (56°C for 30 minutes) test sera and a positive control serum to appropriate wells. Incubate at 34°C for 20 hours in a humid box.
  vii) Measure zone diameters, and calculate areas of haemolysis after the area of the well has been deducted.

• Interpretation of the results
  For results to be valid, positive and negative control sera should give results consistent with those expected on the basis of prior experience. Areas of haemolysis for the control sera should be clear and intra-laboratory variation should be no more than 5% for the control serum. Results may be expressed as mm² or as a ratio of the control serum value. Sera giving positive results in the control plate should be adsorbed with sheep RBCs. For diagnostic purposes, acute and convalescent sera should be tested in duplicate on the same plate. Increase in zone areas produced by convalescent serum compared with acute serum is evidence of infection. The increase in area deemed to be significant depends on the reproducibility of the test within the laboratory, but should be equivalent to a twofold or more increase in antibody concentration. This area can be calculated from a standard curve generated from a dilution series of a standard antiserum.

C. REQUIREMENTS FOR VACCINES AND DIAGNOSTIC BIOLOGICALS

Equine influenza virus vaccines consist of inactivated whole viruses or their subunits, with or without adjuvant. Live attenuated virus and canary pox vectored vaccines have recently become available commercially in some countries. Requirements for such vaccines may be anticipated to differ in some particulars from the following, which are appropriate to inactivated vaccines.

Immunity generated by inactivated vaccines administered via the intramuscular route is reliant on stimulation of circulating antibody to the HA, which neutralises virus; some products have been shown to stimulate antibody in respiratory secretions. Critically the integrity and conformation of the HA should be maintained during inactivation procedures to ensure that the vaccine stimulates appropriate neutralising antibody. This can be tested by use of an immunological assay such as SRD (single radial diffusion), which measures immunologically active HA capable of reacting with specific anti-HA antibodies. The immunogenicity of the vaccine can be confirmed by measurement of HA antibody stimulated in small animal models or the target species.

Although canary pox-vectored vaccines, also administered via the intramuscular route, have the potential for stimulating cytotoxic T cell (CTL) immunity through their ability to present antigen via endogenous processing, they currently include only the HA gene, which is not known to be a CTL target. Their action is therefore reliant on stimulation of antibody to HA. Infectious titre of the recombinant canary pox virus carrying equine influenza HA genes is used as an in vitro measure of potency and immunogenicity is assessed by measurement of antibody stimulated in the target species.

Antibody to HA as measured by SRH, stimulated by inactivated whole virus, subunit or canary pox-vectored vaccine correlates well with protection against infection in an experimental challenge model system (20). In contrast, a cold-adapted temperature-sensitive mutant used as a live attenuated vaccine replicates in the upper respiratory tract and does not stimulate high levels of circulating antibody to HA but nevertheless provides protection against challenge infection. Immunity is presumed to be mediated through mucosal or cellular responses rather than circulating antibody. As with the vectored vaccine, in-process control testing is reliant on measurement of the infectious virus titre.

Guidelines for the production of veterinary vaccines are given in Chapter 1.1.8 Principles of veterinary vaccine production. The guidelines given here and in Chapter 1.1.8 are intended to be general in nature and may be supplemented by national and regional requirements.
1. Seed management

a) Characteristics of the seed

An ongoing surveillance programme by OIE and WHO Reference Laboratories aimed at providing information on suitable vaccine strains is being co-ordinated by the OIE Reference Laboratory (Newmarket) (21). Recommendations on vaccine strains made by the Expert Surveillance Panel will be published annually in the OIE Bulletin.

H7N7: Many vaccines still contain an H7N7 strain. However, the Expert Surveillance Panel has recommended that the H7N7 component should be omitted as there have been few reports of infections with this subtype have been substantiated during the past 20 years.

H3N8: Antigenic variants of H3N8 viruses co-circulate (5) and it is important to include a strain or strains that are epidemiologically relevant as recommended by the OIE Expert Surveillance Panel, as published in the OIE Bulletin.

b) Method of culture

Virus strains may be obtained from OIE Reference Laboratories (see Table given in Part 3 of this Terrestrial Manual). Viruses selected as vaccine strains should be described in terms of origin and passage history. The strains are propagated in the allantoic cavity of 10-day-old embryonated hens' eggs or cell cultures, such as MDCK. All manipulations must be conducted separately for each strain. Viral growth is monitored by HA tests. Passaged virus is identified by serological tests, such as HI or SRH or by PCR using specific primers. If vaccine virus is grown in cell culture, antigenic studies with ferret sera and MAb should be undertaken to ensure that variant viruses have not been selected during passage to prepare master and working seed viruses. Master and working seed viruses are divided into aliquots and stored in freeze-dried form at –20°C or at –70°C following testing for extraneous agents. Records of storage conditions should be maintained.

The master seed lot of each vaccine strain selected should be processed at one time to assure a uniform composition, tested for extraneous agents, and fully characterised. Antisera or MAbs for use in HI tests to characterise vaccine strains may be obtained from OIE and WHO Reference Laboratories.

Working seed lots are derived from a master seed lot and should be of uniform composition, free from extraneous agents, and fully characterised. Aliquots of the working seed are used for production of vaccine.

Master and working seed lots should be prepared in specific pathogen free eggs or, as a minimum, in eggs derived from a healthy flock.

If MDCK cells are used to propagate vaccine virus, master cell lines should be established and stored in liquid nitrogen, and should be tested for freedom from extraneous agents according to National Control Authority Guidelines.

Examination of seed viruses for extraneous agents including mycoplasmas and other equine viruses should be performed by appropriate techniques, including inoculation of susceptible tissue cultures and examination for cytopathic effect or application of fluorescent antibodies for antigen detection.

The presence of other common equine respiratory pathogens, e.g. equine herpesviruses 1, 2, 4, equine picornaviruses, equine viral arteritis, and equine adenoviruses, should be specifically excluded.

The absence of bacteria should be confirmed by standard sterility tests and toxicity tests in small animals.

c) Validation as a vaccine

For each vaccine strain, a prototype batch should be prepared to establish its suitability as a vaccine strain, i.e. purity and safety should be confirmed by standard techniques. The ability of seed-lot viruses to grow to high titre and generate sufficient antigenic mass to stimulate adequate antibody responses in the target species, should be confirmed.

Additionally, vaccine virus derived in MDCK cells should be fully characterised to ensure that antigenic variants have not arisen during the culture process, such that the vaccine virus is no longer representative of the original isolate.
2. Method of manufacture

Production is based on a seed-lot system that has been validated with respect to the characteristics of the vaccine strains. Where eggs are used, each strain of virus is inoculated separately into the allantoic cavity of 9–11-day-old embryonated hens’ eggs from a healthy flock. The eggs are incubated at a suitable temperature for 2–3 days, and the allantoic fluid is collected. Alternatively, each strain is inoculated separately into MDCK cell cultures. The viral suspensions of each strain are collected separately and inactivated. If necessary, they may be purified. Suitable adjuvants and antimicrobial preservatives may be added.

Monovalent virus pools should be inactivated as soon as possible after their preparation, by a method approved by the National Control Authority. If formalin (37% formaldehyde) or beta-propiolactone (2-oxetanone) is used, the concentration by volume should not exceed 0.2%. Ideally, pools should be held at 4°C and should be inactivated within 5 days of harvest. Inactivation of the vaccine must be demonstrated. A suitable method consists of inoculating 0.2 ml of undiluted monovalent pool and 1/10 and 1/100 dilutions of the monovalent pool into the allantoic cavities of groups of fertile eggs (10 eggs in each group), and incubating the eggs at 33–37°C for 3 days. At least 8 of the 10 eggs should survive at each dosage level. A volume of 0.5 ml of allantoic fluid is harvested from each surviving egg. The fluid harvested from each group is pooled, and 0.2 ml of each of the three pools is inoculated, undiluted, into a further group of 10 fertile eggs. Haemagglutinin activity should not be detected in these new groups of eggs. In some countries, the requirement that 80% of the eggs should survive during incubation may be impossible to satisfy, in which case the National Control Authority should then specify a modified requirement to be satisfied. Before inactivation, samples should be collected for bacterial and fungal sterility tests.

Monovalent material may be concentrated and purified by high-speed centrifugation or other suitable methods approved by the National Control Authority, either before or after the inactivation procedure. It is important to concentrate and purify the virus under optimum conditions, e.g. temperatures that preserve its antigenic properties.

The monovalent virus pool shall be shown not to contain viable influenza virus when tested by inoculation of embryonated hens’ eggs, by a method approved by the National Control Authority. Alternatively, the satisfactory inactivation can also be demonstrated by inoculating monolayers of MDCK cells.

3. In-process control

Relevant in-process controls should be applied before and after inactivation and before and after concentration and purification.

In-process controls include: (a) identity of virus strains (tested by HI); (b) sterility; (c) virus titre; (d) haemagglutinin content (tested by chicken RBCs agglutinating units, CCA [chick cell agglutination]); and (e) immunologically active HA (tested by SRD or another suitable immunochemical method).

• Single radial diffusion test

SRD is a reliable method for measuring immunologically active HA in terms of µg HA, and is used routinely for potency testing human influenza vaccines (32).

The potency of inactivated equine influenza vaccine depends on the concentration of immunologically active haemagglutinin (22, 30, 31).

Assessment of the antigenic content of the vaccine by CCA alone may be misleading, as the sensitivity of this assay is a reflection of the ability of virus strains to agglutinate RBCs. Disruption of virus may lead to an apparent increase in HA as measured by CCA. The CCA assay does not provide a measure of the antigenic properties of the HA (HA may retain its properties to bind to RBCs while losing its ability to stimulate antibody).

The composition of some equine influenza vaccines is unusual in that products may contain more than one variant of the H3N8 subtype. In this situation, it is not possible to judge the potency of individual H3N8 components from serological tests performed on sera collected from horses or small animals vaccinated with the final product, because of cross-reactivity between the two isolates of the same subtype. Thus, it is important that a reliable method, such as SRD, be used to measure the potency of individual components before and after inactivation and prior to mixing and formulation with adjuvant.

In the SRD test, virus preparations are compared with a calibrated reference preparation of known HA content. Antigens are allowed to diffuse through a gel containing an antiserum specific for a particular HA. The distance diffused by the antigen before precipitation by the antibody incorporated in the gel is directly related to the concentration of haemagglutinin in the antigen preparations (19).
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Standard reagents for SRD testing are available from the WHO International Laboratory for Biological Standards. Reagents for A/eq/Prague/56 (H7N7), and the H3N8 strains A/eq/Miami/63, A/eq/Kentucky/81, A/eq/Newmarket/1/93 (American lineage) and A/eq/Newmarket/2/93 (European lineage) are currently available.

4. Batch control

a) Sterility

Tests for sterility and freedom from contamination of biological materials may be found in Chapter 1.1.9.

b) Safety

i) Using no fewer than three horses, each horse is inoculated intramuscularly (at two different sites) with the dose of vaccine specified by the manufacturer; these inoculations are repeated 2–4 weeks later. The animals are kept under observation for 10 days after the second set of injections. No abnormal local or systemic reaction should ensue.

ii) If vaccine is to be used in mares, safety should be demonstrated by giving two doses of vaccine to no fewer than two pregnant mares at the prescribed interval within the trimester for which the vaccine is recommended.

c) Potency

Following mixing of viral antigens and adjuvants, aliquots should be potency tested in vivo using horses and guinea-pigs or a suitable alternative immunochemical assay. Adjuvants cause interference in quantitative in-vitro tests, such as CCA and SRD, although SRD may be used on the final product as a qualitative assay to demonstrate the presence of antigen for each vaccine strain. For repeated batch tests, only guinea-pigs or a suitable alternative immunochemical assay are used, subject to agreement of the National Control Authority.

i) Serological responses in horses

For a valid in-vivo potency test, naive seronegative horses must be selected for vaccination. Young horses or ponies (not less than 6 months old) should be screened for the presence of antibody using H7N7 and H3N8 viruses including recently isolated viruses relevant to the area in which the horses were reared. If HI tests are used for screening, H3N8 viruses should be treated with Tween 80/ether to maximise the sensitivity of the test. Alternatively, SRH may be used to establish the seronegative status of animals.

To test a vaccine for efficacy in horses, inject a volume corresponding to one vaccine dose by the recommended route into each of five susceptible seronegative horses. After the period recommended between the first and second doses, as stated on the label, a volume of vaccine corresponding to the second dose of vaccine is injected into each horse.

Three blood samples are collected from each animal, the first at the time of the first vaccination, the second 1 week after the first vaccination, and the third 2 weeks after the second vaccination.

The serological assay used to measure the antibody response to the viruses contained in the vaccine must be standardised for a valid in-vivo potency test, therefore the SRH assay (see Section B.2.b) is preferred. Standard sera for the quality control of equine influenza vaccines are available from the European Pharmacopoeia. These sera should be tested in parallel with the test sera to ensure that the test is valid with respect to sensitivity; the values obtained should not vary by more than 20% from the SRH values assigned in an international collaborative study (18). Due to poor repeatability and reproducibility of the HI test, no HI titre could be assigned to these sera.

The antibody value measured by SRH should not be less than 150 mm². This is higher than the value required in the European Pharmacopoeia Monograph for inactivated equine influenza vaccines (85 mm²) as this value is not considered to be protective. If the value found for any horse after the first vaccination indicates that there has been an anamnestic response, the result is not taken into account. A supplementary test is carried out, as described above, replacing the horses that showed an anamnestic response with an equal number of new animals.

If the HI test is used, the antibody titre of each serum taken after the second vaccination in each test should not be less than 1/64 (calculated for the original serum, taking into account the predilution of 1/8). Alternatively, the antibody levels stimulated by the vaccine under test should be shown to be at

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2 National Institute for Biological Standards and Control, Blanche Lane, South Mimms, Potters Bar, Hertfordshire EN6 3QG, UK.

3 Serum to A/eq/Newmarket/1/77 (Catalogue number E0850010), A/eq/Newmarket/1/93 (E0850021) and A/eq/Newmarket/2/93 (E0850022).
least equal to the antibody levels stimulated by a standard vaccine tested in parallel that has been shown previously to protect horses against challenge infection.

ii) Challenge studies in horses

It may be desirable in certain cases to undertake challenge studies in horses to demonstrate potency, particularly if vaccines are being assessed for their ability to protect against antigenically dissimilar viruses. Challenge studies may be carried out by exposing six vaccinated horses/ponies to an aerosol of virulent influenza virus no fewer than 2 weeks after the second dose of vaccine. Comparisons of clinical signs, virus excretion and serological responses are made with a group of no fewer than four unvaccinated control animals challenged at the same time (19, 20). The timing of the challenge procedure will reflect the claims to be made on the data sheet regarding duration of immunity.

If tests for potency in horses have been carried out with satisfactory results on a representative batch of vaccine, these tests may be omitted as a routine control on other batches of vaccine prepared using the same seed-lot system, subject to agreement by the National Control Authority.

iii) Serological responses of guinea-pigs

Inject each of no fewer than five guinea-pigs free from specific antibodies with one vaccine dose. Collect blood samples 21 days later, and test the serum by SRH or HI (see Sections B.2.a and B.2.b). Perform the tests of each serum using, respectively, the antigen(s) prepared from the strain(s) used in the production of the vaccine. The antibody titre of each serum in each test should not be less than the titre stimulated by a standard vaccine that has been shown to stimulate protective levels of antibody in horses.

d) Duration of immunity

Where claims for duration of immunity are made on the data sheet, these should be supported with data on the duration of protective levels of antibody maintained in horses vaccinated according to the recommended schedule. Antibody levels quoted as protective should be validated in challenge studies (see Section C.4.c.ii) or by comparison with published reports.

e) Stability

Vaccines should be stored at 5±3°C and protected from light. The shelf life quoted on the data sheet should be demonstrated by testing the potency of aliquots over time using the guinea-pig potency test (see Section C.4.c.iii).

f) Preservatives

Preservatives are not normally included.

g) Precautions (hazards)

The contents of each opened vial should be used within 1 hour of opening. Aseptic precautions should be observed during administration, and only healthy horses should be vaccinated. Occasionally, transient local and/or general reactions may occur, and rest may be advisable for 24–48 hours after vaccination.

5. Tests on the final product

a) Safety

Tests are performed as described in Section C.4.b.i. Once safety has been demonstrated on a prototype batch, safety testing in pregnant mares may be omitted for routine testing of subsequent batches of the final product.

b) Potency

See Section C.4.c.iii. As a minimum, serological testing in guinea-pigs should be performed on each batch of the final product.

c) Maintaining epidemiologically relevant strains in vaccines

To enable vaccine manufacturers to respond quickly to recommendations from the Expert Surveillance Panel to update vaccine strains, the Committee for Veterinary Medicinal Products for the European Agency for the Evaluation of Medicinal Products has developed a fast-track licensing system to be used when vaccine strains are updated (9).
REFERENCES


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**NB:** There are OIE Reference Laboratories for Equine influenza Table in Part 3 of this *Terrestrial Manual* or consult the OIE Web site for the most up-to-date list: www.oie.int.
CHAPTER 2.5.8.

EQUINE PIROPLASMOsis

SUMMARY

Equine piroplasmosis is a tick-borne protozoal disease of horses, mules, donkeys and zebra. The aetiological agents are blood parasites named Theileria equi and Babesia caballi. Theileria equi was previously designated as Babesia equi. Infected animals may remain carriers of these parasites for long periods and act as sources of infection for ticks, which act as vectors. The parasites are found inside the red blood cells of the infected animals.

The introduction of carrier animals into areas where tick vectors are prevalent can lead to an epizootic spread of the disease.

Identification of the agent: Infected horses can be identified by demonstrating the parasites in stained blood or organ smears during the acute phase of the disease. Romanovsky-type staining methods, such as Giemsa, give the best results. In carrier animals, low parasitaemias make it extremely difficult to detect parasites, especially in the case of B. caballi infections, although they may sometimes be demonstrated by using a thick blood smear technique.

Paired merozoites joined at their posterior ends are a diagnostic feature of B. caballi infection. The parasites in the erythrocytes measure 2 × 5 µm. The merozoites of T. equi are less than 2–3 µm long, and are pyriform, round or ovoid. A characteristic of T. equi is the arrangement of four pear-shaped merozoites forming a tetrad known as a ‘Maltese cross’.

Molecular techniques for the detection of T. equi and B. caballi based on species-specific polymerase chain reaction (PCR) assays, targeting the 18S rRNA gene, have been developed and continue to expand. These tests have been shown to be highly specific and sensitive and promise to play an increasing role in the diagnosis of infections.

Serological tests: Infections in carrier animals are best demonstrated by testing their sera for the presence of specific antibodies.

Currently, the indirect fluorescent antibody (IFA) test and the competitive enzyme-linked immunosorbertent assay (C-ELISA) are the primary tests used for qualifying horses for importation. The complement fixation (CF) test, for many years the primary test, has been replaced by the IFA and C-ELISA. These tests have proven to be more effective at detecting long-term infected animals and animals treated with antiparasitic drugs; these animals may be CF negative but still be infected. The IFA test and C-ELISA have been shown to be highly specific for each of the two species of piroplasmosis agents involved. Indirect ELISAs may also be used to detect antibodies to both species in infected horses, although cross-reactions between T. equi and B. caballi occur. Application of recombinant T. equi and B. caballi merozoite proteins in diagnostic assays appear to be very promising in the accurate determination of equine piroplasmosis infection.

Requirements for vaccines and diagnostic biologicals: There are no biological products available.

A. INTRODUCTION

Equine piroplasmosis is a tick-borne protozoal disease of horses, mules, donkeys and zebra. The aetiological agents of equine piroplasmosis are Theileria equi and Babesia caballi. Twelve species of Ixodid ticks in the genera Dermacentor, Rhizophus and Hylomma have been identified as transstadial vectors of B. caballi and T. equi, while eight of these species were also able to transmit B. caballi infections transovarially (7, 36, 44, 45).
Infected animals may remain carriers of these blood parasites for long periods and act as sources of infection for tick vectors.

The parasites occur in southern Europe, Asia, countries of the Commonwealth of Independent States, Africa, Cuba, South and Central America, and certain parts of the southern United States of America. *Theileria equi* has also been reported from Australia (but, apparently never established itself in this region), and is now believed to have a wider general distribution than *B. caballi*.

During the life cycle of *Babesia*, initially the sporozoites invade red blood cells (RBCs) where they transform into trophozoites (10, 37). In this situation the trophozoites grow and divide into two round, oval or pear-shaped merozoites. The mature merozoites are now capable of infecting new RBCs and the division process is then repeated.

*Babesia caballi*: the merozoites in the RBCs are pear-shaped, 2–5 µm long and 1.3–3.0 µm in diameter (27). The paired merozoites joined at their posterior ends are considered to be a diagnostic feature of *B. caballi* infection (32).

*Theileria equi*: the merozoites of this organism are relatively small, less than 2–3 µm long (27), and are pyriform, round or ovoid. A characteristic of *T. equi* is the arrangement of four pear-shaped merozoites, measuring about 2 µm in length, forming a tetrad known as the ‘Maltese cross’ arrangement (14).

In *T. equi* infection it has been shown that sporozoites inoculated into horses via a tick bite invade the lymphocytes (41). The sporozoites undergo development in the cytoplasm of these lymphocytes and eventually form *Theileria*-like schizonts. Merozoites released from these schizonts enter RBCs. The taxonomic position of *T. equi* has been controversial and only relatively recently has it been redescribed as a *Theileria* (33). Further support for the close relation with *Theileria* spp. also comes from the homology found between 30 and 34 kDa *T. equi* surface proteins and similar sized proteins of various *Theileria* spp. (22, 24). However, the position of *T. equi* in phylogenetic trees based on the small subunit ribosomal RNA genes is variable and mostly appear as a sister clade of the Theilerids (6, 35) leading some to suggest that *T. equi* is ancestral to the Theilerids (6) or a different group altogether (3). The clinical signs of equine piroplasmosis are often nonspecific, and the disease can easily be confused with other conditions.

Piroplasmosis can occur in peracute, acute and chronic forms. The acute cases are more common, and are characterised by fever that usually exceeds 40°C, reduced appetite and malaise, elevated respiratory and pulse rates, congestion of mucous membranes, and faecal balls that are smaller and drier than normal.

Clinical signs in subacute cases are similar. In addition, affected animals show loss of weight, and fever is sometimes intermittent. The mucous membranes vary from pale pink to pink, or pale yellow to bright yellow. Petechiae and/or ecchymoses may also be visible on the mucous membranes. Normal bowel movements may be slightly depressed and the animals may show signs of mild colic. Mild oedematous swelling of the distal part of the limbs sometimes occurs.

Chronic cases usually present nonspecific clinical signs such as mild inappetence, poor performance and a drop in body mass. The spleen is usually found to be enlarged on rectal examination.

A rare peracute form where horses are found either dead or moribund has been reported (28).

### B. DIAGNOSTIC TECHNIQUES

#### 1. Identification of the agent

Infected horses may be identified by demonstrating the parasites in stained blood, optimally collected from superficial skin capillaries, or organ smears during the acute phase of the disease. Romanovsky-type staining methods, such as the Giemsa method, usually give the best results (43). However, even in acute clinical cases of *B. caballi* infection, the parasitaemia is very low and difficult to detect. Experienced workers sometimes use a thick blood smear technique (30) to detect very low parasitaemia. Thick films are made by placing a small drop (approximately 50 µl) of blood on to a clean glass slide which is then air-dried, heat fixed at 80°C for 5 minutes, and stained in 5% Giemsa for 20–30 minutes.

An accurate identification of the species of the parasite is sometimes desirable, as mixed infections of *T. equi* and *B. caballi* probably occur frequently.

Identification of equine piroplasmosis in carrier animals by means of blood smear examination is not only very difficult but also inaccurate and therefore serological methods are preferred for this (see below). However, false-
negative or false-positive reactions may be encountered in the course of serological tests (8, 9, 49). In such cases, the passage of whole blood, although a cumbersome and expensive exercise, is a very useful technique to determine the true position. Large quantities of whole blood (500 ml) are transfused into a susceptible, preferably splenectomised, horse. This animal is then kept under close observation for clinical signs of disease. Diagnosis is confirmed by the presence of parasites in its RBCs.

In an additional technique, a specific uninfected tick vector is fed on a suspect animal, and the organism can then either be identified in the tick itself or through the transmission of the organism by the tick vector to another susceptible animal.

Success in the establishment of in-vitro cultures of \textit{T. equi} and \textit{B. caballi} may be one alternative to supplement the methods described above, in order to identify carriers of the parasites (15, 16, 53, 54). \textit{Babesia caballi} parasites were successfully cultured from the blood of two horses that tested negative by the complement fixation (CF) test (16). Similarly, \textit{T. equi} could be cultured from horses that did not show any patent parasitaemia at the time of the initiation of the cultures (53, 54).

Molecular techniques for the detection of \textit{T. equi} and \textit{B. caballi} have been described. These methods are based on species-specific polymerase chain reaction (PCR) assays, which mainly target the 18S rRNA gene (4, 6, 40). Further refinements to the technique includes nested PCR (38), loop-mediated isothermal amplification (LAMP) (1) with reported increased sensitivity; a highly sensitive reverse line blot assay (RLB) and multiplex PCR for simultaneous detection and identification of \textit{Theileria} and \textit{Babesia} species in horses (2, 35).

2. Serological tests

It is extremely difficult to diagnose the organisms in carrier animals by means of the microscopic examination of blood smears. Furthermore, it is by no means practical on a large scale. The serological testing of animals is therefore recommended as a preferred method of diagnosis, especially when horses are destined to be imported into countries where the disease does not occur, but the vector is present.

Sera should be collected and dispatched to diagnostic laboratories in accordance with the specifications of that laboratory. Horses for export that have been subjected to serological tests and shown to be free from infection, should be kept free of ticks to prevent accidental infections.

A number of serological techniques have been used in the diagnosis of piroplasmosis, such as the CF test, the indirect fluorescent antibody (IFA) test and the enzyme-linked immunosorbent assay (ELISA). In addition, a simple and rapid immunochromatographic test for \textit{T. equi} has also recently been described and might be a very useful test for the mass screening of serum samples (17).

a) Indirect fluorescent antibody test (a prescribed test for international trade)

The IFA test has been successfully applied to the differential diagnosis of \textit{T. equi} and \textit{B. caballi} infections (29). The recognition of a strong positive reaction is relatively simple, but any differentiation between weak positive and negative reactions requires considerable experience in interpretation. A detailed description of the protocol of the IFA test has been given (29, 34). An example of an IFA protocol is given below.

- **Antigen production**

  Blood for antigen is obtained from horses with a rising parasitaemia, ideally 2–5%. Carrier animals that have already produced antibodies are not suitable for antigen production. Blood (about 15 ml) is collected into 235 ml of phosphate buffered saline (PBS), pH 7.2. The RBCs are washed three times in cold PBS (1000 \text{g} for 10 minutes at 4°C). The supernatant fluid and the white cell layer are removed after each wash. After the last wash, the packed RBCs are reconstituted to the initial volume with 4% bovine serum albumin fraction V made up in PBS, i.e. the original packed cell volume = 30%, so that one-third consists of RBCs. If the original RBC volume is 15 ml, then 5 ml of packed RBCs + 10 ml of 4% bovine albumin in PBS constitutes the antigen. After thorough mixing, the antigen is placed on to prepared wells on a glass slide using a template or a syringe (34). Alternatively, the cells can be spread smoothly on to microscope slides, covering the entire slide with an even, moderately thick film. These slides are allowed to dry, wrapped in soft paper and sealed in plastic bags or wrapped in aluminium foil, and stored at –20°C for up to 1 year.

- **Test procedure**

  i) Each sample of serum is tested against an antigen of \textit{B. caballi} and of \textit{T. equi}.

  ii) Prior to use, the frozen antigen slides are removed from storage at –20°C and incubated at 37°C for 10 minutes.
iii) The antigen smears are then removed from their protective covering and fixed in cold dry acetone (–20°C) for 1 minute. Commercially produced slides are available that are pre-fixed.

iv) If smears were prepared on the whole slide surface, squares (14–21 in number, i.e. 2–3 rows of 7 each) are formed on the antigen smears with nail varnish or rapidly drying mounting medium (i.e. Cystoseal).

v) Test, positive and negative control sera are diluted from 1/80 to 1/1280 in PBS. Negative and positive control sera are included in each test.

vi) Sera are applied (10 µl each) at appropriate dilutions to the different wells or squares on the antigen smear, incubated at 37°C for 30 minutes, and washed several times in PBS and once in water.

vii) An anti-horse immunoglobulin prepared in rabbits and conjugated with fluorescein isothiocyanate (this conjugate is available commercially) is diluted in PBS and applied to the smear, which is then incubated and washed as before.

viii) After the final wash, two drops of a solution containing equal parts of glycerin and PBS are placed on each smear and mounted with a cover-slip.

ix) The smear is then examined under the microscope for the fluorescing parasites. Sera diluted 1/80 or more that show strong fluorescence are usually considered to be positive, although due consideration is also given to the patterns of fluorescence of the positive and negative controls.

b) Enzyme-linked immunosorbent assay (a prescribed test for international trade)

In recent years a number of recombinant antigens for the use in ELISAs have been described. Recombinant *T. equi* (EMA-1; EMA-2; Be82 and Be158) and *B. caballi* proteins (RAP-1; Bc48; Bc134) have been produced in *Escherichia coli* (12, 13, 18, 20, 21, 25, 46) or in insect cells by baculovirus (52, 47). Recombinant antigens produced in *E. coli* or by baculovirus have the obvious advantage of avoiding the need to infect horses for antigen production, and of eliminating the cross-reactions that have been experienced in the past with the crude ELISA antigens. They also provide a consistent source of antigen for international distribution and standardisation.

Indirect ELISAs using EMA-2 and BC48 have shown high sensitivity and specificity in detecting antibodies in infected horses (18, 19, 47). Initial results from these tests are promising and further validation of the assays is underway.

A competitive inhibition ELISA (C-ELISA) using EMA-1 protein and a specific monoclonal antibody (MAb) that defines this merozoite surface protein epitope, have been used in a C-ELISA for *T. equi* (25). This C-ELISA overcomes the problem of antigen purity, as the specificity of this assay depends only on the specificity defined by the MAb *T. equi* epitope. A 94% correlation was shown between the C-ELISA and the CF test in detecting antibodies to *T. equi*. Sera that gave discrepant results were evaluated for their ability to immunoprecipitate 35S-methionine-labelled *in-vitro* translated products of *T. equi* merozoite mRNA. Samples that were C-ELISA positive and CF test negative clearly precipitated multiple *T. equi* proteins. However, immunoprecipitation results with serum samples that were C-ELISA negative and CF test positive were inconclusive (26). This C-ELISA for *T. equi* was also recently validated in Morocco and Israel, giving a concordance of 91% and 95.7% with the IFA test, respectively (39, 42). A similar C-ELISA has been developed using the recombinant *B. caballi* rhoptry-associated protein 1 (RAP-1) and an MAb reactive with a peptide epitope of a 60 kDa *B. caballi* antigen (21). The results of 302 serum samples tested with this C-ELISA and the CF test showed a 73% concordance. Of the 72 samples that were CF test negative and C-ELISA positive, 48 (67%) were shown to be positive on the IFA test, while four of the five samples that tested CF test positive and C-ELISA negative were positive on the IFA test (21).

A test protocol for an equine piroplasmosis C-ELISA has been described and used for additional validation studies (23, 51). The apparent specificity of the *T. equi* and *B. caballi* C-ELISAs lay between 99.2% and 99.5% using sera from 1000 horses presumed to be piroplasmosis-free. One thousand foreign-origin horses of unknown infection status were tested by the C-ELISA and the CF test with an apparent greater sensitivity of the C-ELISA. The results were 1.1% (*T. equi*) and 1.3% (*B. caballi*) more seropositive animals detected by the C-ELISA than by the CF test; the additional positive results were confirmed by IFA testing. A similar study of 645 foreign-origin horses tested for import and pre-import purposes used heat-treated sera (58°C for 30 minutes), and resulted in 3.6% (*T. equi*) and 2.1% (*B. caballi*) more seropositive animals detected by the C-ELISA than by the CF test. Both C-ELISAs were highly reproducible well-to-well, plate-to-plate, and day-to-day, with overall variances of ± 1.2% and ±1.6% for the *T. equi* and *B. caballi* tests, respectively.

The C-ELISA protocol is given below.
A detailed description of antigen production and a test protocol has been given by the National Veterinary Services Laboratories of the United States Department of Agriculture (USDA) (51). A commercial kit is now available that is based on the same antigens and monoclonal antibodies.

- **Solutions**

  **Antigen coating buffer:** prepare the volume of antigen coating buffer required using the following amounts of ingredients per litre: 2.93 g sodium bicarbonate; 1.59 g sodium carbonate; sufficient ultra-pure water to dissolve, and make up to 1 litre with ultra-pure water. Adjust to pH 9.6.

  **C-ELISA wash solution (high salt diluent):** prepare the volume of C-ELISA wash solution required by using the following amounts of ingredients per litre: 29.5 g sodium chloride; 0.22 g monobasic sodium phosphate; 1.19 g dibasic sodium phosphate; 2.0 ml Tween 20; sufficient ultra-pure water to dissolve, and make up to 1 litre with ultra-pure water. Mix well. Adjust pH to 7.4. Sterilise by autoclaving at 121°C.

- **Antigen production**

  Frozen transformed *E. coli* culture is inoculated at a 1/10,000 dilution into any standard non-selective bacterial growth broth (e.g. Luria broth) containing added carbenicillin (100 µg/ml) and isopropyl-thiogalactoside (IPTG, 1 mM). Cultures are incubated on an orbital shaker set at 200 rpm at 37°C overnight. Cells grown overnight are harvested by centrifugation (5000 g for 10 minutes), washed in 50 mM Tris/HCl and 5 mM ethylene diamine tetra-acetic acid (EDTA) buffer, pH 8.0, and harvested again as before. (Antigen is available from the National Veterinary Services Laboratories, P.O. Box 844, Ames, Iowa 50010, USA.)

  Cells are resuspended to 10% of the original volume in the Tris/EDTA buffer to which 1 mg/ml of lysozyme has been added, and incubated on ice for 20 minutes. Nonidet P-40 detergent (NP-40) is then added to a final 1% concentration (v/v), vortexed, and the mixture is incubated on ice for 10 minutes. The material is next sonicated four times for 30 seconds each time at 100 watts, on ice, allowing 2 minutes between sonications for the material to remain cool. The sonicate is centrifuged at 10,000 g for 20 minutes. The resulting supernatant is dispensed in 0.5 ml aliquots in microcentrifuge tubes and may then be stored at −70°C for several years. The presence of heterologous host bacterial antigens does not interfere with the binding of specific equine anti-piroplasma antibodies or the binding of the paired MAbs to their respective expressed recombinant antigen epitopes and is confirmed by the following procedures. The antigen-containing supernatants are quality controlled by titrating them with their paired MAbs and with reference monospecific equine antisera to verify both an adequate level of expression and complete specificity for the homologous species of piroplasmosis agent. Normal serum (negative serum) controls must not interfere with binding of the MAbs or positive equine reference sera to the expressed antigen preparation.

- **Test procedure**

  i) Microtitration plates are prepared by coating the wells with 50 µl of either *T. equi* antigen or *B. caballi* antigen diluted in antigen-coating buffer. The dilution used is determined by standard serological titration techniques. The plate is sealed with sealing tape, stored overnight at 4°C, and frozen at −70°C. Plates can be stored at −70°C for up to 6 months.

  ii) The primary anti-*T. equi* or anti-*B. caballi* MAb and secondary antibody-peroxidase conjugate is diluted as directed by the manufacturer at the time of use in the C-ELISA, with antibody-diluting buffer (supplied with the test kit).

  iii) Plates are thawed at room temperature, the coating solution is decanted, and the plates are washed twice with C-ELISA wash solution.

  iv) The serum controls and test serum samples are diluted 1/2 with serum-diluting buffer before 50 µl of sera is added to wells. Each unknown serum sample is tested in single or duplicate wells. Positive control sera and blanks are tested in duplicate while negative controls are tested in triplicate on different parts of the plate. Plates are incubated covered, at room temperature (21–25°C) for 30 minutes in a humid chamber, and then washed three times in C-ELISA wash solution.

  v) All wells then receive 50 µl/well of diluted primary anti-*T. equi* or anti-*B. caballi* MAb. (The MAb is produced in a cell culture bioreactor and is available from the National Veterinary Services Laboratories, P.O. Box 844, Ames, Iowa 50010, USA.) Plates are incubated covered for 30 minutes at room temperature (21–25°C) in a humid chamber, and then washed three times in C-ELISA wash solution.

  vi) Diluted secondary peroxidase anti-murine IgG (50 µl/well) conjugate is added to each well. Plates are incubated covered for 30 minutes at room temperature (21–25°C) in a humid chamber, and then washed three times in C-ELISA wash solution.
vii) Chromogenic enzyme substrate (50 µl/well) is added to all wells, and plates are incubated for 15 minutes at room temperature (21–25°C) during colour development.

viii) The colour development is stopped by adding 50 µl of stop solution to all wells and the plates are read immediately on a plate reader.

ix) The plates are read at 620, 630 or 650 nm wavelength (OD). The average OD is calculated for the duplicate wells for all control sera and blank wells. For a valid test, the mean of the negative controls must produce an OD >0.300 and <2.000. The mean positive control sera must produce an inhibition of ≥40%.

x) Per cent inhibition [%I] is calculated as follows: %I = 100 – [(Sample OD × 100) ÷ (Mean negative control OD)].

xi) If a test samples produces ≥40% inhibition it is considered positive. If the test sample produces <40% inhibition it is considered negative.

c) Complement fixation

The CF test has been used in the past by some countries to qualify horses for importation (48). A detailed description of antigen production and a test protocol has been given, for example by the USDA (7, 9, 50). The CF test may not identify all infected animals, especially those that have been drug-treated or that produce anti-complementary reactions, or because of the inability of IgG(T) (the major immunoglobulin isotype of equids) to fix guinea-pig complement (31). Therefore the IFA test and C-ELISA have replaced the CF as the prescribed tests for international trade.

An example of a CF test protocol is given below.

- **Solutions**

  *Alsever’s solution:* prepare 1 litre of Alsever’s solution by dissolving 20.5 g glucose; 8.0 g sodium citrate; 4.2 g sodium chloride in sufficient distilled water. Adjust to pH 6.1 using citric acid, and make up the volume to 1 litre with distilled water. Sterilise by filtration.

  *Stock veronal buffer (5×):* dissolve the following in 1 litre of distilled water: 85.0 g sodium chloride; 3.75 g sodium 5,5 diethyl barbituric; 1.68 g magnesium chloride (MgCl₂ 6H₂O); 0.28 g calcium chloride. Dissolve 5.75 g of 5,5 diethyl barbituric acid in 0.5 litre hot (near boiling) distilled water. Cool this acid solution and add to the salt solution. Make up to 2 litres with distilled water and store at 4°C. To prepare a working dilution, add one part stock solution to four parts distilled water. The final pH should be from 7.4 to 7.6.

- **Antigen production**

  Blood is obtained from horses with a high parasitaemia (e.g. 3–7% parasitaemia for B. caballi and 60–85% for T. equi), and mixed with equal volumes of Alsever’s solution as an anticoagulant. The plasma/Alsever’s supernatant and buffy coat are removed when the RBCs have settled to the bottom of the flask. The RBCs are washed several times with cold veronal buffer and then disrupted. The antigen is recovered from the lysate by centrifugation at 30,900 g for 30 minutes.

  The recovered antigen is washed several times in cold veronal buffer by centrifugation at 20,000 g for 15 minutes. Polyvinyl pyrrolidone 40,000 (1–5% w/v) is added as a stabiliser and the preparation is mixed on a magnetic stirrer for 30 minutes, strained through two thicknesses of sterile gauze, dispensed into 2 ml volumes and freeze-dried. The antigen can then be stored at below –50°C for several years.

- **Test procedure**

  i) The specificity and potency of each batch of antigen should be checked against standard antisera of known specificity and potency. Optimal antigen dilutions are also determined in a preliminary checkerboard titration.

  ii) Test sera are inactivated for 30 minutes at 58°C (donkey and mule sera are inactivated at 62.5°C for 35 minutes) and tested in dilutions of 1/5 to 1/5120. Veronal buffer is used for all dilutions.

  iii) Complement is prepared and titrated spectrophotometrically to determine the 50% haemolytic dose (C'H₅₀) (45) and used in the test at five times C'H₅₀. The haemolytic system consists of equal parts of a 2% sheep (RBC) suspension and veronal buffer with 5 minimum haemolytic doses (MHDs) of haemolysin (amboceptor) (50). Some laboratories use twice the 100% haemolytic dose, which gives equivalent sensitivity.
iv) The test has been adapted to microtitration plates (11). The total volume of the test is 0.125 ml, made up of equal portions (0.025 ml) of antigen, complement (five times C'H50) and diluted serum. Incubation is performed for 1 hour at 37°C.

v) A double portion (0.05 ml) of the haemolytic system is added and the plates are incubated for a further 45 minutes at 37°C with shaking after 20 minutes.

vi) The plates are centrifuged for 1 minute at 200 g before being read over a mirror.

vii) A lysis of 50% is recorded as positive, with the titre being the greatest serum dilution giving 50% lysis. A titre of 1/5 is regarded as positive. A full set of controls (positive and negative sera) must be included in each test as well as control antigen prepared from normal (uninfected) horse RBCs.

Anticomplementary samples are examined by the IFA test. Donkey sera are frequently anticomplementary.

C. REQUIREMENTS FOR VACCINES AND DIAGNOSTIC BIOLOGICALS

No biological products are available currently.

REFERENCES


* * *

**NB:** There is an OIE Reference Laboratories for Equine piroplasmosis (see Table in Part 3 of this *Terrestrial Manual* or consult the OIE Web site for the most up-to-date list: [www.oie.int](http://www.oie.int)).
CHAPTER 2.5.9.

EQUINE RHINOPNEUMONITIS

SUMMARY

Equine rhinopneumonitis (ER) is a collective term for any one of several highly contagious, clinical disease entities of equids that may occur as a result of infection by either of two closely related herpesviruses, equid herpesvirus-1 and -4 (EHV-1 and EHV-4).

Infection by either EHV-1 or EHV-4 is characterised by a primary respiratory tract disease of varying severity that is related to the age and immunological status of the infected animal. Infections by EHV-1 in particular are capable of progression beyond the respiratory mucosa to cause the more serious disease manifestations of abortion, perinatal foal death, or neurological dysfunction.

Identification of the agent: The standard method of identification of the herpesviral agents of ER continues to be laboratory isolation of the virus from appropriate clinical or necropsy material, followed by seroconfirmation of its identity. The viruses can be isolated in equine cell culture from nasopharyngeal samples taken from horses during the febrile stage of respiratory tract infection, from liver, lung, spleen, or thymus of aborted fetuses and early foal deaths, and from the leukocyte fraction of the blood of animals with acute EHV-1 disease. Positive identification of viral isolates as EHV-1 or EHV-4 can be achieved by immunofluorescence with type-specific monoclonal antibodies.

A rapid presumptive diagnosis of rhinopneumonitis abortion can be achieved by direct immunofluorescent detection of viral antigen in cryostat sections of tissues from aborted fetuses, using conjugated polyclonal antiserum.

Sensitive and reliable methods for EHV-1/4 detection by polymerase chain reaction or immunoperoxidase staining have been developed and are useful adjuncts to standard virus cultivation techniques for diagnosis of ER.

Post-mortem demonstration of histopathological lesions of EHV-1 in tissues from aborted fetuses, cases or perinatal foal death or in the central nervous system of neurologically affected animals complements the laboratory diagnosis of ER.

Serological tests: Because most horses will possess some level of antibody to EHV-1/4, the demonstration of specific antibody in the serum collected from a single blood sample is not sufficient for a positive diagnosis of recent, active ER. Paired, acute and convalescent sera from animals suspected of being infected with EHV-1 or EHV-4 can be tested for a four-fold or greater rise in virus-specific antibody titre by either virus neutralisation, or enzyme-linked immunosorbent assay, or complement fixation.

Requirements for vaccines and diagnostic biologicals: Both live attenuated and inactivated viral vaccines of varying composition are commercially available for use in assisting in the control of ER. While vaccination is helpful in reducing the incidence of abortion in mares, and in ameliorating the severity of clinical signs of respiratory infection in young horses, it should not be considered to be a substitute for strict adherence to the well established tenets of sound management practices known to reduce the risk of rhinopneumonitis. Revaccination at frequent intervals is recommended with each of the products, as the duration of vaccine-induced immunity is relatively short.

Standards for production and licensing of both attenuated and inactivated EHV vaccines are established by appropriate veterinary regulatory agencies in the countries of vaccine manufacture and use. A single set of internationally recognised standards for ER vaccines is not available. In
each case, however, vaccine production is based on the system of a detailed outline of production employing a well characterised cell line and a master seed lot of vaccine virus that has been validated with respect to virus identity, safety, virological purity, immunogenicity, and the absence of extraneous microbial agents.

A. INTRODUCTION

Equine rhinopneumonitis (ER) is an historically derived term that describes a constellation of several disease entities of horses that may include respiratory disease, abortion, neonatal foal pneumonitis, or myeloencephalopathy (1, 2, 5, 7). The disease has been recognised for over 60 years as a threat to the international horse industry, and is caused by either of two members of the Herpesviridae family, equid herpesvirus-1 and -4 (EHV-1 and EHV-4). EHV-1 and EHV-4 are closely related alphaherpesviruses of horses with nucleotide sequence identity within individual homologous genes ranging from 55% to 84%, and amino acid sequence identity from 55% to 96% (13, 14). The two herpesviruses are enzootic in all countries in which large populations of horses are maintained as part of the cultural tradition or agricultural economy. There is no recorded evidence that the two herpesviruses of ER pose any health risks to humans working with the agents.

ER is highly contagious among susceptible horses, with viral transmission to cohort animals occurring by inhalation of aerosols of virus-laden respiratory secretions. Extensive use of vaccines has not eliminated EHV infections, and the world-wide annual financial burden from these equine pathogens is immense.

In horses under 3 years of age, clinical ER usually takes the form of an acute, febrile respiratory illness that spreads rapidly through the group of animals. The viruses infect and multiply in epithelial cells of the respiratory mucosa. Signs of infection become apparent 2–8 days after exposure to virus, and are characterised by fever, inappetence, depression, and nasal discharge. The severity of respiratory disease varies with the age of the horse and the level of immunity resulting from previous vaccination or natural exposure. Subclinical infections with EHV-1/4 are common, even in young animals. Although mortality from uncomplicated ER is rare and complete recovery within 1–2 weeks is the normal pattern, the respiratory infection is a frequent and significant cause of interrupted schedules among horses assembled for training, racing, or competitive equestrian events. Fully protective immunity resulting from infection is of short duration, and convalescent animals are susceptible to reinfection by EHV-1/4 after several months. Although reinfections by the two herpesviruses cause less severe or clinically inapparent respiratory disease, the risks of subsequent abortion and/or central nervous system (CNS) disease are not eliminated. The greatest clinical threats to individual breeding, racing, or pleasure horse operations posed by ER are the potential abortigenic and neurological sequelae of EHV-1 respiratory infection.

B. DIAGNOSTIC TECHNIQUES

1. Identification of the agent (3)

Because ER is a highly contagious disease with the potential for occurring as explosive outbreaks with high mortality from abortigenic or neurological sequelae, rapid diagnostic methods are important. Although several rapid and innovative diagnostic techniques based on enzyme-linked immunosorbent assay (ELISA), polymerase chain reaction (PCR), immunohistochemical staining with peroxidase, or nucleic acid hybridisation probes have been recently described, their use is often restricted to specialised reference laboratories, and thus the method of choice for diagnosis of ER by diagnostic virology laboratories handling many routine samples continues to be the traditional methodology of cell culture isolation followed by sero-identification of the isolated viruses. Successful laboratory isolation of EHV-1/4 depends on strict adherence to proper methods for both sample collection and laboratory processing.

a) Collection of samples

Samples of nasopharyngeal exudate for virus isolation are best obtained from horses during the very early, febrile stages of the respiratory disease, and are collected via the nares by swabbing the nasopharyngeal area with a 5 × 5 cm gauze sponge attached to the end of a 50 cm length of flexible, stainless steel wire encased in latex rubber tubing. A guarded uterine swab devise can also be used. After collection, the swab should be removed from the wire and transported immediately to the virology laboratory in 3 ml of cold (not frozen) fluid transport medium (serum-free MEM [minimal essential medium] with antibiotics). Virus infectivity can be prolonged by the addition of bovine serum albumin or gelatine to 0.1% (w/v).

Virological examination of fetal tissues from suspect cases of EHV-1 abortion is most successful when performed on aseptically collected samples of liver, lung, thymus, and spleen. The tissue samples should be transported to the laboratory and held at 4°C until inoculated into tissue culture. Samples that cannot be processed within a few hours should be stored at -70°C. In ante-mortem cases of EHV-1 neurological
disease, the virus can often be isolated from the leukocyte fraction of the blood of acutely infected horses or, less often, from the nasopharynx of the affected animal or cohort animals. For attempts at virus isolation from blood leukocytes, a 20 ml sample of sterile blood, collected in citrate, or heparin anticoagulant (EDTA [ethylene diamine tetra-acetic acid] should not be used as it can destroy the cell cultures). The samples should be transported without delay to the laboratory on ice, but not frozen. Although the virus has, on occasion, been isolated from post-mortem cases of EHV-1 neurological disease by culture of samples of brain and spinal cord, such attempts to isolate virus are often unsuccessful; however, they maybe useful for PCR examination.

b) Virus isolation

For efficient primary isolation of EHV-4 from horses with respiratory disease, equine-derived cell cultures must be used. Both EHV-1 and EHV-4 may be isolated from nasopharyngeal samples using primary equine fetal kidney cells or cell strains of equine fibroblasts derived from dermal (E-Derm) or lung tissue. EHV-1 can be isolated on other cell types, as will be discussed later. The nasopharyngeal swab and its accompanying 3 ml of transport medium are transferred into the barrel of a sterile 10 ml syringe. Using the syringe plunger, the fluid is squeezed from the swab into a sterile tube. A portion of the expressed fluid is then filtered through a sterile, 0.45 µm membrane syringe filter unit into a second sterile syringe tube. Filtration will decrease bacterial contamination, but may also lower virus titre. Recently prepared cell monolayers in 25 cm² tissue culture flasks are inoculated with 0.5 ml of the filtered, as well as the unfiltered, nasopharyngeal swab extract. Cell monolayers in multiwell plates incubated in a 5% CO₂ environment may also be used. Virus is allowed to attach by incubating the inoculated monolayers at 37°C on a platform rocker for 1.5–2 hours. Monolayers of uninoculated control cells should be incubated in parallel with sterile transport medium only.

At the end of the attachment period, the inocula are removed and the monolayers are rinsed twice with phosphate buffered saline (PBS) to remove virus-neutralising antibody that may be present in the nasopharyngeal secretions. After addition of 5 ml of supplemented maintenance medium (MEM containing 2% fetal calf serum [FCS] and twice the standard concentrations of antibiotics [penicillin, streptomycin, gentamicin, and amphotericin B]), the flasks are incubated at 37°C. The use of positive control virus samples to validate the isolation procedure carries the risk that this may lead to eventual contamination of diagnostic specimens. This risk can be minimised by using routine precautions and good laboratory technique, including the use of biosafety cabinets, inoculating positive controls after the diagnostic specimens, decontaminating the surfaces in the hood while the inoculum is adsorbing and using a positive control of relatively low titre. Inoculated flasks should be inspected daily by microscopy for the appearance of characteristic herpesvirus cytopathic effect (CPE) (focal rounding, increase in refractility, and detachment of characteristic herpesvirus cytopathic effect (CPE) (focal rounding, increase in refractility, and detachment of cell monolayers). CPE is observed.

A number of cell types may be used for isolation of EHV-1 from the tissues of aborted fetuses or from post-mortem cases of neurological disease (e.g. rabbit kidney [RK-13], baby hamster kidney [BHK-21], Madin–Darby bovine kidney [MDBK], pig kidney [PK-15], etc.), but equine-derived cell cultures are most sensitive and must be used if the infrequent cases of EHV-4 abortion are to be detected. Around 10% (w/v) pooled tissue homogenates of liver, lung, thymus, and spleen (from aborted fetuses) or of CNS tissue (from cases of neurological disease) are used for virus isolation. These are prepared by first mincing small samples of tissue into 1 mm cubes in a sterile Petri dish with dissecting scissors, followed by macerating the tissue cubes further in serum-free culture medium with antibiotics using a mechanical tissue grinder (e.g. Ten-Broek or Stomacher). After centrifugation at 1200 g for 10 minutes, the supernatant is removed and 0.5 ml is inoculated into duplicate cell monolayers in 25 cm² flasks. Following incubation of the inoculated cells at 37°C for 1.5–2 hours, the inocula are removed and the monolayers are rinsed twice with PBS. After addition of 5 ml of supplemented maintenance medium, the flasks are incubated at 37°C for up to 1 week or until viral CPE is observed.

Culture of peripheral blood leukocytes for the presence of EHV-1 can be attempted in horses during the early stages of the myeloencephalopathy. Buffy coats may be prepared from unclotted blood by centrifugation at 600 g for 15 minutes, and the buffy coat is taken after the plasma has been carefully removed. The buffy coat is then layered onto Ficoll 1,090, centrifuged at 400 g for 20 minutes in and the leukocyte-rich interface is then layered onto Ficoll 1.077 and centrifuged in the same way. The PBMC interface (without most granulocytes) is washed twice in PBS (300 g for 10 minutes) and resuspended in 1 ml of MEM containing 2% FCS. Then, 0.5 ml of the resuspended cell suspension is added to each of the duplicate monolayers of equine fibroblast, equine fetal or RK-13 cell monolayers in 25 cm² flasks containing 8–10 ml freshly added maintenance medium. The flasks are incubated at 37°C for 7 days; the inoculum is not removed. Because CPE may be difficult to detect in the presence of the massive inoculum of leukocytes, each flask of cells is freeze–thawed after 7 days of incubation and the contents centrifuged at 300 g for 10 minutes. Finally, 0.5 ml of the cell-free, culture medium supernatant is transferred to freshly made cell monolayers that are just subconfluent. These are incubated and observed for viral CPE for at least 5–6 days before discarding as negative.
c) **Seroconfirmation of virus identity**

The basis for identification of any herpesvirus isolate recovered from specimens submitted from suspected cases of ER is its immunoreactivity with specific antisera. Specific identification of an isolate as EHV-1 or EHV-4 can be quickly and simply accomplished by immunofluorescent (FA) detection of viral antigen in the infected cell culture using type-specific monoclonal antibodies (MAbs), which are available from OIE Reference Laboratories for equine rhinopneumonitis. The test, which is type-specific and accurate, can be performed on a small aliquot of infected cells from the same container inoculated with clinical or post-mortem material. An isolate made in a laboratory that lacks MAbs or FA capability can be confirmed as EHV1/4 by virus neutralization using a virus-specific polyclonal antiserum or by the PCR (see section B.1.f).

Cell monolayers infected with the isolate are removed by scraping from the flask when at least 75% CPE is evident. The cells are pelleted from the culture medium and resuspended in 0.5 ml of PBS. 50 µl of the cell suspension is placed into two wells of a multiwell microscope slide, air-dried, and fixed for 10 minutes with 100% acetone. Control cell suspensions (uninfected, EHV-1 infected, or EHV-4 infected) are also spotted into each of two wells of the same slide. Control cells may be prepared in advance and stored frozen in small aliquots. A drop of an appropriate dilution of MAb specific for EHV-1 is added to one well of each cell pair, and a drop of MAb specific for EHV-4 is added to each of the other wells. After 30 minutes' incubation at 37°C in a humid chamber, unreacted antibody is removed by two 10-minute washes with PBS. MAbs bound to viral antigen can be detected with goat anti-mouse IgG conjugated with fluorescein isothiocyanate (FITC). A drop of diluted conjugate is added to each well and, after 30 minutes at 37°C, the wells are again washed twice with PBS. Cells are examined with a fluorescence microscope, and positive fluorescence with the antibody of appropriate specificity indicates the virus type.

d) **Virus detection by direct immunofluorescence**

Direct immunofluorescent detection of EHV-1 antigens in samples of post-mortem tissues collected from aborted equine fetuses provides an indispensable method to the veterinary diagnostic laboratory for making a rapid preliminary diagnosis of herpesvirus abortion (9). Side-by-side comparisons of the immunofluorescent and cell culture isolation techniques on more than 100 cases of equine abortion have provided evidence that the diagnostic reliability of direct immunofluorescent staining of fetal tissues obtained at necropsy approaches that of virus isolation attempts from the same tissues. In the United States of America (USA), specific and potent polyclonal antiserum to EHV-1, prepared in swine and conjugated with FITC, is provided to veterinary diagnostic laboratories for this purpose by the National Veterinary Services Laboratories of the United States Department of Agriculture (USDA). The antiserum cross-reacts with EHV-4 and hence is not useful for serotyping. Freshly dissected samples (5 × 5 mm pieces) of fetal tissue (lung, liver, thymus, and spleen) are frozen, sectioned on a cryostat at −20°C, mounted on to microscope slides, and fixed with 100% acetone. After air-drying, the sections are incubated at 37°C in a humid atmosphere for 30 minutes with an appropriate dilution of the conjugated swine antibody to EHV-1. Unreacted antibody is removed by two washes in PBS, and the tissue sections are then covered at 37°C in a humid atmosphere for 30 minutes with an appropriate dilution of the conjugated swine antibody to EHV-1. Unreacted antibody is removed by two washes in PBS, and the tissue sections are then covered with aqueous mounting media and a cover-slip, and examined for fluorescent cells indicating the presence of EHV antigen. Each test should include a positive and negative control consisting of sections from known EHV-1 infected and uninfected fetal tissue.

e) **Virus detection by immunoperoxidase staining**

Enzyme immunohistochemical (IH) staining methods (e.g. immunoperoxidase) have been developed recently as procedures for detecting EHV-1 antigen in paraffin-embedded tissues of aborted equine fetuses or neurologically affected horses (12, 20). Such ancillary IH techniques for antigen detection may facilitate identification of the virus in archival tissue samples or in clinical cases in which traditional laboratory methods for EHV-1 detection have been unsuccessful. Immunoenzymatic staining for EHV-1 is particularly useful for the simultaneous evaluation of morphological lesions and the identification of the infectious agent. Immunoperoxidase staining for EHV-1 or EHV-4 may also be done on infected cell monolayers (16). Adequate controls must be included with each immunoperoxidase test run for evaluation of both the method specificity and antibody specificity.

f) **Virus detection by polymerase chain reaction**

The PCR can be used for rapid amplification and diagnostic detection of nucleic acids of EHV-1 and -4 in clinical specimens, paraffin-embedded archival tissue, or inoculated cell cultures (4, 10, 11, 17, 18). A variety of type-specific PCR primers have been designed to distinguish between the presence of EHV-1 and EHV-4. The correlation between PCR and virus isolation techniques for diagnosis of EHV-1 or EHV-4 is high (17). Diagnosis of ER by PCR is rapid, sensitive, and does not depend on the presence of infectious virus in the clinical sample. It now forms an integral part of a range of diagnostic tests currently available for ER, each with its own advantages and limitations.

For diagnosis of active infection by EHV, PCR methods are most reliable with samples from aborted fetuses or from nasopharyngeal swabs and peripheral blood leukocytes of foals and yearlings; they are most useful
in explosive epizootics of abortion or respiratory tract disease in which a rapid identification of the virus is critical for guiding management strategies. PCR examinations of spinal cord and brain tissue, as well as PBMC, are important in seeking a diagnosis on a horse with neurological signs. However, the interpretation of the amplification by PCR of genomic fragments of EHV-1 or EHV-4 in lymph nodes or trigeminal ganglia from adult horses is complicated by the high prevalence of latent EHV-1 and EHV-4 DNA in such tissues (19).

A simple multiplex PCR assay for simultaneous detection of both EHV-1 and EHV-4 has been described (18). A more sensitive protocol for nested PCR detection of EHV-1 or EHV-4 in clinical or pathological specimens (nasal secretions, blood leukocytes, brain and spinal cord, fetal tissues, etc.) is described here (4). This procedure has been used successfully; however, the technology in this area is changing rapidly and other simpler more sensitive techniques are becoming available.

i) *Prepare template DNA from test specimens:* Following sample homogenisation and cell (and virion) lysis in the presence of a chaotropic salt, nucleic acids bind selectively to silica or cationic resin substrates. Substrate-bound nucleic acids are purified in a series of rapid wash steps followed by recovery with low-salt elution. The reagents for performing such steps for rapid nucleic acid isolation are available in kit format from a number of commercial sources (e.g. High Pure PCR Template Preparation Kit, Roche Molecular Biochemicals, Indianapolis, USA; QIAamp DNA Kit, Qiagen, Valencia, USA).

ii) *Nested primer sequences specific for EHV-1* (Based on those described in reference 4):

- BS-1-P1 = 5’-TCT-ACC-CCT-AGC-ACG-ACG-CTC-3’ (917–936)
- gB1-R-2 = 5’-ACG-CTG-TCG-ATG-TCG-TAA-AAC-CTG-AGA-G-3’ (2390–2363)
- BS-1-P3 = 5’-CTT-TAG-CGG-TGA-TGT-GGA-AT-3’ (1377–1396)
- gB1-R-a = 5’-AAG-TAG-CGC-TTC-TGA-TTG-AGG-3’ (2147–2127)

iii) *Nested primer sequences specific for EHV-4* (4):

- BS-4-P1 = 5’-TCT-ATT-GAG-TTG-GCT-ATG-CT-3’ (1705–1724)
- BS-4-P2 = 5’-TCC-TGG TTG-TTA-TTG-GGT-AT-3’ (2656–2637)
- BS-4-P3 = 5’-TGT-TTC-CGC-CAC-TCT-TGA-CG-3’ (1857–1876)
- BS-4-P4 = 5’-ACT-GCC-TCT-CCC-ACC-TTA-CC-3’ (2456–2437)

iv) *PCR conditions for first stage amplification:* Specimen template DNA (1–2 µg in 2 µl) is added to a PCR mixture (total volume of 50 µl) containing 1 × PCR buffer (50 mM KCl, 10 mM Tris/HCl, pH 9.0, 0.1% Triton X-100), 200 µM of each dNTP, 2.5 mM MgCl₂, 2.0 µM of each outer primer (BS-1-P1 and gB1-R-2 for EHV-1 detection and, in a separate reaction mixture, BS-4-P1 and BS-4-P2 for EHV-4 detection) and 0.5 µ Taq DNA polymerase. Cycling parameters are: initial denaturation at 94°C for 4 minutes; 40 cycles of 94°C for 30 seconds, 60°C for 30 seconds, and 72°C for 90 seconds; with a final extension at 72°C for 10 minutes. Separate reaction mixtures containing either known viral DNA or no DNA (water) should be prepared and amplified as positive and negative controls.

v) *PCR conditions for second stage (nested) amplification:* Two µl of a 1/10 dilution of the first amplification product is added to a fresh PCR mixture (total volume of 50 µl) containing 1 × PCR buffer, 200 µM of each dNTP, 2.5 mM MgCl₂, 2.0 µM of each nested primer (BS-1-P3 and gB1-R-a for EHV-1 detection and, in a separate reaction mixture, BS-4-P3 and BS-4-P4 for EHV-4 detection) and 0.5 µ Taq DNA polymerase. Cycling parameters are: initial denaturation at 94°C for 4 minutes; 40 cycles of 94°C for 30 seconds, 60°C for 30 seconds, and 72°C for 1 minute; with a final extension at 72°C for 10 minutes.

vi) *Gel analysis of amplified products:* 10 µl of each final amplified product, including controls, is mixed with 2 µl of 6 × loading dye and electrophoresed on a 1.5% agarose gel in Tris/acetate or Tris-Borate running buffer, along with a 100 base pairs (bp) DNA ladder. The gel is stained with ethidium bromide and viewed by UV transillumination for amplified products of either 770 bp for EHV-1 or 580 bp for EHV-4.

**g) Histopathology**

Histopathological examination of sections of formalin-fixed, paraffin-embedded tissues from aborted fetuses or from neurologically affected horses is an essential part of the laboratory diagnosis of these two clinical manifestations of ER. In aborted fetuses, typical herpetic intranuclear inclusion bodies present within bronchiolar epithelium or in cells at the periphery of areas of hepatic necrosis are pathognomonic lesions for EHV-1. The characteristic, but not pathognomonic, microscopic lesion associated with EHV-1 neuropathy is a degenerative thrombotic vasculitis of small blood vessels in the brain or spinal cord.
(perivascular cuffing and infiltration by inflammatory cells, endothelial proliferation and necrosis, and thrombus formation).

2. Serological tests

Because of the ubiquity of the viral agents of ER and the high seroprevalence among horses in most parts of the world, the demonstration of a negative antibody titre to EHV-1/4 by serological testing of horses designated for export is not part of present veterinary regulations that seek to prevent international spread of infectious diseases of horses. Serological testing can, however, be a useful adjunct procedure for assisting in the diagnosis of ER in horses. Serodiagnosis of ER is based on the demonstration of significant increases in antibody titres in paired sera taken during the acute and convalescent stages of the disease. The results of tests performed on sera from a single collection date are, in most cases, impossible to interpret with any degree of confidence. The initial (acute phase) serum sample should be taken as soon as possible after the onset of clinical signs, and the second (convalescent phase) serum sample should be taken 2–4 weeks later. ‘Acute phase’ sera from mares after abortion or from horses with EHV-1 neurological disease may already contain maximal titres of EHV-1 antibody, with no increase in titres detectable in sera collected at later dates. In such cases, serological testing of paired serum samples from clinically unaffected cohort members of the herd for rising antibody titres against EHV-1/4 may provide information useful for retrospective diagnosis of ER within the herd. Finally, the serological detection of antibodies to EHV-1 in heart or umbilical cord blood or other fluids of equine fetuses can be of diagnostic value in rare cases of virologically negative fetuses aborted as a result of EHV-1 infection; in some cases, the EHV 1/4 genome maybe identified from these tissues by PCR.

Serum antibody levels to EHV-1/4 may be determined by ELISA (8), virus neutralisation (VN) (15), or complement fixation (CF) tests (15). Because there are no internationally recognised reagents or standardised techniques for performing any of the serological tests for detection of EHV-1/4 antibody, antibody titre determinations on the same serum may differ from one laboratory to another. Furthermore, all of the serological tests mentioned detect antibodies that are cross-reactive between EHV-1 and EHV-4. Nonetheless, the demonstration, by any of the tests, of a four-fold or greater rise in antibody titre to EHV-1 or EHV-4 during the course of a clinical illness provides serological confirmation of recent infection with one of the viruses. The ELISA and CF test have the advantage that they provide results faster and do not require cell culture facilities. Recently, a type-specific ELISA that can distinguish between antibodies to EHV-1 and EHV-4 was developed and made commercially available (6). The microneutralisation test is a widely used and sensitive serological assay for detecting EHV-1/4 antibody and will thus be described here.

a) Virus neutralisation test

This serological test is most commonly performed in flat-bottom 96-well microtitre plates (tissue culture grade) using a constant dose of virus and doubling dilutions of equine test sera. At least two replicate wells for each serum dilution are required. Serum-free MEM is used throughout as a diluent. Virus stocks of known titre are diluted just before use to contain 100 TCID$_{50}$ (50% tissue culture infective dose) in 25 µl. Monolayers of E-Derm or RK-13 cells are monodispersed with EDTA/trypsin and resuspended at a concentration of $5 \times 10^5$/ml. Note that RK-13 cells can be used with EHV-1 but do not give clear CPE with EHV-4. Antibody positive and negative control equine sera and controls for cell viability, virus infectivity, and test serum cytotoxicity, must be included in each assay. End-point VN titres of antibody are calculated by determining the reciprocal of the highest serum dilution that protects 100% of the cell monolayer from virus destruction in both of the replicate wells.

A suitable test procedure is as follows:

i) Inactivate test and control sera for 30 minutes in a water bath at 56°C.

ii) Add 25 µl of serum-free MEM to all wells of the microtitre assay plates.

iii) Pipette 25 µl of each test serum into duplicate wells of both rows A and B of the plate. The first row serves as the serum toxicity control and the second row as the first dilution of the test. Make doubling dilutions of each serum starting with row B and proceeding to the bottom of the plate by sequential mixing and transfer of 25 µl to each subsequent row of wells. Six sera can be assayed in each plate.

iv) Add 25 µl of the appropriately diluted EHV-1 or EHV-4 virus stock to each well (100 TCID$_{50}$/well) except those of row A, which are the serum control wells for monitoring serum toxicity for the indicator cells. Note that the final serum dilutions, after addition of virus, run from 1/4 to1/256.

v) A separate control plate should include titration of both a negative and positive horse serum of known titre, cell control (no virus), virus control (no serum), and a virus titration to calculate the actual amount of virus used in the test.

vi) Incubate the plates for 1 hour at 37°C in 5% CO$_2$ atmosphere.

vii) Add 50 µl of the prepared E-Derm or RK-13 cell suspension ($5 \times 10^5$ cells/ml) in MEM/10% FCS to each well.
viii) Incubate the plates for 4–5 days at 37°C in an atmosphere of 5% CO₂ in air.

ix) Examine the plates microscopically for CPE and record the results on a worksheet. Alternatively, the cell monolayers can be scored for CPE after fixing and staining as follows: after removal of the culture fluid, immerse the plates for 15 minutes in a solution containing 2 mg/ml crystal violet, 10% formalin, 45% methanol, and 45% water. Then, rinse the plates vigorously under a stream of running tap water.

x) Wells containing intact cell monolayers stain blue, while monolayers destroyed by virus do not stain. Verify that the cell control, positive serum control, and serum cytotoxicity control wells stain blue, that the virus control and negative serum control wells are not stained, and that the actual amount of virus added to each well is between 10^{1.5} and 10^{2.5} TCID₅₀. Wells are scored as positive for neutralisation of virus if 100% of the cell monolayer remains intact. The highest dilution of serum resulting in complete neutralisation of virus (no CPE) in both duplicate wells is the end-point titre for that serum.

xi) Calculate the neutralisation titre for each test serum, and compare acute and convalescent phase serum titres from each animal for a four-fold or greater increase.

C. REQUIREMENTS FOR VACCINES AND DIAGNOSTIC BIOLOGICALS

Both live attenuated and inactivated vaccines are available as licensed, commercially prepared products for use as prophylactic aids in reducing the burden of disease in horses caused by EHV-1/4 infection. Clinical experience has demonstrated that none of the vaccine preparations should be relied on to provide an absolute degree of protection from ER. Multiple doses, repeated annually, of each of the currently marketed ER vaccines are recommended by their respective manufacturers, with vaccination schedules that vary with the particular vaccine.

Guidelines for the production of veterinary vaccines are given in Chapter 1.1.8 Principles of veterinary vaccine production. The guidelines given here and in Chapter 1.1.8 are intended to be general in nature and may be supplemented by national and regional requirements.

At least sixteen vaccine products for ER, each containing different permutations of EHV-1, EHV-4, and the two subtypes of equine influenza virus, are currently marketed by five veterinary biologicals manufacturers.

The clinical indications stated on the product label for use of the several available vaccines for ER are either herpesvirus-associated respiratory disease, abortion, or both. Only four vaccine products have met the regulatory requirements for claiming efficacy in providing protection from herpesvirus abortion as a result of successful vaccination and challenge experiments in pregnant mares. None of the vaccine products has been conclusively demonstrated to prevent the occurrence of neurological disease sometimes associated with EHV-1 infection.

1. Seed management
   a) Characteristics and culture
      The master seed virus (MSV) for ER vaccines must be prepared from strains of EHV-1 and/or EHV-4 that have been positively and unequivocally identified by both serological and genetic tests. Seed virus must be propagated in a cell line approved for equine vaccine production by the appropriate regulatory agency. A complete record of original source, passage history, medium used for propagation, etc., shall be kept for the master seed preparations of both the virus(es) and cell stock(s) intended for use in vaccine production. Permanently stored stocks of both MSV and master cell stock (MCS) used for vaccine production must be demonstrated to be pure, safe and, in the case of MSV, also immunogenic. Generally, the fifth passage from the MSV and the twentieth passage from the MCS are the highest allowed for vaccine production. Results of all quality control tests on master seeds must be recorded and made a part of the licensee's permanent records.
   b) Validation as a vaccine
      i) Purity
         Tests for master seed purity include prescribed procedures that demonstrate the virus and cell seed stocks to be free from bacteria, fungi, mycoplasmas, and extraneous viruses. Special tests must be performed to confirm the absence of equine arteritis virus, equine infectious anaemia virus, equine influenza virus, equine herpesvirus-2, -3, and -5, equine rhinovirus, the alphaviruses of equine encephalomyelitis, bovine viral diarrhoea virus (BVDV – common contaminant of bovine serum), and porcine parvovirus (PPV – potential contaminant of porcine trypsin). The purity check should also include the exclusion of the presence of EHV-1 from EHV-4 MSV and vice versa.
      ii) Safety
         Samples of each lot of MSV to be used for preparation of live attenuated ER vaccines must be tested for safety in horses determined to be susceptible to the virulent wild-type virus, including pregnant
mares in the last 4 months of gestation. Vaccine safety must be demonstrated in a ‘safety field trial’ in horses of various ages from three different geographical areas. The safety trial should be conducted by independent veterinarians using a prelicensing batch of vaccine. EHV-1 vaccines making a claim for efficacy in controlling abortion must be tested for safety in a significant number of late gestation pregnant mares, using the vaccination schedule that will be recommended by the manufacturer for the final vaccine product.

iii) Immunogenicity
Tests for immunogenicity of the EHV-1/4 MSV stocks should be performed in horses on an experimental test vaccine prepared from the highest passage level of the MSV allowed for use in vaccine production. The test for MSV immunogenicity consists of vaccination of horses with low antibody titres to EHV-1/4, with doses of the test vaccine that will be recommended on the final product label. Second serum samples should be obtained and tested for significant increases in neutralising antibody titre against the virus, 21 days after the final dose.

iv) Efficacy
An important part of the validation process is the capacity of a prelicensing lot of the ER vaccine to provide a significant level of clinical protection in horses from the particular disease manifestation of EHV-1/4 infection for which the vaccine is offered, when used under the conditions recommended by the manufacturer's product label. Serological data are not acceptable for establishing the efficacy of vaccines for ER. Efficacy studies must be designed to ensure appropriate randomisation of test animals to treatment groups, blinding of the recording of clinical observations, and the use of sufficient numbers of animals to permit statistical evaluation for effectiveness in prevention or reduction of the specified clinical disease. The studies should be performed on fully formulated experimental vaccine products (a) produced in accordance with, (b) at or below the minimum antigenic potency specified in, and, (c) produced with the highest passage of MSV and MCS allowed by the approved ‘Outline of Production’ (see Section C.2). Vaccine efficacy is demonstrated by vaccinating a minimum of 20 EHV-1/4-susceptible horses possessing serum neutralising antibody titres ≤32, followed by challenge of the vaccinates and ten nonvaccinated control horses with virulent virus. A significant difference in the clinical signs of ER must be demonstrated between vaccinates and nonvaccinated control horses. The vaccination and challenge study must be performed on an identical number of pregnant mares and scored for abortion if the vaccine product will make a label usage claim ‘for prevention of’ or ‘as an aid in the prevention of’ abortion caused by EHV-1.

2. Method of manufacture
A detailed protocol of the methods of manufacture to be followed in the preparation of vaccines for ER must be compiled, approved, and filed as an Outline of Production with the appropriate licensing agency. Specifics of the methods of manufacture for ER vaccines will differ with the type (live or inactivated) and composition (EHV-1 only, EHV-1 and EHV-4, EHV-4 and equine influenza viruses, etc.) of each individual product, and also with the manufacturer.

3. In-process control
Cells, virus, culture medium, and medium supplements of animal origin that are used for the preparation of production lots of vaccine must be derived from bulk stocks that have passed the prescribed tests for bacterial, fungal, and mycoplasma sterility; nontumorgenicity; and absence of extraneous viral agents.

4. Batch control
Each bulk production lot of ER vaccine must pass tests for sterility, safety, and immunogenic potency.

a) Sterility
Samples taken from each batch of completed vaccine are tested for bacteria, fungi, and mycoplasma contamination. Procedures to establish that the vaccine is free from extraneous viruses are also required; such tests should include inoculation of cell cultures that allow detection of the common equine viruses, as well as techniques for the detection of BVDV and PPV in ingredients of animal origin used in the production of the batch of vaccine.

b) Safety
Tests to assure safety of each production batch of ER vaccine must demonstrate complete inactivation of virus (for inactivated vaccines) as well as a level of residual virus-killing agent that does not exceed the maximal allowable limit (e.g. 0.2% for formaldehyde). Safety testing in laboratory animals is also required.
c) **Potency**

Batch control of antigenic potency for EHV-1 vaccines only may be tested by measuring the ability of dilutions of the vaccine to protect hamsters from challenge with a lethal dose of hamster-adapted EHV-1 virus. Although potency testing on production batches of ER vaccine may also be performed by vaccination of susceptible horses followed by either viral challenge or assay for seroconversion, the recent availability of virus type-specific MAbs has permitted development of less costly and more rapid *in-vitro* immunoassays for antigenic potency. The basis for such *in-vitro* assays for ER vaccine potency is the determination, by use of the specific MAbs, of the presence of at least the minimal amount of viral antigen within each batch of vaccine that correlates with the required level of protection (or seroconversion rate) in a standard animal test for potency.

d) **Duration of immunity**

Tests to establish the duration of immunity to EHV-1/4 achieved by immunisation with each batch of vaccine are not required. The results of many reported observations indicate that vaccination-induced immunity to EHV-1/4 is not more than a few months in duration; these observations are reflected in the frequency of revaccination recommended on ER vaccine product labels.

e) **Stability**

At least three production batches of vaccine should be tested for shelf life before reaching a conclusion on the vaccine’s stability. When stored at 4°C, inactivated vaccine products generally maintain their original antigenic potency for at least 1 year. Lyophilised preparations of the live virus vaccine are also stable during storage for 1 year at 4°C. Following reconstitution, live virus vaccine is unstable and cannot be stored without loss of potency.

5. Tests on the final product

Before release for labelling, packaging, and commercial distribution, randomly selected filled vials of the final vaccine product must be tested by prescribed methods for freedom from contamination and safety in laboratory test animals.

a) **Safety**

See Section C.4.b.

b) **Potency**

See Section C.4.c.

**REFERENCES**


* * *

NB: There are OIE Reference Laboratories for Equine rhinopneumonitis; see Table in Part 3 of this Terrestrial Manual or consult the OIE Web site for the most up-to-date list: www.oie.int).
SUMMARY

Equine viral arteritis (EVA) is a contagious viral disease of equids caused by equine arteritis virus (EAV), an RNA virus classified in the family Arteriviridae. Only one major serotype of the virus has been identified so far. Equine arteritis virus is found in horse populations in many countries worldwide. Although infrequently reported in the past, confirmed outbreaks of EVA appear to be on the increase.

The majority of naturally acquired infections with EAV are subclinical. Where present, clinical signs of EVA can vary in range and severity. The disease is characterised principally by fever, depression, anorexia, dependent oedema, especially of the limbs, scrotum and prepuce in the stallion, conjunctivitis, an urticarial-type skin reaction, abortion and, rarely, a fulminating pneumonia, enteritis or pneumo-enteritis in young foals. Apart from mortality in young foals, the case-fatality rate in outbreaks of EVA is very low. Affected horses almost invariably make complete clinical recoveries. A long-term carrier state can occur in a variable percentage of infected stallions, but not in mares, geldings or sexually immature colts.

Identification of the agent: EVA cannot be differentiated clinically from a number of other respiratory and systemic equine diseases. Diagnosis of EAV infection is based on virus isolation, detection of nucleic acid or viral antigen, or demonstration of a specific antibody response. Virus isolation should be attempted from appropriate clinical or post-mortem specimens in rabbit, equine, or monkey kidney cell culture. The identity of isolates of EAV should be confirmed by neutralisation test, reverse-transcription polymerase chain reaction (RT-PCR) assay, or by immunocytochemical methods, namely indirect immunofluorescence or avidin–biotin–peroxidase techniques.

Detection and identification of EAV nucleic acid in suspect cases of the disease can also be attempted using the RT-PCR assay and appropriate viral-specific RNA primers.

Where mortality is associated with a suspected outbreak of EVA, a wide range of tissues should be examined for histological evidence of panvasculitis that is especially pronounced in the small arteries throughout the body. The characteristic vascular lesions present in the mature animal are not a notable feature in EVA-related abortions. In such cases, equine arteritis viral antigens may be visualised by immunohistochemical examination of placental and various fetal tissues.

Serological tests: A variety of serological tests, including virus neutralisation (VN), complement fixation (CF), indirect fluorescent antibody, agar gel immunodiffusion, the enzyme-linked immunosorbent assay (ELISA), and the fluorescent microsphere immunoassay assay (MIA) have been used for the detection of antibody to EAV. The tests currently in widest use are the complement-enhanced VN test and the ELISA. The VN test is a very sensitive and highly specific assay of proven value in diagnosing acute infection and in seroprevalence studies. Several ELISAs have been developed, none of which have been as extensively validated as the VN test though some appear to offer comparable specificity and close to equivalent sensitivity. The CF test is less sensitive than either procedure, but it can be used for diagnosing recent infection.

Requirements for vaccines and diagnostic biologicals: Two commercial tissue culture vaccines are currently available against EVA. One is a modified live virus (MLV) vaccine prepared from virus that has been attenuated for horses by multiple serial transfers in primary equine and rabbit cell cultures. It has been shown to be safe and protective for stallions and nonpregnant mares. Vaccination of foals under 6 weeks of age and of pregnant mares in the final 2 months of gestation is contraindicated. There is no evidence of back reversion to virulence of the vaccine virus following its use in the field over more than 20 years. The second vaccine is an inactivated,
adjuvanted product prepared from virus grown in equine cell culture that can be used in nonbreeding and breeding horses. In the absence of appropriate safety data, the vaccine is not currently recommended for use in pregnant mares.

A. INTRODUCTION

Equine viral arteritis (EVA) is a contagious viral disease of equids caused by equine arteritis virus (EAV), a positive-sense, single-stranded RNA virus, and the prototype member of the genus Arterivirus, family Arteriviridae, order Nidovirales (10). Epizootic lymphangitis pinkeye, fièvre typhoide and rotlaufseuche are some of the descriptive terms used in the past to refer to a disease that clinically very closely resembled EVA. The natural host range of EAV would appear to be restricted to equids, although very limited evidence would suggest it may also include new world camels, viz. alpacas and llamas (63). The virus does not present a human health hazard (59). EAV is present in the horse population of many countries world-wide (59). There has been an increase in the incidence of EVA in recent years that has been linked to the greater frequency of movement of horses and use of transported semen (2, 59).

While the majority of cases of acute infection with EAV are subclinical, certain strains of the virus can cause disease of varying severity (59). Typical cases of EVA can present with all or any combination of the following clinical signs: fever, depression, anorexia, leukopenia, dependent oedema, especially of the limbs, scrotum and prepuce of the stallion, conjunctivitis, ocular discharge, supra or periorbital oedema, rhinitis, nasal discharge, a local or generalised urticarial skin reaction, abortion, stillbirths and, rarely, a fulminating pneumonia, enteritis or pneumo-enteritis in young foals. Regardless of the severity of clinical signs, affected horses almost invariably make complete recoveries. The case-fatality rate in outbreaks of EVA is very low; mortality is usually only seen in very young foals, particularly those congenitally infected with the virus (37, 59, 62), and very rarely in otherwise healthy adult horses.

EVA cannot be differentiated clinically from a number of other respiratory and systemic equine diseases, the most common of which are equine influenza, equine herpesvirus 1 and 4 infections, infection with equine rhinitis A and B viruses, equine adenoviruses and streptococcal infections, with particular reference to purpura haemorrhagica. The disease also has clinical similarities to equine infectious anaemia, African horse sickness fever, cases of Hendra virus infection, Getah virus infection and toxicosis caused by hoary alyssum (Berteroa incana). After infection, EAV replicates in macrophages and circulating monocytes (18) and is shed in various secretions/excretions of acutely infected animals, in especially high concentration from the respiratory tract (42).

A variable percentage of acutely infected stallions later become long-term carriers in the reproductive tract and constant semen shedders of the virus (59, 60). The carrier state, which has been shown to be androgen dependent, has been found in the stallion, but not in the mare, gelding or sexually immature colt (59). Unequivocal evidence of the carrier state has only been found in stallions serologically positive for antibodies to the virus (60). While temporary down-regulation of circulating testosterone levels using a GnRH antagonist or by immunisation with GnRH would appear to have expedited clearance of the carrier state in some stallions, the efficacy of either treatment strategy has yet to be fully established. Concern has been expressed that such a therapeutic approach could be used to deliberately mask existence of the carrier state.

B. DIAGNOSTIC TECHNIQUES

1. Identification of the agent

a) Virus isolation

Where an outbreak of EVA is suspected, or when attempting to confirm a case of subclinical EAV infection, virus isolation should be attempted from nasopharyngeal and conjunctival swabs, unclotted blood samples, and semen from stallions considered to be possible carriers of the virus (59). To optimise the chances of virus isolation, the relevant specimens should be obtained as soon as possible after the onset of fever in affected horses. There is evidence that heparin can inhibit the growth of EAV in rabbit kidney cells (RK-13 cell line) (1), and therefore, its use as an anticoagulant is contraindicated as it may interfere with isolation of the virus from whole blood. Acid citrate dextrose or ethylenediaminetetraacetic acid (EDTA) are the anticoagulants of choice to use in obtaining unclotted blood samples. Where EVA is suspected in cases of mortality in young foals or older animals, isolation of EAV can be attempted from a variety of tissues, especially the lymphatic glands associated with the alimentary tract and related organs, and also the lungs, liver and spleen (42). In outbreaks of EVA-related abortion and/or cases of stillborn foals, placental and fetal fluids and a wide range of placental, lymphoreticular and other fetal tissues (especially lung) can be productive sources of virus (59).

Swabs for attempted isolation should be immersed in a suitable viral transport medium and these, together with any fluids or tissues collected for virus isolation and/or reverse-transcription polymerase chain reaction

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Chapter 2.5.10. – Equine viral arteritis

(RT-PCR) testing should be shipped either refrigerated or frozen in an insulated container to the laboratory, preferably using an overnight delivery service. Unclotted blood samples must be transported refrigerated but not frozen. Where possible, specimens should be submitted to a laboratory with established competency to test for this infection.

Although reportedly not always successful in natural cases of EAV infection (43, 59), virus isolation should be attempted from clinical specimens or necropsied tissues using rabbit, equine or monkey kidney cell culture (43, 57, 59). Selected cell lines, e.g. RK-13 (ATCC CCL-37), LLC-MK2 (ATCC CCL-7), and primary horse or rabbit kidney cell culture can be used, with RK-13 cells being the cell system of choice (57). Experience over the years has shown that primary isolation of EAV from semen can present more difficulty than from other clinical specimens or from infected tissues unless an appropriate cell culture system is used. Several factors have been shown to influence primary isolation of EAV from semen in RK-13 cells (49). Higher isolation rates have been obtained using 3–5-day-old monolayers, a large inoculum size in relation to the cell surface area in the inoculated flasks or multiwell plates, and most importantly, the incorporation of carboxymethyl cellulose (medium viscosity, 400–800 cps) in the overlay medium. It should be noted that most RK-13 cells, including ATCC CCL-37, are contaminated with bovine viral diarrhoea virus, the presence of which appears to enhance sensitivity of this cell system for the primary isolation of EAV, especially from semen. There is considerable evidence to indicate that primary isolation rates of EAV may be increased by using RK-13 cells of high passage history1 (57).

Inoculated cultures are examined daily for the appearance of viral cytopathic effect (CPE), which is usually evident within 2–6 days. In the absence of visible CPE, culture supernatants should be subinoculated on to confluent cell monolayers after 4–7 days. While the vast majority of isolations of EAV are made on the first passage in cell culture, a small minority only become evident on the second or subsequent passages in vitro (59, 60). The identity of isolates of EAV can be confirmed in a one-way neutralisation test, by standard RT-PCR or real-time RT-PCR assay (2, 12, 51, 53) or by an immunocytochemical method (36), namely indirect immunofluorescence (16) or the avidin–biotin–peroxidase (ABC) technique (36). A polyclonal rabbit antiserum has been used to identify EAV in infected cell cultures. Mouse monoclonal antibodies (MAbs) to the nucleocapsid protein (N) (2, 40) and major envelope glycoprotein (GP5) of EAV (2, 17) and a monospecific rabbit antiserum to the unglycosylated envelope protein (M) (2, 39, 40) have also been developed and these can detect various strains of the virus in RK-13 cells as early as 12–24 hours after infection (2, 36).

- **Virus isolation from semen (a prescribed test for international trade)**

There is considerable evidence that short- and long-term carrier stallions shed EAV constantly in the semen, but not in respiratory secretions or urine; nor has it been demonstrated in the buffy coat (peripheral blood mononuclear cells) of such animals (59, 60). Stallions should first be blood tested using the virus neutralisation (VN) test or an appropriately validated enzyme-linked immunosorbent assay (ELISA) or other serological test procedure. Virus isolation should be attempted from the semen of stallions serologically positive (titre ≥1/4) for antibodies to EAV that do not have a certified history of vaccination against EVA with confirmation that they were serologically negative (titre <1/4) at time of initial vaccination. Virus isolation is also indicated in the case of shipped semen where the serological status and possible vaccination history of the donor stallion is not available. It is recommended that virus isolation from semen be attempted from two samples, which can be collected on the same day, on consecutive days or after an interval of several days or weeks. There is no evidence that the outcome of attempted virus isolation from particular stallions is influenced by the frequency of sampling, the interval between collections or time of the year. Isolation of EAV should be carried out preferably on portion of an entire ejaculate collected using an artificial vagina or a condom and a teaser or phantom mare. When it is not possible to obtain semen by this means, a less preferable alternative is to collect a dismount sample at the time of breeding. Care should be taken to ensure that no antiseptics/disinfectants are used in the cleansing of the external genitalia of the stallion prior to collection. Samples should contain the sperm-rich fraction of the ejaculate with which EAV is associated as the virus is not present in the pre-sperm fraction of semen (59, 60). Immediately following collection, the semen should be refrigerated on crushed ice or on freezer packs for transport to the laboratory with a minimum of delay. Where there is likely to be a delay in submitting a specimen for testing, the semen can be frozen at or below –20°C for a short period before being dispatched to the laboratory. Freezing a sample has not been found to mitigate against isolation of EAV from the semen of a carrier stallion. In situations where it is not feasible to determine the carrier status of a stallion by virus isolation or RT-PCR procedures, the stallion can be test bred to two seronegative mares, which are checked for seroconversion to the virus 28 days after breeding (59).

- **Test procedure**

  i) On receipt in the laboratory, it should be noted whether each semen sample is frozen, chilled or at ambient temperature. Every sample should be checked to ensure that it contains the sperm-rich

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1 Such a line (RK-13-KY) is available from Dr P.J. Timoney, Maxwell H. Gluck Equine Research Center, University of Kentucky, Lexington, Kentucky 40546-0099, United States of America (E-mail address: ptimoney@uky.edu).
fraction of the ejaculate. This can be established by microscopic examination of a wet-mount preparation of a sample. Additionally, specimens of ejaculate should be visually inspected for colour and presence of gross particulate contamination. If a semen specimen is contaminated with blood, which can result from trauma to the external genitalia of the stallion at time of collection, a repeat sample should be requested as testing such a specimen from a serologically positive stallion may compromise the reliability of the virus isolation result.

ii) Although no longer considered an essential step, pretreatment of semen before inoculation into cell culture by short-term sonication (for three 15-second cycles); facilitates effective mixing and dispersion of a sample.

iii) After removal of culture medium, 3–5-day-old confluent monolayer cultures of RK-13 cells, either in 25 cm² tissue culture flasks or multiwell plates, are inoculated with serial decimal dilutions (10⁻¹–10⁻³) of seminal plasma in tissue culture maintenance medium containing 2% fetal bovine serum and antibiotics. An inoculum of 1 ml per 25 cm² flask is used and no fewer than two flasks per dilution of seminal plasma are inoculated. Inoculum size and number of wells inoculated per dilution of a specimen should be pro-rated where multiwell plates are used. Appropriate dilutions of a virus positive control semen sample or virus control of known titre diluted in culture medium should be included in each test.

iv) The flasks are closed, lids replaced on multiwell plates and inoculated cultures gently rotated to disperse the inoculum over the cell monolayers.

v) Inoculated cultures are then incubated for 1 hour at 37°C either in an aerobic incubator or an incubator containing a humidified atmosphere of 5% CO₂ in air, depending on whether flasks or multiwell plates are used.

vi) Without removing any of the inoculum or washing the cell monolayers, the latter are overlaid with 0.75% carboxymethyl cellulose containing medium with antibiotics.

vii) The flasks or plates are reincubated at 37°C and checked microscopically for viral CPE, which is usually evident within 2–6 days.

viii) In the absence of visible CPE, culture supernatants are subinoculated onto 3–5 day-old confluent cell monolayer cultures of RK-13 cells after 5–7 days. After removal of the overlay medium, monolayers are stained with 0.1% formalin-buffered crystal violet solution.

The identity of any virus isolates should be confirmed by VN, immunofluorescence (16) or ABC technique, using a monospecific antiserum to EAV or MAbs to the structural proteins, N or GP5 of the virus (2, 18, 36, 37), or by standard RT-PCR (2, 6, 12, 27, 51, 53) or real-time RT-PCR assay (5, 38, 64).

In the one-way neutralisation test, serial decimal dilutions of the virus isolate are tested against a neutralising MAb or monospecific antiserum prepared against the prototype Bucyrus strain of EAV (ATCC VR 796) and also a serum negative for neutralising antibodies to the virus. Corresponding titrations of the prototype Bucyrus virus with the same reference antibody reagents are included as test controls. The test is performed in either 25 cm² tissue culture flasks or multiwell plates. Appropriate quantities of the known EAV positive and negative antibody reagents are inactivated for 30 minutes in a water bath at 56°C and diluted 1/4 in phosphate buffered saline, pH 7.2; then 0.3 ml of diluted antibody reagent is dispensed into five tubes for each virus isolate to be tested. Serial decimal dilutions (10⁻¹–10⁻³) of each virus are made in Eagles Minimal Essential Medium containing 10% fetal bovine serum, antibiotics and 10% freshly diluted guinea-pig complement. Then, 0.3 ml of each virus dilution is added to the tubes containing positive and negative antibody reagents. The tubes are shaken and the virus/antibody mixtures are incubated for 1 hour at 37°C. The mixtures are then inoculated onto 3–5-day-old confluent monolayer cultures of RK-13 cells, either in 25 cm² flasks or multiwell plates, using two flasks or wells per virus dilution. Each flask is inoculated with 0.25 ml of virus/antibody mixture; the inoculum size is pro-rated where multiwell plates are used. Inoculated flasks or plates are incubated for 2 hours at 37°C; gently rocking after 1 hour to disperse the inoculum over the cell monolayers. Without removing any of the inoculum or washing the cell monolayers, the latter are overlaid with 0.75% carboxymethyl cellulose containing medium and incubated for 4–5 days at 37°C, either in an aerobic incubator or an incubator containing a humidified atmosphere of 5% CO₂ in air. After removal of the medium, monolayers are stained with 0.1% formalin-buffered crystal violet solution. Plaques are counted and the virus infectivity titre is determined both in the presence and absence of EAV antibodies using the Spearman–Kärber method (34). Confirmation of the identity of an isolate is based on a reduction in plaque count of at least 10² logs of virus in the presence of antibody positive serum against the Bucyrus strain of EAV.

The vast majority of EAV isolates from carrier stallions are made in the first passage in cell culture using the described test procedure (59, 60). The occurrence of nonviral cytotoxicity or bacterial contamination of specimens is not considered significant problems when attempting isolation of this virus from stallion semen. Nonviral cytotoxicity, if observed, usually affects monolayers inoculated with the 10⁻¹ and, much less frequently, the 10⁻² dilution of seminal plasma. Treatment of seminal plasma with polyethylene glycol (Mol. wt 6000) prior to inoculation has been used with some success in overcoming this problem (25). The method described involves the addition of polyethylene glycol to the 10⁻¹–10⁻³ dilutions of seminal plasma.
b) Nucleic acid recognition methods

The standard two-step RT-PCR, single-step RT-PCR and real-time RT-PCR (rRT-PCR) assays are gaining greater acceptance and being more widely used as an alternative to virus isolation in cell culture for the detection of EAV in diagnostic materials. The RT-PCR-based assays provide a means of identifying virus-specific RNA in clinical specimens, namely nasopharyngeal swab filtrates, buffy coats, raw and extended semen and urine, and in post-mortem tissue samples (5, 6, 27, 55, 56, 64). Standard two-step RT-PCR, single-step RT-PCR, RT-nested PCR (RT-nPCR), and one tube TaqMan® rRT-PCR assays have been developed and evaluated for the detection of various strains of the virus in tissue culture fluid, semen and nasal secretions (5, 6, 12, 27, 38, 50, 51, 53, 55, 56, 64, 66). These assays targeted six different open reading frames (ORFs) in the EAV genome (ORFs 1b, 3–7). However, there is considerable variation in the sensitivity and specificity among RT-PCR assays incorporating different primer pairs targeting various ORFs (6). Results comparable to virus isolation have been obtained with some but not all standard single-step RT-PCR, two-step RT-PCR, RT-nPCR or one tube TaqMan® rRT-PCR assays (5, 6, 9, 27, 38, 51, 53, 56, 66). Compared with traditional virus isolation, these RT-PCR-based assays are frequently more sensitive, less expensive and considerably more rapid to perform, the majority taking less than 24 hours to complete. In addition, RT-PCR assays have the advantage of not requiring viable virus for performance of the test. The one-tube rRT-PCR assay for EAV provides a simple, rapid and reliable method for the detection and identification of viral nucleic acid in equine semen and tissue culture fluid (5, 38). The one tube rRT-PCR has the following important advantages over the standard two-step RT-PCR: 1) eliminating the possibility of cross contamination between samples with previously amplified products as the sample tube is never opened; and 2) reducing the chance of false-positive reactions because the rRT-PCR product is detected with a sequence-specific probe. Because of the high sensitivity of the RT-PCR assay, however, and in the absence of appropriate safeguards in the laboratory, there is the potential for cross-contamination between samples, giving rise to false-positive results. For example, the RT-nPCR assay, while it provides enhanced sensitivity for the detection of EAV, it also increases the likelihood of false-positive results (6). The risk of cross-contamination is greater using the RT-nPCR assay because of the second PCR amplification step involving the product from the first RT-PCR reaction. To minimise the risk of cross-contamination, considerable care needs to be taken, especially during the steps of RNA extraction and reaction setup. Relevant EAV positive and negative template controls and, where appropriate, nucleic acid extracted from the tissue culture fluid of uninfected cells, need to be included in each RT-PCR assay. Thus, in many circumstances, use of the single-step RT-PCR or the one tube rRT-PCR assay would largely circumvent the problems associated with cross contamination.

Primer selection is critical to the sensitivity of the RT-PCR assay with primers (and probe in the case of the rRT-PCR assay) preferably designed from the most conserved region(s) of the EAV genome. Comparative nucleotide sequence analysis has shown that ORF 1b (encodes the viral polymerase), ORF 6 (M protein) and 7 (N protein) are more conserved than the other ORFs among EAV strains so far analysed from North America and Europe (3, 4, 38, 64). The most conserved gene among different strains of EAV is ORF7 and primers specific for ORF7 (and probe for rRT-PCR) have detected a diversity of strains of the virus of European and North American origin (5, 6, 38, 53, 56). Furthermore, the use of multiple primer pairs specific for different ORFs 1b ([forward: 5'-GAT-GTC-TAT-GCT-CCA-TCA-TT-3' and reverse: 5'-GGC-GTA-GGC-TCC-AAT-TGA-A-3')] (12) and/or [forward: 5'-CCT-GAG-ACA-CTG-CAC-GT-3’ and reverse 5’-CCT-GAT-GCC-ACA-TGG-AAT-GA-3’] (27), ORF 6 ([forward: 5'-CTG-AGG-TAT-GGG-AGC-CAT-AG-3’ and reverse: 5’-CCA-GGC-AAA-AGC-AGA-AAA-CC-3’]) (51) and ORF 7 ([forward 5’-ATG-GCG-TGA-AGA-CGA-TCA-CG-3’ and reverse 5’-AGA-ATA-TCC-ACG-TCT-TAC-GGC-3’] (53) also markedly increases the likelihood of detecting North American and European strains of EAV in the RT-PCR assay. The two primer pairs specific for ORF 1b are suitable for use in the RT-nPCR assay (6, 12, 27). While the RT-PCR has been found to be highly sensitive for viral nucleic acid detection in raw semen, there is evidence to that it is not of equivalent reliability when tested extended or cryopreserved semen of very low virus infectivity (66).

In addition to the foregoing RT-PCR assays, 2 TaqMan® fluorogenic probe-based one-tube rRT-PCR assays have been described for the detection of EAV nucleic acid (5); primers ([forward: 5’-GGC-GAC-
AGC-CTA-CAA-GCT-ACA-3', reverse: 5'-CGG-CAT-CTG-CAG-TGA-GTG-A-3' and probe [5'FAM-TTG-CGG-ACC-CGC-ATC-TGA-CCA-A-TAMRA-3'] and (64); primers [forward: 5'-GTA-CAC-CGC-AGT-TGG-TAA-CA-3', reverse: 5'-ACT-TCA-ACA-TGA-CGC-CAC-AC-3'] and probe [5'FAM-TGG-CTC-ATT-CAC-TGC-AGA-TGC-CGG-TAMRA-3']). It should be noted, however, that genomic variation among field isolates of EAV could reduce the sensitivity of both RT-PCR and rRT-PCR assays, even when the primers and probe are based on the most conserved region of the EAV genome (ORF 7 [38]).

In the absence of general agreement on a complete consensus or universal primer set for EAV, and since no RT-PCR assay can determine the actual infectivity of a sample, there is a value to performing virus isolation in conjunction with RT-PCR or rRT-PCR for the identification of virus in clinical or post-mortem specimens.

Strains of EAV isolated from different regions of the world have been classified into different phylogenetic groups by sequence analysis of the GP3, GP5 and M envelope protein genes (ORFs 3, 5 & 6 respectively) and the nucleocapsid (N) protein gene (ORF 7 [2, 8, 13, 54, 65]). The GP5 gene has been found to be most useful and reliable for this purpose (8, 54, 65). The relationships between strains demonstrated by nucleotide sequencing are a useful molecular epidemiological tool for tracing the origin of outbreaks of EVA (2, 4, 65).

c) Histopathological and immunohistochemical methods

Where mortality is associated with a suspected outbreak of EVA, a wide range of tissues should be examined for histological evidence of panvasculitis that is especially pronounced in the small arteries throughout the body, particularly in the caecum, colon, spleen, associated lymphatic glands and adrenal cortex (16, 18, 33). The presence of a disseminated necrotising arteritis involving endothelial and medial cells of affected vessels is considered to be pathognomonic of EVA. The characteristic vascular lesions present in the mature animal are not, however, a prominent feature in many cases of EAV-related abortion (32).

EAV antigen can be identified in various tissues of EVA-affected animals either in the presence or absence of lesions (18). Antigen has been demonstrated in lung, heart, liver and spleen and the placenta of aborted fetuses (18, 56). Immunohistochemical examination of biopsied skin specimens has also been investigated as a means of confirming acute EAV infection. Though of some value, it is not entirely reliable for the diagnosis of the disease. Viral antigen can be detected within the cytoplasm of infected cells by immunofluorescence using conjugated equine polyclonal anti-EAV serum (16), or by the ABC technique using mouse MAb to the GP5 (28) or N proteins of the virus (18, 37, 40, 56).

2. Serological tests

A variety of serological tests including neutralisation (microneutralisation [52] and plaque reduction [41] (VN)), the complement fixation (CF) test (22), the indirect fluorescent antibody test (16), the agar gel immunodiffusion (16), the ELISA (11, 14, 30, 31, 35, 48) and the fluorescent microsphere immunoassay (MIA) (Go, pers. comm.) have been used to detect antibody to EAV.

The test currently in widest international use to diagnose infection, carry out seroprevalence studies, and test horses for export, is a microneutralisation test in the presence of complement. It has also been used to screen fetal heart blood for the retrospective diagnosis of cases of EVA-related abortion (56). Apart from the VN test, the CF test has been used for diagnosing recent EAV infection as complement-fixing antibodies are relatively short-lived in duration (22). In contrast, neutralising antibody titres to EAV frequently persist for several years after natural infection (59). Although a number of ELISAs have been developed (11, 14, 30, 31, 35, 48), none has as yet been as extensively validated as the VN, though some appear to offer nearly comparable sensitivity and specificity (11, 30, 31, 48). Unlike the VN test, a positive reaction in the ELISA is not necessarily reflective of the protective immune status of an individual horse to EAV as both non-neutralising and neutralising antibodies are involved.

Antiserum to unpurified EAV has been prepared in horses and in rabbits using conventional immunisation protocols. Also, mouse monoclonal and monospecific rabbit antibodies have been developed to the nucleocapsid protein (N) major envelope glycoprotein (GP5), and unglycosylated envelope protein (M) of EAV (7, 17, 28, 39, 40).

OIE Standard Sera for EAV are available2 and these can facilitate international standardisation of the microneutralisation test and ELISA.

2 Available from Dr P.J. Timoney, Maxwell H. Gluck Equine Research Center, Dept of Veterinary Science, University of Kentucky, Lexington, Kentucky 40546-0099, United States of America; E-mail address: ptimoney@uky.edu.
Only one major serotype of EAV has been recognised so far (41, 59). This is represented by the prototype Bucyrus strain (ATCC VR 796). The reference virus used in the EAV VN test is the CVL-Bucyrus (Weybridge) strain. Virus stock is grown in the RK-13 cell line, clarified of cellular debris by low-speed centrifugation and stored in aliquots at –70°C. Several frozen aliquots are thawed and the infectivity of the stock virus is determined by titration in RK-13 cells.

**a) Virus neutralisation (a prescribed test for international trade)**

The VN test is used to screen stallions for evidence of EAV infection and to determine whether there is a need to attempt virus detection in semen using cell culture or RT-PCR assay. It is also used for diagnostic purposes to confirm infection in suspect cases of EVA. The VN test procedure in current widest use is that developed by the National Veterinary Service Laboratories of the United States Department of Agriculture (52). It is important to obtain a sterile blood sample as bacterial contamination of serum can interfere with the test result. It is recommended that the test be carried out in RK-13 cells using the approved CVL-Bucyrus (Weybridge) strain of EAV as reference virus (20). Although originally derived from the prototype Bucyrus virus, the passage history of the CVL (Weybridge) strain is not fully documented. The sensitivity of the VN test for detection of antibodies to EAV can be significantly influenced by several factors, especially the source and passage history of the strain of virus used (20, 21). The CVL-Bucyrus (Weybridge) strain and the highly attenuated MLV vaccine strain of EAV are of comparable sensitivity for detecting low-titred positive sera, especially from EVA-vaccinated horses. Efforts are continuing to bring about greater uniformity in the testing protocol and serological results among laboratories providing the VN or other comparable serological assays for this infection.

**Test procedure**

- **Sera** are inactivated for 30 minutes in a water bath at 56°C (control sera, only once).
- Serial twofold dilutions of the inactivated test sera in serum-free cell culture medium (25 µl volumes) are made in a 96-well, flat-bottomed, cell-culture grade microtitre plate starting at a 1/2 serum dilution and using duplicate rows of wells for each serum to be tested. Most sera are screened initially at a 1/4 and 1/8 serum dilution (i.e. final serum dilution after addition of an equal volume of the appropriate dilution of stock virus to each well). Positive samples at the 1/8 dilution can, if desired, be retested and titrated out for end-point determination. Individual serum controls, together with negative and known low- and high-titre positive control sera must also be included in each test.
- A dilution of stock virus to contain from 100 to 300 TCID_{50} (50% tissue culture infective dose) per 25 µl is prepared using as diluent, serum-free cell culture medium containing antibiotics and fresh guinea-pig or rabbit complement at a final concentration of 10%.
- 25 µl of the appropriate dilution of stock virus is added to every well containing 25 µl of each serum dilution, except the test serum control wells.
- A virus back titration of the working dilution of stock virus is included, using four wells per tenfold dilution, to confirm the validity of the test results.
- The plates are covered and shaken gently to facilitate mixing of the serum/virus mixtures.
- The plates are incubated for 1 hour at 37°C in a humid atmosphere of 5% CO\textsubscript{2} in air.
- A suspension of cells from 3–5-day-old cultures of RK-13 cells are prepared using a concentration that will ensure confluent monolayers in the microtitre plate wells within 18–24 hours after seeding.
- 100 µl of cell suspension is added to every well, the plates covered with plate lids or sealed with tape and shaken gently.
- The plates are incubated at 37°C in a humid atmosphere of 5% CO\textsubscript{2} in air.
- The plates are read microscopically for nonviral CPE after 12–18 hours and again for viral CPE after 48–72 hours' incubation. The validity of the test is confirmed by establishing that the working dilution of stock virus contained 30–300 TCID_{50} virus and that the titres of the positive serum controls are within 0.3 log\textsubscript{10} units of their predetermined titres.

A serum dilution is considered to be positive if there is an estimated 75% or preferably a 100% reduction in the amount of viral CPE in the serum test wells compared with that present in the wells of the lowest virus control dilution. End-points are then calculated using the Spearman–Kärber method (34). A titre of 1/4 or greater is considered to be positive. A negative serum should only have a trace (less than 25%) or no virus neutralisation at the lowest dilution tested. Antibody titres may, on occasion, be difficult to define as partial neutralisation may be observed over a range of several serum dilutions. Infrequently, sera will be encountered that cause toxic changes in the lower dilutions tested. In such cases it may not be possible to
C. REQUIREMENTS FOR VACCINES AND DIAGNOSTIC BIOLOGICALS

A number of experimental and commercial vaccines have been developed against EVA. Currently, there are two commercially available vaccines, both tissue-culture derived. The first is a modified live virus (MLV) vaccine prepared from virus that has been attenuated for horses by multiple serial transfers in equine and rabbit cell cultures (19, 41). This vaccine is licensed for use in stallions, nonpregnant mares and in nonbreeding horses. Whereas nonbreeding horses can be vaccinated at any time, stallions and mares should be vaccinated not less than 3 weeks prior to breeding. The vaccine is not recommended for use in pregnant mares, especially in the last 2 months of gestation, nor in foals under 6 weeks of age unless in the face of significant risk of exposure to natural infection. The vaccine is commercially available in the USA and Canada. It has also been used in New Zealand, subject to ministerial controls, to aid in that country’s EVA eradication programme.

The second commercially available vaccine against EVA is an inactivated product prepared from virus grown in equine cell culture, which is filtered, chemically inactivated and then combined with a metabolisable adjuvant. The vaccine is commercially available in the USA and Canada. It has also been used in New Zealand, subject to ministerial controls, to aid in that country’s EVA eradication programme.
An additional inactivated vaccine against EVA has been developed in Japan for use should an outbreak of EVA occur in that country (24). It is an aqueous formalin-inactivated vaccine that has been shown to be safe and effective for use in nonbreeding and breeding horses. For optimal immunisation with this vaccine, horses require a primary course of two injections given at an interval of 4 weeks, with a booster dose administered every 6–12 months. As the vaccine is currently not commercially available, no details can be provided on its production.

Guidelines for the production of veterinary vaccines are given in Chapter 1.1.8 Principles of veterinary vaccine production. The guidelines given here and in Chapter 1.1.8 are intended to be general in nature and may be supplemented by national and regional requirements.

1. Seed management

a) Characteristics of the seed

Both MLV and inactivated commercial vaccines are derived from the prototype Bucyrus strain of EAV (ATCC VR 796). Available evidence points to the existence of only one major serotype of the virus, and strain variation is not considered to be of significance in relation to vaccine efficacy (41, 59).

In the case of the MLV vaccine, the prototype virus was attenuated by serial passage in primary cultures of horse kidney (HK-131), rabbit kidney (RK-111), and a diploid equine dermal cell line, ATCC CCL57 (ECID-24) (19, 29, 41). The indications from the use of this vaccine are that the virus is safe and immunogenic between its 80th and 111th passage in primary rabbit kidney (19, 29, 41, 44, 45, 61).

The inactivated adjuvanted vaccine is prepared from the unattenuated prototype Bucyrus strain of EAV (ATCC VR 796) that has been plaque purified and in its fourth serial passage in the diploid equine dermal cell line (ECID-4). After growth in cell culture, the virus is then purified by filtration before being chemically inactivated and adjuvanted.

Suitable lots of master seed virus for each vaccine should be maintained in liquid nitrogen or its equivalent.

b) Method of culture

The virus for both MLV and inactivated vaccines should be grown in a stable cell culture system, such as equine dermal cells, using an appropriate medium supplemented with sterile bovine serum or bovine serum albumin as replacement for bovine serum in the growth medium. Cell monolayers should be washed prior to virus inoculation to remove traces of bovine serum. Extensive virus growth as evidenced by the appearance of cytopathic changes in 80–100% of the cells should be obtained within 2–3 days.

c) Validation as a vaccine

In the case of both MLV and inactivated vaccines, the respective virus strains should be grown in an appropriate cell culture system that has been officially approved for vaccine production and confirmed to be free from extraneous bacteria, fungi, mycoplasmas and viruses (46). The identity of the vaccine virus in the master seed should be confirmed by neutralisation with homologous anti-EAV serum. Incomplete neutralisation of EAV by homologous horse or rabbit antisera has been scientifically documented (46, 52) and is a problem when screening master seed virus for extraneous viruses and when attempting to confirm the identity of the vaccine virus. The problem has been circumvented by reducing the infectivity titre of the master seed virus below that required for seed virus production before conducting a neutralisation test on the diluted virus. Virus/serum mixtures are tested for residual live virus by serial passage in cell culture. No evidence of cytopathic viruses, haemadsorbing viruses, or noncytopathic strains of bovine virus diarrhoea virus should be found, based on attempted virus isolation in cell culture. If cells of equine origin are used, they should be confirmed to be free from equine infectious anaemia virus. The newer technologies of PCR and antigen-capture ELISA may be used as adjuncts to virus isolation in screening for adventitious agents.

The MLV vaccine has been shown to be both safe and effective for use in stallions and nonpregnant mares (44, 45, 61). Although not recommended by the manufacturer for pregnant mares, especially in the last 2 months of gestation, the vaccine has been used to immunise pregnant mares in the face of high risk of natural exposure to EAV, with minimal, if any, reported adverse effects. Vaccination confers a high level of protective immunity that persists for at least several years (29, 41, 59). Based on experimental studies and extensive field use of the vaccine since 1985, there is no evidence of back reversion to virulence of the vaccine virus, nor of recombination of the vaccine virus with naturally occurring strains of EAV. Furthermore, there is no confirmed evidence that the attenuated strain of EAV in the current vaccine localises and sets up the carrier state in the reproductive tract of the vaccinated stallion (45, 59, 60, 61).

The commercial inactivated vaccine has been shown to be nonreactive and safe for use in healthy nonbreeding and breeding horses. Transient local reactions may be observed in less than 10% of horses.
vaccinated with the inactivated vaccine. Limited field studies of this vaccine indicate that it is immunogenic, stimulating a satisfactory degree of immunity, the duration of which has yet to be reported.

Although there are no published reports on the efficacy of either commercial vaccine in preventing establishment of the carrier state in the stallion, an experimental aqueous formalin inactivated vaccine against EVA has been shown to prevent virus persistence in the reproductive tract of vaccinated stallions following subsequent challenge with EAV (23).

2. Method of manufacture

Both the MLV and inactivated vaccines are produced by cultivation of the respective seed viruses in an equine dermal cell system. Cell monolayers should be washed prior to inoculation with seed virus to remove traces of bovine serum in the growth medium. Inoculated cultures should be maintained on an appropriate maintenance medium. Harvesting of infected cultures should take place when almost the entire cell sheet shows the characteristic CPE. Supernatant fluid and cells are harvested and clarified of cellular debris and unwanted material by filtration. In the case of the inactivated vaccine, the purified virus is then chemically inactivated and adjuvanted with a metabolisable adjuvant.

3. In-process control

The MLV and inactivated vaccines should be produced in a stable cell line that has been tested for identity and confirmed to be free from contamination by bacteria, fungi, mycoplasmas or other adventitious agents. In addition to the preproduction testing of the master seed virus for each vaccine and the cell line for adventitious contaminants, the cell cultures infected with the respective vaccine viruses should be examined macroscopically for evidence of microbial growth or other extraneous contamination during the incubation period. If growth in a culture vessel cannot be reliably determined by visual examination, subculture, microscopic examination, or both should be carried out.

4. Batch control

a) Sterility

Tests for sterility and freedom from contamination of biological materials may be found in Chapter 1.1.9.

b) Safety

In the case of both MLV and inactivated vaccines, each production lot of vaccine should be checked for extraneous bacterial, fungal and mycoplasmal contaminants. The vaccine should be safety tested by the intramuscular inoculation of at least two horses seronegative for neutralising antibodies to EAV with one vaccine dose of lyophilised virus each (46). None of the inoculated horses should develop any clinical signs of disease other than mild pyrexia during the ensuing 2-week observation period. In addition, nasopharyngeal swabs should be collected daily from each horse for attempted virus isolation; white blood cell counts and body temperatures should also be determined on a daily basis. No significant febrile or haematological changes should supervene following vaccination (45, 59, 61). Limited shedding of vaccine virus by the respiratory route for at most 7 days may be demonstrated in the occasional vaccinated horse (61). There is no evidence of persistence of the vaccine virus in the reproductive tract after vaccination of stallions (45, 60, 61).

To ensure complete inactivation of the vaccine virus, each serial lot of the inactivated vaccine should be checked for viable virus by three serial passages in equine dermal cells and by direct fluorescent antibody staining with specific EAV conjugate before being combined with adjuvant. This should be followed by a safety test in guinea-pigs and mice.

c) Potency

Potency of the vaccine in the final containers is determined by plaque infectivity assay in monolayer cultures of equine dermal cells and by a vaccination challenge test in horses (46). The vaccine must be tested in triplicate in cell culture, the mean infectivity titre calculated and the dose rate determined on the basis that each dose of vaccine shall contain not less than $3 \times 10^4$ plaque-forming units of attenuated EAV. The in-vivo potency of the MLV and inactivated vaccines is evaluated in a single vaccination challenge test using 17–20 vaccinated and 5–7 control horses or in two tests each comprising ten vaccinates and five controls.

The viral antigen concentration in the inactivated vaccine is over one-thousand times the concentration of viral antigen present in the MLV vaccine.
d) **Duration of immunity**

Detectable neutralising antibody titres to EAV should develop in the majority of horses within 1–2 weeks of vaccination with the MLV vaccine (44, 45, 58, 59, 61). Reported responses to primary vaccination have been variable in a couple of studies. In one stallion vaccination study, there was a rapid fall in antibody titres with a significant number of animals reverting to seronegativity 1–3 months after vaccination (61). On the other hand, other studies have been characterised by an excellent durable response, with persistence of high VN levels for at least 1–2 years (58). Revaccination with this vaccine results in an excellent anamnestic response, with the development of high antibody titres that remain relatively undiminished for several years (59).

Experimental studies have shown that most horses vaccinated with the inactivated vaccine develop low to moderate neutralising antibody titres to EAV by day 14 after the second vaccination. There is no published information on the duration of immunity conferred by this vaccine.

e) **Stability**

The lyophilised MLV vaccine can be stored for at least 3–4 years at 2–7°C without loss in infectivity, provided it is kept in the dark (29). Infectivity is preserved for much longer periods if vaccine is frozen at –20°C or below. Once rehydrated, however, the vaccine should be used within 1 hour or else destroyed. The inactivated vaccine is stored as a liquid suspension at 2–8°C, with no loss of potency for at least 1 year, provided it is protected from light.

f) **Preservatives**

The preservatives added to the MLV and inactivated vaccines are neomycin, polymyxin B and amphotericin B.

g) **Precautions (hazards)**

Pregnant mares should not be vaccinated with the MLV vaccine during the last 2 months of gestation, as there is a risk, albeit minimal, of fetal invasion by the vaccine virus. The possibility of a vaccinally induced anaphylactic reaction, though very rare, could result from the administration of either the MLV or inactivated vaccine. In the absence of appropriate safety data, the inactivated vaccine is currently not recommended for use in pregnant mares.

5. **Tests on the final product**

a) **Safety**

With the exception of the inactivated vaccine, which needs to be sterility tested a second time to ensure freedom from contamination, no further safety tests are required on the inactivated or MLV vaccines.

b) **Potency**

No potency tests additional to those conducted on each production lot of the MLV or inactivated vaccines are required on either final product.

**REFERENCES**


Chapter 2.5.10. — Equine viral arteritis


*  *

**NB:** There are OIE Reference Laboratories for Equine viral arteritis Table in Part 3 of this *Terrestrial Manual* or consult the OIE Web site for the most up-to-date list: www.oie.int).
CHAPTER 2.5.11.

GLANDERS

SUMMARY

Glanders is a contagious and fatal disease of horses, donkeys, and mules, and is caused by infection with the bacterium *Burkholderia mallei* (the name recently changed from *Pseudomonas mallei* and was previously classified as *Pfeifferella, Loefflerella, Malleomyces* or *Actinobacillus*). The disease causes nodules and ulcerations in the upper respiratory tract and lungs. A skin form also occurs, known as ‘farcy’. Control of glanders requires testing of suspect clinical cases, screening of apparently normal equids, and elimination of positive reactors. It is transmitted to humans and all infected/contaminated or potentially infected/contaminated material must be handled in a laboratory that meets the requirements for Containment Group 3 pathogens.

Identification of the agent: Smears from fresh material may reveal Gram-negative nonsporulating, nonencapsulated rods. The presence of a capsule-like cover has been demonstrated by electron microscopy. The bacteria grow aerobically and prefer media that contain glycerol. Unlike the *Pseudomonas* species and the closely related bacterium *Burkholderia pseudomallei*, *Burkholderia mallei* is nonmotile. Guinea-pigs are highly susceptible, and males can be used, if strictly necessary, to recover the organism from a heavily contaminated sample. Commercially available biochemical identification kits lack diagnostic sensitivity. Specific monoclonal antibodies and polymerase chain reaction (PCR) as well as real-time PCR assays are available. The latter have also been evaluated in recent outbreaks.

Mallein and serological tests: The mallein test is a sensitive and specific clinical test for hypersensitivity against *Burkholderia mallei*. Mallein, a water soluble protein fraction of the organism, is injected subcutaneously, intradermo-palpebrally or given by eyedrop. In infected animals, the skin or the eyelid swells markedly within 1–2 days. Complement fixation test and enzyme-linked immunosorbent assays are the most accurate and reliable serological tests for diagnostic use. A rose bengal plate agglutination test has recently been developed in Russia; it has been validated in Russia only.

Requirements for vaccines and diagnostic biologicals: There are no vaccines. Mallein purified protein derivative is currently available commercially from the Central Veterinary Control and Research Institute, 06020 Etilk, Ankara, Turkey.

A. INTRODUCTION

Glanders is a bacterial disease of perissodactyls or odd-toed ungulates with zoonotic potential known since ancient times. It is caused by the bacterium *Burkholderia mallei* (the name recently changed from *Pseudomonas mallei* (45) and has been classified in the past as *Pfeifferella, Loefflerella, Malleomyces* or *Actinobacillus*). Outbreaks of the disease may occur in felines living in the wild or in zoological gardens. Susceptibility to glanders has been proved in camels, bears, wolves and dogs. Carnivores may become infected by eating infected meat, but cattle and pigs are resistant (22). Small ruminants may also be infected if kept in close contact to glanderoius horses (42). Glanders in the acute form occurs most frequently in donkeys and mules with high fever and respiratory signs (swollen nostrils, dyspnoea, and pneumonia); death occurs within a few days. In horses, glanders generally takes a more chronic course and they may survive for several years. Chronic and subclinical ‘occult’ cases are dangerous sources of infection due to permanent or intermittent shedding of bacteria (42).

In horses, inflammatory nodules and ulcers develop in the nasal passages and give rise to a sticky yellow discharge, accompanied by enlarged firm submaxillary lymph nodes. Stellate scarring follows upon healing of the
ulcers. The formation of nodular abscesses in the lungs is accompanied by progressive debility, febrile episodes, coughing and dyspnoea. Diarrhoea and polyuria can also occur. In the skin form ('farcy'), the lymphatics are enlarged and nodular abscesses ('buds') of 0.5–2.5 cm develop, which ulcerate and discharge yellow oily pus. Nodules are regularly found in the liver and spleen. Discharges from the respiratory tract and skin are infective, and transmission between animals, which is facilitated by close contact, by inhalation, ingestion of contaminated material (e.g. from infected feed and water troughs), or by inoculation (e.g. via a harness) is common. The incubation period can range from a few days to many months (23, 42).

Glanders is transmissible to humans by direct contact with diseased animals or infected/contaminated material. In the untreated acute disease, 95% mortality can occur within 3 weeks (25). However, survival is possible if the infected person is treated early and aggressively with multiple systemic antibiotic therapies (17, 33). A chronic form with abscessation also occurs (25). When handling suspect or known infected animals or fomites, stringent precautions should be taken to prevent self-infection or transmission of the bacterium to other equids. Laboratory samples should be securely packaged, kept cool and shipped as outlined in Chapter 1.1.1 Collection and shipment of diagnostic specimens. All manipulations with potentially infected/contaminated material must be performed in a laboratory that meets the requirements for Containment Group 3 pathogens as outlined in Chapter 1.1.2 Biosafety and biosecurity in the veterinary microbiology laboratory and animal facilities.

Glanders has been eradicated from many countries by statutory testing, elimination of infected animals, and import restrictions. It persists in some Asian, African and South American countries. It can be considered a re-emerging disease and may be imported by pet or racing equids into glanders free areas (26).

**B. DIAGNOSTIC TECHNIQUES**

1. **Identification of the agent**

Cases for specific glanders investigation should be differentiated on clinical grounds from other chronic infections of the nasal mucosae or sinuses, and from strangles (Streptococcus equi infection), ulcerative lymphangitis (Corynebacterium pseudotuberculosis), pseudotuberculosis (Yersinia pseudotuberculosis) and sporotrichosis (Sporotrichium spp.). Glanders should be excluded positively from suspected cases of epizootic lymphangitis (caused by Histoplasma farciminosum), with which it has many clinical similarities. In humans in particular, glanders should be distinguished from melioidosis (B. pseudomallei infection), which is caused by an organism with close similarities to B. mallei (22).

a) **Morphology of Burkholderia mallei**

The organisms are fairly numerous in smears from fresh lesions, but in older lesions they are scanty (41). They should be stained by methylene blue or Gram stain. The organisms are mainly extracellular, fairly straight Gram-negative rods with rounded ends, 2–5 μm long and 0.3–0.8 μm wide with granular inclusions of various size. They often stain irregularly and do not have a readily visible capsule, under the light microscope, or form spores. The presence of a capsule-like cover has been established by electron microscopy. This capsule is composed of neutral carbohydrates and serves to protect the cell from unfavourable environmental factors. Unlike other organisms in the Pseudomonas group and its close relative Burkholderia pseudomallei, Burkholderia mallei has no flagellae and are therefore nonmotile (19, 31). The organisms are difficult to demonstrate in tissue sections, where they may have a beaded appearance (21). In culture media, they vary in appearance depending on the age of the culture and type of medium. In older cultures, there is much pleomorphism. Branching filaments form on the surface of broth cultures (26).

b) **Cultural characteristics**

It is preferable to attempt isolation from unopened uncontaminated lesions (21). The organism is aerobic and facultatively anaerobic only in the presence of nitrate (8, 19), growing optimally at 37°C (20). It grows well, but slowly, on ordinary culture media, 72-hour incubation of cultures is recommended; glycerol enrichment is particularly useful. After a few days on glycerol agar, there is a confluent, slightly cream-coloured growth that is smooth, moist, and viscous. With continued incubation, the growth thickens and becomes dark brown and tough. It also grows well on glycerol potato agar and in glycerol broth, on which a slimy pellicle forms. On plain nutrient agar, the growth is much less luxuriant, and growth is poor on gelatin (34). In samples not obtained under sterile conditions B. mallei is regularly overgrown by other bacteria.

Alterations to characteristics may occur in vitro, so fresh isolates should be used for identification reactions. Litmus milk is slightly acidified by B. mallei, and coagulation may occur after long incubation. The organism reduces nitrates. Although some workers have claimed that glucose is the only carbohydrate that is fermented (slowly and inconstantly), other workers have shown that if an appropriate medium and indicator are used, glucose and other carbohydrates, such as arabinose, fructose, galactose and mannose, are
consistently fermented by \textit{B. mallei} (6). Indole is not produced, horse blood is not haemolysed and no diffusible pigments are produced in cultures (19). A commercial laboratory test kit (e.g. API [Analytical Profile Index] system: Analytab Products, BioMerieux or Biollog [Hayward, California]) can be used for easy confirmation that an organism belongs to the \textit{Pseudomonas} group. In general, commercially available systems are not suited to unambiguously identifying members of the steadily growing number of species of the genus \textit{Burkholderia} (9). Lack of motility is then of special relevance. A bacteriophage specific for \textit{B. mallei} is available (43).

All culture media prepared should be subjected to quality control and must support growth of the suspect organism from a small inoculum. The reference strain should be cultured in parallel with the suspicious samples to ensure that the tests are working correctly.

In contaminated samples, supplementation of media with substances that inhibit the growth of Gram-positive organisms (e.g. crystal violet, proflavine) has proven to be of use, as has pretreatment with penicillin (1,000 units/ml for 3 hours at 37°C) (22). A selective medium has been developed (44) composed of polymyxin E (1,000 units), bacitracin (250 units), and actidione (0.25 mg) incorporated into nutrient agar (100 ml) containing glycerine (4%), donkey or horse serum (10%), and ovine haemoglobin or tryptone agar (0.1%).

Outside the body, the organism has little resistance to drying, heat, light or chemicals, so that survival beyond 2 weeks is unlikely (25). Under favourable conditions, however, it can probably survive a few months. \textit{Burkholderia mallei} can remain viable in tap water for at least 1 month (34). For disinfection, benzalkonium chloride or 'roccal' (1/2,000), sodium hypochlorite (500 ppm available chlorine), iodine, mercuric chloride in alcohol, and potassium permanganate have been shown to be highly effective against \textit{B. mallei} (20). Phenolic disinfectants are less effective.

c) Laboratory animal inoculation

Guinea-pigs, hamsters and cats have been used for diagnosis when necessary. If isolation in a laboratory animal is considered unavoidable, suspected material is inoculated intraperitoneally into a male guinea-pig. As this technique has a sensitivity of only 20%, the inoculation of at least five animals is recommended (25). Positive material will cause a severe localised peritonitis and orchitis (the Strauss reaction). The number of organisms and their virulence determines the severity of the lesions. Additional steps are used when the test material is heavily contaminated (11). The Strauss reaction is not specific for glanders, and other organisms can elicit it. Bacteriological examination of infected testes should confirm the specificity of the response obtained.

d) Confirmation by polymerase chain reaction and real-time PCR

In the past few years, several PCR and real-time PCR assays for the identification of \textit{Burkholderia mallei} have been developed (2, 13, 32, 36, 38, 39), but only a PCR and a real-time PCR assay were evaluated using samples from a recent outbreak of glanders in horses (30, 37). These two assays will be described in more detail here. However, the robustness of these assays will have to be demonstrated in the future by interlaboratory studies. The guidelines and precautions outlined in Chapter 1.1.5 Validation and quality control of polymerase chain reaction methods used for the diagnosis of infectious diseases, have to be taken into account.

- **DNA preparation**

Single colonies are transferred from an agar plate to 200 µl lysis buffer (5× buffer D [PCR Optimization Kit, Invitrogen, DeShelp, The Netherlands; 1/5 diluted in ultra-pure water]; 0.5% Tween 20 [ICI, American Limited, Merck, Hohenbrunn, Germany]; 2 mg/ml proteinase K [Roche Diagnostics, Mannheim, Germany]). After incubation at 56°C for 1 hour and inactivation for 10 minutes at 95°C, 2 and 4 µl of the cleared lysate are used as template in the PCR or the real-time PCR assay, respectively.

Tissue samples of horses (skin, liver, spleen, lung, and conchae) inactivated and preserved in formalin (48 hours, 10% v/v) are cut with a scalpel into pieces of 0.5 × 0.5 cm (approximately 500 mg). The specimens are washed twice in deionised water (10 ml), incubated over night in sterile saline at 4°C, and minced using liquid nitrogen, a mortar and a pestle. Total DNA is prepared from 50 mg tissue using the QIAamp Tissue Kit™ according to the manufacturer's instructions (Qiagen, Hilden, Germany). DNA is eluted with 80 µl dH2O of which 4 µl are used as template.

- **PCR assay (30)**

The assay may have to be adapted to the PCR instrument used with minor modifications to the cycle conditions and the concentration of the chemicals used.
The oligonucleotides used in ref. 30 are designed based on differences of the fliP sequences from *B. mallei* ATCC 23344T (accession numbers NC_006350, NC_006351) and *B. pseudomallei* K96243 (accession numbers NC_006348, NC_006349). Primers Bma-fliP-f (5'-TCA-GGT-TTG-TAT-GTC-GCT-CGG-3') and Bma-fliP-r (5'-CTA-GGT-GAA-GCT-CTG-CGC-GAG-3') are used to amplify a 989 bp fragment. PCR is done using 50 µl ready-to-go mastermix (Eppendorf, Hamburg, Germany) and 15 pmol of each primer. Thermal cycling conditions are 94°C for 30 seconds; 65°C for 30 seconds and 72°C for 60 seconds. This cycle is repeated for 35 times. A final elongation step is added (72°C for 7 minutes). Visualisation of the products is done by UV light after agarose gel (1% w/v in TAE buffer) electrophoresis and staining with ethidium bromide. No template controls containing PCR-grade water instead of template and positive controls containing DNA of *B. mallei* have to be included in each run to detect contamination by amplicons of former runs or amplification failure.

The linear range of the assay was determined to cover concentrations from 240 pg to 70 fg bacterial DNA/reaction. The lower limit of detection defined as the lowest amount of DNA that was consistently detectable in three runs with eight measurements each is 60 fg DNA or four genome equivalents (95% probability). The intra-assay variability of the fliP PCR assay for 35 pg DNA/reaction is 0.68 % (based on Ct values) and for 875 fg 1.34%, respectively. The inter-assay variability for 35 pg DNA/reaction is 0.89% (based on Ct values) and for 875 fg DNA 2.76 %, respectively.

e) Other methods

The genome of the *Burkholderia mallei* type strain ATCC 23344T was sequenced in 2004 (27). Several genomes of other isolates followed and revealed a wide genomic plasticity. Passages in different host species or culture media may provoke considerable sequence alterations (29). The loss of the ability to produce LPS and/or capsule polysaccharide upon ongoing culture due to mutation is a well known fact and results in reduced or absent virulence and influences serologic tests (26). Several molecular typing techniques have successfully been introduced. Simple molecular techniques like PCR-restriction fragment length polymorphism (35) and pulsed field gel electrophoresis (5) can be used for further discrimination of isolates. Ribotyping using restriction enzymes *Pst*I and *EcoR*I in combination with an *E. coli* 18-mer rDNA probe produced 17 distinct ribotypes within 25 *B. mallei* isolates (12). These techniques are still the in-house tests of specialised laboratories as an extensive strain collection is necessary. Multilocus sequence typing (MLST) can be done with purified DNA so there is no need for excessive cultivation of the agent or the keeping of strain collections. Web-based analysis might even enhance diagnostics (10). No specific histopathology features can be described for lesions caused by *B. mallei*. For immunohistochemical analysis, *B. mallei* specific hyperimmune sera of rabbits can be used.
2. **Mallein and serological tests**

**a) The mallein test (a prescribed test for international trade)**

The mallein purified protein derivative (PPD), which is available commercially, is a solution of water-soluble protein fractions of heat-treated *B. mallei*. The test depends on infected horses being hypersensitive to mallein. Advanced clinical cases in horses and acute cases in donkeys and mules may give inconclusive results requiring additional methods of diagnosis to be employed (1).

- **The intradermo-palpebral test**

This is the most sensitive, reliable and specific test for detecting infected perissodactyls or odd-toed ungulates, and has largely displaced the ophthalmic and subcutaneous tests (3): 0.1 ml of concentrated mallein PPD is injected intradermally into the lower eyelid and the test is read at 24 and 48 hours. A positive reaction is characterised by marked oedematous swelling of the eyelid, and there may be a purulent discharge from the inner canthus or conjunctiva. This is usually accompanied by a rise in temperature. With a negative response, there is usually no reaction or only a little swelling of the lower lid.

- **The ophthalmic test**

This is less reliable than the intradermo-palpebral test. A few drops of mallein are instilled into the eye at the canthus. In an infected animal, the eyelids, and sometimes the side of the face, become swollen and there may be a little discharge from the eye. The reaction may also occur to a lesser extent in the opposite eye.

- **The subcutaneous test**

This test interferes with subsequent serological diagnosis and so the other two mallein tests are preferred. Also, the test may not be acceptable in some countries. The horse’s temperature has to be under 102°F (38.8°C) on the day before the test, at the time of the injection, and at 9, 12 and 15 hours after the injection. A 10 cm square skin patch in the middle of the neck is clipped and disinfected; 2.5 ml of dilute mallein are injected subcutaneously into the centre of the patch. With a positive test, the horse develops a pyrexia of 104°F (40.0°C) or over during the first 15 hours, and a firm painful swelling with raised edges develops within 24 hours at the injection site. In nonglandered horses, there is no, or minimal, transient local swelling. Doubtful reactors may be retested after 14 days using a double dose of mallein.

**b) Complement fixation test (a prescribed test for international trade)**

Although not as sensitive as the mallein test, the CF test is an accurate serological test that has been used for glanders diagnosis for many years (3). It is reported to be 90–95% accurate, serum being positive within 1 week of infection and remaining positive in the case of exacerbation of the chronic process (34). Recently, however, the specificity of CF testing has been questioned (26).

- **Antigen preparation (16)**

  i) Flasks of beef infusion broth with 3% glycerol are inoculated with log-phase growth *B. mallei* and incubated at 37°C for 8–12 weeks.

  ii) The cultures are inactivated by exposing the flasks to flowing steam (100°C) for 60 minutes.

  iii) The clear supernatant is decanted and filtered. The filtrate is heated again by exposure to live steam for 75 minutes on 3 consecutive days, and clarified by centrifugation.

  iv) The clarified product is concentrated to one-tenth the original volume by evaporation on a steam or hot water bath.

  v) Concentrated antigen is bottled in brown-glass bottles to protect from light and stored at 4°C. Antigen has been shown to be stable for at least 10 years in this concentrated state.

  vi) Lots of antigen are prepared by diluting the concentrated antigen 1/20 with sterile physiological saline with 0.5% phenol. The diluted antigen is dispensed into brown-glass vials and store at 4°C. The final working dilution is determined by a block titration. The final working dilution for CF test use is made at the time the CF test is performed.

The resulting antigen is primarily lipopolysaccharide. An alternative procedure is to use young cultures by growing the organism on glycerol–agar slopes for 12 hours and washing off with normal saline. A suspension of the culture is heated for 1 hour at 70°C and the heat-treated bacterial suspension is used as antigen. The disadvantage of this antigen preparation method is that the antigen contains all the bacterial cell components. The antigen should be safety tested by inoculating blood agar plates.
Chapter 2.5.11. — Glanders

- **Test procedure (24)**
  i) Serum is diluted 1/5 in veronal (barbiturate) buffered saline containing 0.1% gelatin (VBSG) or CFD (complement fixation diluent – available as tablets) without gelatine.

  ii) Diluted serum is inactivated for 30 minutes at 56°C. The USDA complement fixation protocol calls for inactivation for 35 minutes (24). (Serum of equidae other than horses should be inactivated at 63°C for 30 minutes.)

  iii) Twofold dilutions of the sera are prepared in 96-well round-bottom microtitre plates.

  iv) Guinea-pig complement is diluted in the chosen buffer and 5 (or optionally 4) complement haemolytic units-50% (CH50) are used.

  v) Sera, complement and antigen are reacted in the plates and incubated for 1 hour at 37°C. (An alternate acceptable procedure is overnight incubation at 4°C.)

  vi) A 2% suspension of sensitised washed sheep red blood cells is added. The USDA protocol calls for confirmation of positive reactions in a tube test using 3% sheep red blood cells (24).

  vii) Plates are incubated for 45 minutes at 37°C, and then centrifuged for 5 minutes at 600 g.

  A sample that produces 100% haemolysis at the 1/5 dilution is negative, 25–75% haemolysis is suspicious, and no haemolysis (100% fixation) is positive. Unfortunately, false-positive results can occur, and *B. pseudomallei* and *B. mallei* cross react and cannot be differentiated by serology (3, 25). Also healthy horses can have a false positive CF reaction for a variable period following a mallein intradermal test.

c) **Enzyme-linked immunosorbent assays**

Both plate and membrane (blot) enzyme-linked immunosorbent assays (ELISAs) have been reported for the serodiagnosis of glanders, but none of these procedures has been shown to differentiate serologically between *B. mallei* and *B. pseudomallei*. Blotting approaches have involved both dipstick dot-blot and electrophoretically separated and transferred western blot methods (14, 40). A competitive ELISA that uses an uncharacterised anti-lipopolysaccharide monoclonal antibody has also been developed and found to be similar to the CF test in performance (15). Continuing development of monoclonal antibody reagents specific for *B. mallei* antigenic components offers the potential for more specific ELISAs in the foreseeable future that will help resolve questionable test results of quarantined imported horses (4, 7, 18, 25). At this time, none of these tests has been validated.

d) **Other serological tests**

The avidin–biotin dot ELISA has been described (40), but has not yet been widely used or validated. The antigen is heat-inactivated bacterial culture that has been concentrated and purified. A dot of this antigen is placed on a nitrocellulose dipstick that is then used to test for antibody against *B. mallei* in equine serum. Using antigen-dotted, preblocked dipsticks, the test can be completed in approximately 1 hour. Serum or whole blood can be used for the test, and partial haemolysis does not impart any background colour to the antigen-coated area on the nitrocellulose. Recently, polysaccharide microarray technology has offered a new promising approach to improve sensitivity in serology (28).

The rose bengal plate agglutination test (RBT) has been described for the diagnosis of glanders in horses and other susceptible animals; this test has been validated in Russia only. The antigen is a heat-inactivated bacterial suspension coloured with rose bengal, which is used in a plate agglutination test.

The accuracy of other agglutination tests and precipitin is unsatisfactory for use in control programmes. Horses with chronic glanders and those in a debilitated condition give negative or inconclusive results.

C. REQUIREMENTS FOR VACCINES AND DIAGNOSTIC BIOLOGICALS

No vaccines are available.

Mallein PPD for use in performing the intradermo-palpebral and ophthalmic tests is produced commercially by the Central Veterinary Control and Research Institute, 06020 Etlik, Ankara, Turkey.

The ID-Leystad has provided the following information on requirements for mallein PPD.

1. **Seed management**

Three strains of *Burkholderia mallei* are employed in the production of mallein PPD, namely Bogor strain (originating from Indonesia), Mukteswar strain (India) and Zagreb strain (Yugoslavia). The seed material is kept...
as a stock of freeze-dried cultures. The strains are subcultured on to glycerol agar at 37°C for 1–2 days. For maintaining virulence and antigenicity, the strains may be passaged through guinea-pigs.

2. Method of manufacture

Dorset–Henley medium, enriched by the addition of trace elements, is used for production of mallein PPD. The liquid medium is inoculated with a thick saline suspension of \( B. \textit{mallei} \), grown on glycerol agar. The production medium is incubated at 37°C for about 10 weeks. The bacteria are then killed by steaming for 3 hours in a Koch’s steriliser. The fluid is then passed through a layer of cotton wool to remove coarse bacterial clumps. The resulting turbid fluid is cleared by membrane filtration, and one part trichloroacetic acid 40% is immediately added to nine parts culture filtrate. The mixture is allowed to stand overnight and a light brownish to greyish precipitate settles.

The supernatant fluid is pipetted off and discarded. The precipitate is centrifuged for 15 minutes at 2500 \( g \) and the layer of precipitate is washed three or more times in a solution of 5% NaCl, pH 3, until the pH is 2.7. The washed precipitate is dissolved by stirring with a minimum of an alkaline solvent. The fluid is dark brown and a pH of 6.7 will be obtained. This mallein concentrate has to be centrifuged thoroughly and the supernatant is diluted with an equal amount of a glucose buffer solution. The protein content of this product is estimated by the Kjeldahl method and freeze-dried after it has been dispensed into ampoules.

3. In-process control

During the period of incubation, the flasks are inspected frequently for any signs of contamination, and suspect flasks are discarded. A typical growth of the \( B. \textit{mallei} \) cultures comprises turbidity, sedimentation, some surface growth with a tendency towards sinking, and the formation of a conspicuous slightly orange-coloured ring along the margin of the surface of the medium.

4. Batch control

Each batch of mallein PPD is tested for sterility, safety, preservatives, protein content and potency.

Sterility testing is performed according to the European Pharmacopoeia guidelines.

The examination for safety is conducted on from five to ten normal healthy horses by carrying out the intradermo-palpebral test. The resulting swelling should be, at most, barely detectable and transient, without any signs of conjunctival discharge.

Preparations containing phenol as a preservative should not contain more than 0.5% (w/v) phenol. The protein content should be not less than 0.95 mg/ml and not more than 1.05 mg/ml.

Potency testing is performed in guinea-pigs and horses. The animals are sensitised by subcutaneous inoculation with a concentrated suspension of heat-killed \( B. \textit{mallei} \) in paraffin oil or incomplete Freund’s adjuvant. Cattle can also be used instead of horses. The production batch is bioassayed against a standard mallein PPD by intradermal injection in 0.1 ml doses in such a way that complete randomisation is obtained.

In guinea-pigs, the different areas of erythema are measured after 24 hours, and in horses the increase in skin thickness is measured by calipers. The results are statistically evaluated, using standard statistical methods for parallel-line assays.

REFERENCES


Chapter 2.5.11. – Glanders


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NB: There is an OIE Reference Laboratory for Glanders Table in Part 3 of this Terrestrial Manual or consult the OIE Web site for the most up-to-date list: www.oie.int.
CHAPTER 2.5.12.

HORSE MANGE

See Chapter 2.9.8. Mange

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There is no chapter on horse pox currently available.

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CHAPTER 2.5.14.

VEnezuelAN equine encephAlomyelitiS

SUMMARY

Venezuelan equine encephalomyelitis (VEE) viruses, of the genus Alphavirus of the family Togaviridae, cause disease ranging from mild febrile reactions to fatal encephalitic zoonoses in Equidae and humans. They are transmitted by haematophagous insects, primarily mammalophilic mosquitoes.

The VEE complex of viruses includes six antigenic subtypes (I–VI). Within subtype I there are five antigenic variants (variants AB–F). Originally, subtypes I-A and I-B were considered to be distinct variants, but they are now considered to be identical (I-AB). Antigenic variants I-AB and I-C are associated with epizootic activity in equids and human epidemics. Historically, severe outbreaks have involved many thousands of human and equine cases. The other three variants of subtype I (I-D, I-E, I-F) and the other five subtypes of VEE (II–VI) circulate in natural enzootic cycles. Equidae serve as amplifying hosts for epizootic VEE strains while enzootic VEE viruses cycle primarily between sylvatic rodents and mosquitoes. Enzootic variants and subtypes have been considered to be nonpathogenic for equids, but can cause clinical disease in humans. During 1993 and 1996 however, limited outbreaks of encephalitis in horses in Mexico were shown to be caused by enzootic VEE viruses of subtype I-E.

Identification of the agent: Diagnosis of VEE virus infection can be confirmed by the isolation, identification, and antigenic classification of the isolated virus.

A presumptive diagnosis of equine encephalomyelitis can be made when susceptible animals in tropical or subtropical areas display clinical signs of encephalomyelitis where haematophagous insects are active. VEE virus can be isolated in cell cultures or in laboratory animals using the blood or serum of febrile animals in an early stage of infection. It is recovered less frequently from the blood or brain tissue of encephalitic animals.

VEE virus can be identified by complement fixation, haemagglutination inhibition, plaque reduction neutralisation (PRN), or immunofluorescence tests using VEE-specific antibodies. Specific identification of epizootic VEE variants can be made by the indirect fluorescent antibody test, or a differential PRN test using subtype- or variant-specific monoclonal antibody, or by nucleic acid sequencing.

Serological tests: Specific antibodies may be demonstrated by PRN tests against epizootic VEE virus variants or by IgM capture enzyme-linked immunosorbent assay. Antibody can also be demonstrated by the haemagglutination inhibition or the complement fixation tests.

Any diagnosis of VEE in an individual that is based on seroconversion in the absence of an epizootic should be made with care. Infections of equids with enzootic VEE viruses produce a low level viraemia accompanied by antibody development, but without clinical disease in most cases. Antibody induced by such subclinical infections may be reactive to epizootic VEE virus variants.

Requirements for vaccines and diagnostic biologicals: The only acceptable vaccines against VEE are an attenuated virus vaccine, made with strain TC-83, or inactivated virus preparations also made from this strain. Attenuated virus is immunogenic when given by intramuscular injection, but sometimes causes adverse reactions in the recipient.

Formalin-inactivated virulent VEE virus preparations should never be used in equids, as residual virulent virus can remain after formalin treatment, and thereby cause severe illness in both animals and humans. Epizootics of VEE have occurred from the use of such formalin-treated viruses.
A. INTRODUCTION

Venezuelan equine encephalomyelitis (VEE) is an arthropod-borne inflammatory viral infection of equines and humans, resulting in mild to severe febrile and, occasionally fatal, encephalitic disease.

VEE viruses form a complex within the genus Alphavirus, family Togaviridae. The VEE virus complex is composed of six subtypes (I–VI). Subtype I includes five antigenic variants (AB–F), of which variants 1-AB and 1-C are associated with epizootic VEE in equids and concurrent epidemics in humans (2–4, 8–10). The epizootic variants 1-AB and 1-C are thought to originate from mutations of the enzootic 1-D serotype (11); 1-AB and 1-C isolates have only been obtained during equine epizootics. The enzootic strains include variants 1-D, 1-E and 1-F of subtype I, subtype II, four antigenic variants (A–D) of subtype III, and subtypes IV–VI. Normally, enzootic VEE viruses do not produce clinical encephalomyelitis in the equine species (9), but in 1993 and 1996 in Mexico, the 1-E enzootic subtype caused limited epizootics in horses. The enzootic variants and subtypes can produce clinical disease in humans (3, 4, 5, 8, 10).

Historically, epizootic VEE was limited to northern and western South America (Venezuela, Colombia, Ecuador, Peru and Trinidad) (4). From 1969 to 1972, however, epizootic activity (variant 1-AB) occurred in Guatemala, El Salvador, Nicaragua, Honduras, Costa Rica, Belize, Mexico, and the United States of America (USA) (Texas). Epizootics of VEE caused by I-AB or I-C virus have not occurred in North America and Mexico since 1972. Recent equine and human isolations of epizootic VEE virus were subtype 1-C strains from Venezuela in 1993, 1995 and 1996 and Colombia in 1995.

The foci of enzootic variants and subtypes are found in areas classified as tropical wet forest, i.e. those areas with a high water table or open swampy areas with meandering sunlit streams. These are the areas of the Americas where rainfall is distributed throughout the year or areas permanently supplied with water. Enzootic viruses cycle among rodents, and perhaps birds, by the feeding of mosquitoes (3, 4, 8, 10). Enzootic VEE strains have been identified in the Florida Everglades (subtype II), Mexico (variant I-E), Central American countries (variant I-E), Panama (variants I-D and I-E), Venezuela (variant I-D), Colombia (variant I-D), Peru (variants I-D, III-C, and III-D), French Guiana (variant III-B and subtype V), Ecuador (variant I-D), Suriname (variant III-A), Trinidad (variant III-A), Brazil (variants I-F and III-A and subtype IV), and Argentina (subtype VI). In an atypical ecological niche, variant III-B has been isolated in the USA (Colorado and South Dakota) in an unusual association with birds (3, 4, 8, 10).

A tentative diagnosis of viral encephalomyelitis in equids can be based on the occurrence of acute neurological disease during the summer in temperate climates or in the wet season in tropical or subtropical climates. These are the seasons of haematophagous insect activity. Virus infection will result in clinical disease in many equids concurrently rather than in isolated cases. Epizootic activity can move vast distances through susceptible populations in a short time (3, 4, 8, 10). Differential diagnoses include eastern or western equine encephalomyelitis (Chapter 2.5.5), Japanese encephalitis (Chapter 2.1.7), West Nile fever (Chapter 2.1.20), rabies (Chapter 2.1.13) and other infectious, parasitic, or non-infectious agents producing similar signs.

Human VEE virus infections have originated by aerosol transmission from the cage debris of infected laboratory rodents and from laboratory accidents. Infections with both epizootic and enzootic variants and subtypes have been acquired by laboratory workers (6). Severe clinical disease or death can occur in humans. Those who handle infectious VEE viruses or their antigens prepared from infected tissues or cell cultures should be vaccinated and shown to have demonstrable immunity in the form of VEE virus-specific neutralising antibody (1, 4). All procedures producing aerosols from VEE virus materials should be conducted in biosafety cabinets at containment level 3 (see Chapter 1.1.2 Biosafety and biosecurity in the veterinary microbiological laboratory and animal facilities) (6, 7).

B. DIAGNOSTIC TECHNIQUES

1. Identification of the agent

A confirmatory diagnosis of VEE is based on the isolation and identification of the virus or on the demonstration of seroconversion. The period of viraemia coincides with the onset of pyrexia within 12–24 hours of infection. Viraemia terminates 5–6 days after infection, and coincides with the production of neutralising antibodies and the appearance of clinical neurological signs. Frequently, VEE viruses cannot be isolated from the brains of infected equids. Blood samples for virus isolation should be collected from febrile animals that are closely associated with clinical encephalitic cases.

Virus may be isolated from the blood or sera of infected animals by inoculating 1–4-day-old mice or hamsters intracerebrally or by the inoculation of other laboratory animals, such as guinea-pigs and weaned mice. It may
also be isolated by the inoculation of various cell cultures including African green monkey kidney (Vero), rabbit kidney (RK-13), baby hamster kidney (BHK-21), or duck or chicken embryo fibroblasts, or by inoculation of embryonated chicken eggs. Details of virus identification techniques are described in Chapter 2.5.5.

Isolates can be identified as VEE virus by complement fixation (CF), haemagglutination inhibition (HI), or plaque reduction neutralisation (PRN) tests, or by immunofluorescence as described in Chapter 2.5.5. The VEE virus isolates can be characterised by the indirect fluorescent antibody or PRN tests using monoclonal antibody or by nucleic acid sequencing. The VEE virus characterisation should be carried out in a reference laboratory (see Table given in Part 3 of this Terrestrial Manual).

2. Serological tests

Diagnosis of VEE virus infection in equids requires the demonstration of specific antibodies in paired serum samples collected in the acute and convalescent phases. After infection, PRN antibodies appear within 5–7 days, CF antibodies within 6–9 days, and HI antibodies within 6–7 days. The second convalescent phase serum sample should be collected 4–7 days after the collection of the first acute phase sample or at the time of death. The serological procedures are described in detail in Chapter 2.5.5. Vaccination history must be taken into account when interpreting any of the VEE serological test results. In horses not recently vaccinated with an attenuated live virus strain, demonstration of VEE-specific serum IgM antibodies in a single serum sample supports recent virus exposure.

Any diagnosis of VEE in an individual that is based on seroconversion in the absence of an epizootic should be made with care. Although enzootic subtypes and variants are nonpathogenic for equids, infection will stimulate antibody production to epizootic VEE virus variants.

C. REQUIREMENTS FOR VACCINES AND DIAGNOSTIC BIOLOGICALS

The acceptable vaccines against VEE infection are an attenuated virus vaccine, strain TC-83, and an inactivated virus preparation made from that strain (3, 4, 8, 10). The inactivated vaccine is now the most widely used, and is marketed in EEE/VEE, EEE/VEE/VEE, EEE/VEE/VEE/tetanus toxoid, and EEE/VEE/VEE/West Nile virus/tetanus toxoid combinations.

Inactivated vaccine should be administered in two doses with an interval of 2–4 weeks between doses. Annual revaccination is recommended.

Attenuated vaccine should be reconstituted with physiological saline and used immediately. Multidose vials are kept on ice while the vaccine is being used. Any vaccine not used within 4 hours of reconstitution should be safely discarded. Foals under 2 weeks of age and pregnant mares should not be vaccinated. Animals are vaccinated subcutaneously in the cervical region with a single dose. Revaccination is not recommended.

NOTE: Formalin-treated preparations of virulent epizootic VEE virus should never be used in equids. Residual virulent virus can remain after formalin treatment, and result in severe illness. Epizootics of VEE have occurred in Central and Southern America from the use of such preparations (8, 12).

1. Seed management¹

a) Characteristics of the seed

Attenuated VEE virus vaccine strain TC-83 originated from the Trinidad donkey strain (a variant of I-AB) of epizootic VEE virus isolated in 1944. This strain was derived by serial passage of the Trinidad donkey strain in fetal guinea pig heart cells. It is safe and immunogenic at the established passage levels, and induces protective immunity in vaccinated equids, although adverse reactions can sometimes occur. The vaccine was originally developed for use in personnel involved in high-risk VEE virus research. Suitable seed lots should be maintained at −70°C in a lyophilised state.

b) Method of culture

The virus is grown in fetal guinea pig heart cell cultures in a suitable medium.

¹ Sections on Seed management, Manufacture, In-process control, and Batch control are taken from the Biotechnology, Biologics, and Environmental Protection Division of the United States Department of Agriculture’s Animal and Plant Health Inspection Service (APHIS).
c) **Validation as a vaccine**

The cells used for vaccine production must be free from bacterial, fungal, mycoplasmal, and viral contamination. VEE virus is identified in batches of vaccine by PRN tests against hyperimmune serum. For inactivated vaccines of cell culture origin, strain TC-83 virus is treated with formaldehyde.

2. **Method of manufacture (see footnote 1)**

Vaccine is produced by harvesting supernatant fluid from fetal guinea pig heart monolayers in which the replication of attenuated VEE virus has occurred. The monolayers are maintained at approximately 37°C. The time of harvesting is determined by the occurrence of characteristic cytopathic changes when approximately 70–100% of the cell sheet is affected, typically 1–3 days after infection. The supernatant fluid is clarified by low speed centrifugation and suitable stabilisers are added to protect the virus during freezing and lyophilisation.

3. **In-process control (see footnote 1)**

Cultures should be examined daily for cytopathic changes. After harvesting, the virus suspension should be tested for the presence of microbial contaminants. Inactivated vaccines derived from attenuated strain TC-83 virus should be checked to exclude the presence of viable virus after formalin treatment.

4. **Batch control (see footnote 1)**

a) **Sterility**

Tests for sterility and freedom from contamination of biological materials may be found in Chapter 1.1.9.

b) **Safety**

Safety tests of the inactivated vaccine are described in Chapter 2.5.5.

Safety tests of the attenuated vaccine are conducted in mice. A 0.5 ml dose is injected intraperitoneally or subcutaneously into each of eight mice, and the animals are kept under observation for 7 days. If adverse reactions attributable to the product occur during this period, the product is considered to be unsatisfactory.

c) **Potency**

Potency tests of the inactivated vaccine are described in Chapter 2.5.5 except that antibody titre in inoculated guinea-pigs will be ≥1/4.

Potency of the attenuated vaccine can be determined by testing in horses. Each of 20 susceptible horses is inoculated subcutaneously with 1 ml of lyophilised vaccine that has a reconstituted virus titre of at least 2.5 log_{10} TCID_{50} (50% tissue culture infective dose) per ml. For a valid test, at least 19 of 20 vaccinated horses must have HI antibody titres of at least 1/20 or serum neutralising antibody titres of at least 1/40 within 21–28 days of vaccination.

When tested at any time within the expiration period following lyophilisation, the product must have a virus titre of 0.7 log_{10} greater than that used to test horses as described above, but no less than 2.5 log_{10} TCID_{50}/dose.

The final product must be free from bacterial, fungal, mycoplasmal, or extraneous viral contaminants.

d) **Duration of immunity**

Comprehensive studies on duration of immunity are not available. An annual revaccination is recommended for the inactivated vaccine. Foals that are vaccinated at under 1 year of age should be revaccinated before the next vector season. Revaccination with the attenuated vaccine is not recommended.

e) **Stability**

The lyophilised vaccine is stable and immunogenic for 3 years if kept refrigerated at 2–7°C. After 3 years, vaccine should be discarded. The vaccines should be used immediately after reconstitution. Multidose vials of the attenuated vaccine should be kept on ice while being used. All unused vaccine should be safely discarded 4 hours after reconstitution.

f) **Preservatives**

The preservatives used are thimerosal at a 1/1000 dilution and antibiotics (neomycin, polymyxin, amphotericin B, and gentamicin).
g) Precautions (hazards)

Pregnant mares and foals under 2 weeks of age should not be vaccinated.

All personnel handling infectious VEE viruses or their antigens prepared from infected tissues or cell cultures should be vaccinated and shown to have demonstrable immunity in the form of VEE virus-specific neutralising antibody. All procedures producing aerosols from VEE virus materials should be conducted in biosafety cabinets with biocontainment and efficient filtration of the exhaust air from the laboratory (6, 7).

5. Tests on the final product

a) Safety and potency

Safety and potency tests are as outlined above under Batch control (Sections C.4.b and C.4.c). The attenuated vaccine must have a virus titre of no less than 2.5 log_{10} TCID_{50}/dose.

REFERENCES


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NB: There is an OIE Reference Laboratory for Venezuelan equine encephalomyelitis (see Table in Part 3 of this Terrestrial Manual or consult the OIE Web site for the most up-to-date list: www.oie.int).
SECTION 2.6.
LAGOMORPHA

CHAPTER 2.6.1.
MYXOMATOSIS

SUMMARY

Myxomatosis is a major disease of the European rabbit caused by the Myxoma virus, a member of the Poxviridae family. The diagnosis of myxomatosis, regardless of its clinical form, depends on the isolation and identification of the virus or the demonstration of its antigens. The presence of a humoral immune response facilitates a retrospective diagnosis of a mild form of the disease, and can provide an indication of the prevalence of infection in a rabbit population. The disease is characterised by gross myxomatous skin lesions.

Identification of the agent: When skin lesions are present on a dead rabbit, the viral antigen may be demonstrated by immunodiffusion tests on lesion fragments. Monolayer cell cultures of rabbit kidney inoculated with lesion material will show the characteristic cytopathic effects of poxviruses. The presence of virus can be confirmed by immunofluorescence and negative-staining electron microscopy.

The inoculation of rabbits with suspect material takes longer to identify infection, but this will serve to confirm the presence of infective virus and indicate its pathogenicity.

Serological tests: Identification and titration of specific antibodies arising from natural infection or from immunisation are done by traditional complement fixation or by a recently developed and more sensitive enzyme-linked immunosorbent assay (ELISA), which is not affected by pro- or anti-complementary factors. The difficulty in obtaining blood samples from representative members of a population can be obviated by collecting blood dried on filter paper; this can later be extracted and examined by the indirect fluorescent antibody test or ELISA. Microcapillary blood sampling can also be used for the ELISA.

Qualitative agar gel immunodiffusion tests have the advantage of detecting both antigen and humoral antibodies.

Requirements for vaccines and diagnostic biologicals: Modified live virus vaccines prepared from fibroma virus or modified Myxoma virus strains are available for immunisation of rabbits.

A. INTRODUCTION

Myxomatosis is a major viral disease of wild and domestic European rabbits (Oryctolagus cuniculus) caused by the Myxoma virus (MV), a member of the Poxviridae family. The aetiological agent was first isolated from a colony of laboratory rabbits in Uruguay in 1898 and identified as a poxvirus in 1927. The natural hosts are two species of leporid: Sylvilagus brasiliensis in South America (South American strains) and Sylvilagus bachmani (Californian strains) in California (7). In its natural hosts, the viral strains produce only a benign fibroma, generalised disease occurring only in juvenile animals. In the European rabbits, two forms of the disease have been identified to date: the nodular (classical) form and the amyxomatous (respiratory) form.

Florid skin lesions and severe immunodysfunction, accompanied by supervening Gram-negative bacterial infections of the respiratory tract, characterise the nodular myxomatosis syndrome caused by a virulent MV strain.
Prototype strains of virus deriving from the Australian and European outbreaks have been designed that characterise the various virulence grades (from grade I to grade V) as determined in laboratory rabbits (8). After infection with a grade I (the most virulent) strain, the first sign of infection is a lump at the site of infection, which increases in size and usually becomes protuberant and ulcerates. An acute blepharo-conjunctivitis and an oedematous swelling of the perineum and scrotum gradually develop. The secondary skin lesions appear on about the sixth or the seventh day (7). Death usually occurs between the eighth and fifteenth day post-infection. After infection with grade II to V strains, the clinical signs are usually the same with the exception that they evolve more slowly and are less severe. When animals survive, the lesions progressively heal. The mortality rate fluctuates between 20 and 100%, according to the viral strain. The natural mode of transmission of the nodular form is by biting insects, but limited transmission from rabbit to rabbit is possible if they are closely confined. This form is mainly observed in small-scale rabbitries (2). The clinical signs of amyxomatous myxomatosis are mainly respiratory, skin nodules being few and small. It might be thought that amyxomatous myxomatosis would not spread via vectors but through direct contact, and would arise predominantly in intensive enclosed rabbitries. However, this last notion must be viewed with caution as the disease has also been observed in wild rabbits. So far, these forms of myxomatosis have been reported only in France (5, 15), Spain (18) and more recently in Belgium (16).

As MV has a very narrow host range (it only infects leporides), there is no health risk to humans.

B. DIAGNOSTIC TECHNIQUES

As the signs of the disease become less distinct with the attenuation of virus strains, the submission of samples for laboratory diagnosis becomes more important. Moreover, the expression of the ectodermotropism is clearly reduced for amyxomatous MV strains, and the clinical diagnosis of the amyxomatous form of myxomatosis is clearly more difficult than for the classical one. The different techniques available vary in their ability to detect MV in typical myxomatous lesions, oedema of the eyelids or genital oedema. Nevertheless, the diagnosis of attenuated typical myxomatosis or of atypical (amyxomatous) forms most often requires the isolation of the virus by inoculation of sensitive cell lines such as the RK-13 cell line (ATCC CCL37) and identification of the virus by immunological methods. In both cases, the agent can also be identified by demonstration of MV nucleic acid by polymerase chain reaction, molecular techniques were not specifically evaluated for diagnosis.

1. Identification of the agent

A portion of lesion (myxoma or pieces of organs or tissues, especially eyelids) is excised with scissors. Myxoma are separated from the epidermis and superficial dermis. This is washed with phosphate buffered saline (PBS) with antibiotics as defined below and homogenised with ground glass at a dilution rate of 1 g tissue/4.5–9.0 ml of PBS. Cells are disrupted by two freeze-thaw cycles, or by ultrasonication to liberate virions and viral antigens. This suspension is centrifuged for 5–10 minutes at 1500 g. The supernatant fluid is used for the tests.

a) Culture

Isolation of the virus in cell culture is accomplished using primary cultures of rabbit kidney (RK) cells, or with established cell lines, such as RK-13, in Opti-MEM containing 2% calf serum, 300 international units (IU)/ml penicillin; 300 µg/ml streptomycin; 100 µg/ml gentamycin; 50 IU/ml nystatin (mycostatin); and 5 µg/ml amphotericin (fungizone). The inoculum consists of the supernatant fluid from a homogenised lesion or oculo-respiratory discharge in Opti-MEM with 2% calf serum and antibiotics. This is removed from the cell layer after 2 hours. The cell layer is washed in a small volume of medium and then replenished with maintenance medium (Opti-MEM).

A cytopathic effect (CPE) typical of poxviruses (14) usually develops after 24–48 hours (37°C and 5% CO₂), but with some strains, it may take up to 7 days for CPE to be observed. According to the viral strain, groups of cells with a confluent cytoplasm form syncytia that vary in size from 2 to 50 or even 100 nuclei together. The nuclei of some cells change, the chromatin forming basophilic aggregations that vary in size and number and give the culture a leopard-skin appearance. Eosinophilic intracytoplasmic inclusions remain discrete, if present at all. Affected cells round up, contract and become pyknotic. They then lyse and become detached from the glass or plastic support. Later, all cells are affected and the cell monolayer detaches completely.

Shope’s fibroma virus at first produces well-defined voluminous masses of rounded cells, which proliferate and pile up (14). At the edge, cells just becoming infected show discrete nuclear changes and acidophilic cytoplasmic inclusions that are numerous at an early stage. The cell layer is destroyed after several days.

b) Immunological methods

Agar gel immunodiffusion (AGID) tests are simple and rapid to perform – results can be obtained within 24 hours. Agar plates are prepared with Noble agar (0.6 g), ethylene diamine tetra-acetic acid (EDTA)
Chapter 2.6.1. — Myxomatosis

(2.5 g), sodium chloride (4.5 g), and distilled water (500 ml) containing thiomersal (merthiolate) at 1/100,000 dilution. Standard antiserum (see below), and the test sample are placed in opposing wells that are 6 mm in diameter and 5 mm apart. Another technique is to deposit a small portion of the lesion directly into the agar, 5 mm away from a filter paper disk impregnated with the antiserum. A number of lines of precipitation, usually up to three, appear within 48 hours, indicating the presence of myxoma viral antigens. Only one line forms in the presence of heterologous reactions with Shope’s fibroma virus.

Indirect fluorescent antibody (IFA) tests can be applied to cultures from 24 hours onwards. IFA tests reveal intracytoplasmic multiplication of virus, without being able to distinguish MV from Shope’s fibroma virus. The inoculation of chicken embryo cells (trypsinised at day 11 of egg incubation) does not result in CPE, but it is useful for detecting the viral antigens by IFA tests.

c) Electron microscopy

Negative-staining electron microscopy (EM) can be applied to a portion of skin lesion. The technique is simple and rapid to perform, giving results in 1 hour. About 1 mm³ of the tissue is laid in a watch glass and three drops of distilled water are added. After 1–2 minutes at room temperature an EM grid coated with formvar and carbon is laid over the liquid. After 1 minute any excess liquid is removed with filter paper and immediately a 2% aqueous solution of ammonium molybdate, pH 7.0, is dropped on to the grid. After 10 seconds the excess liquid is removed with filter paper and the grid is prepared for the electron microscope. In a positive case, typical poxvirus particles can be seen. MV cannot be distinguished from Shope’s fibroma virus using this method.

d) Inoculation tests

Rabbit intradermal inoculation also offers a means of identifying the virus through its special characteristics and pathogenicity (virulence grade, classical or amyxomatous forms). It should be avoided if possible but has the advantage of being a gauge of virulence, from the type of inflammation in lesions (local or systemic infection) to the extent of lesions and survival time, and can distinguish Shope’s fibroma virus (with its simple fibromatous local lesion) from MV (capable of causing generalised infection in adults). Rabbits should be of a domestic breed, weighing approximately 2 kg, unvaccinated and previously tested for the absence of antibodies (14).

The inoculum may be the supernatant fluid from a homogenised lesion (with antibiotics) or the product of a cell culture. Between 0.1 and 0.2 ml is administered intradermally behind the ear or into the dorso-lumbar region, which has previously been depilated. The inoculum may be assayed by injecting serial dilutions in saline buffer at one site for each dilution. A primary lesion will appear at the sites within 2–5 days, followed by conjunctivitis. Using five sites for each dilution allows a 50% infective dose (ID₅₀) to be obtained. If the animal survives, the disease can be confirmed serologically after 15 days.

2. Serological tests

Antibodies develop within 8–13 days. In the nonlethal forms and in vaccinated rabbits, the titre is highest after 20–60 days; it declines thereafter, disappearing after 6–8 months in the absence of reinfection (serological response evaluated by use of the complement fixation [CF] test) (19).

Various serological tests may be used, but agar gel immunodiffusion (AGID), CF, IFA and enzyme-linked immunosorbent assay (ELISA), (in order of increasing sensitivity) are the most appropriate tests for international trade and other applications. These tests require standard antigens and antisera. The antigen can be prepared from the Lausanne strain, or some antigenically related strain, propagated in rabbits or cell cultures.

• Preparation of standard reagents (AGID, CF and IFA tests)
  • Preparation of antigen

Myxomatous lesions are removed from rabbits at 6–7 days after inoculation and homogenised in veronal buffer to a dilution of 1/5. The antigen is the supernatant fluid that is obtained following centrifugation (5–10 minutes, 1500 g). Any anticomplementary activity is abolished by adding 0.6% chloroform. The antigen fluid can be frozen at –30°C or –70°C for stock purposes or used directly in CF tests after titration against a standard antiserum.

Antigen is made from cell cultures using the RK-13 cell line. RK-13 (rabbit kidney cells CCL-37) are cultured in Dulbecco’s modified Eagle’s medium (DMEM, Gibco) containing penicillin and streptomycin and 10% fetal calf serum (FCS) for 48 hours before infection. Cells are cultured at 37°C in 5% CO₂. Cells are infected by the MV strain at a multiplicity of infection (m.o.i.) of 1–5. After incubation for 2 hours at 37°C, the inoculum is removed and the cells are maintained in DMEM with 5% FCS for 48 hours at 37°C with 5% CO₂. The
monolayer is harvested about 48 hours after infection, when the cells clearly show CPE (80%), and is centrifuged (1000 g). The supernatant fluid is retained. Infected cells are frozen and thawed three times to release additional virus and the viral suspension is clarified at 1000 g. The newly harvested supernatant is added to the original supernatant. The final supernatant fluid is the antigen, and is stored at –20°C or –70°C (for longer conservation). It is titrated in cell cultures before use.

• **Titration of standard antigen by complement fixation test**

  i) Inactivate standard antiserum for 30 minutes in a water bath at 56°C. No international standard serum for myxomatosis is available, but internal positive standards should be prepared and the titre estimated in the appropriate range, using the CF test or ELISA. After this, the following procedure is used to standardise batches of antigen.

  ii) Make doubling dilutions of standard serum in calcium/magnesium/veronal buffer (CMV) (BIOMERIEUX, ref 72171), pH 7.2, from 1/2 to 1/4096, using a 96-well round-bottomed microtitre plate, one row (column) per dilution and 25 µl per well.

  iii) Using tubes, make doubling dilutions of antigen in CMV, from 1/10 to 1/1280.

  iv) Add 25 µl of the first antigen dilution to each well in the first line of the plate. Similarly add succeeding dilutions of antigen to subsequent lines of the plate so that a checkerboard titration of antigen and antibody is created.

  v) Add 25 µl (6 H50 units [50% haemolysis]) of complement per well.

  vi) Incubate the plate, covered with a plastic film, for 1 hour at 37°C or 14 hours at 4°C.

  vii) Add 50 µl per well of the haemolytic system (2.5% sheep red blood cells [RBCs] and an equal volume of anti-sheep RBC serum diluted both in CMV) (for optimal working dilution of anti-sheep RBC, follow the recommendations of the producer; alternatively it should be determined individually for each lot of serum used).

  viii) Cover the plate again and incubate for 30 minutes at 37°C.

  ix) Read the highest dilution of antigen giving complete haemolysis (H100) with the highest dilution of standard serum. There is 1 antigenic unit (AgU) in 25 µl of antigen of this dilution.

• **Myxoma virus titration of infectious particles in cell cultures**

  i) MV is diluted from 10^{-1} to 10^{-5} in DMEM + antibiotics + 2% FCS.

  ii) Confluent RK-13 cells in P6 (Falcon plates) are infected in triplicate with 200 µl of serial dilutions of interest and incubated at 37°C for 2 hours in a 5% CO₂ incubator.

  iii) Inoculum is then removed and 2 ml of DMEM + 5% FCS is added to each well.

  iv) Incubation is performed at 37°C in a 5% CO₂ incubator for 2 days.

  v) Medium is then removed and replaced by solid medium with 1% LMP agarose and 2% FCS.

  vi) Incubation is performed for 1 or 2 days more, then plaques are counted without coloration for each dilution (coloration is possible with an overlay containing neutral red).

  vii) After average calculation, the titre of the viral suspension is expressed in plaque-forming units (PFU) per ml.

• **Preparation and titration of standard positive serum**

  For the standard antiserum, an adult serologically negative rabbit is vaccinated with an attenuated strain of MV, or with the Shope’s fibroma virus. After 3–4 weeks, the rabbit is inoculated intradermally with virulent myxoma virus (Lausanne strain or a related one) (5 × 10^3 PFU). Serum is obtained 3 weeks later and titrated by the CF test or ELISA. If the titre is >1/640 or > 1/1000, respectively for CF test and ELISA, the animal is bled and the serum is stored at –20°C.

  a) **Complement fixation test**

  CF tests (19) are done in tubes or in microtitre plates (6) by conventional methods, recording 100% or 50% haemolysis. This is the standard method at the present time.

  i) Titrate the complement in haemolysis tubes, in the presence of 1 AgU, in order to determine the H50 unit.


ii) Inactivate the test serum and the positive and negative control sera in a water bath for 30 minutes at 56°C.

iii) Make doubling dilutions of test and control sera in CMV, from 1/4 to 1/1024, using a 96-well round-bottomed microtitre plate and 25 µl per well. Use the first well for the initial 1/4 dilution and the second as a serum control (anticomplementary control at 1/4 dilution). Provide antigen (without serum), complement, and RBC control wells (see below) (two wells of each).

iv) Add 1 AgU of MV antigen in 25 µl per well (except to serum, complement and RBC control wells), then add 6 H50 of complement in 25 µl per well (except to RBC control wells).

v) Incubate the plates, covered with plastic film, for 14 hours (overnight) at 4°C.

vi) Add 50 µl per well of the haemolytic system

vii) Cover the plates again and incubate for 30 minutes at 37°C.

viii) Prepare H100, H75, H50, H25 haemolysis controls using complement controls (H100) and CMV.

ix) Read after centrifugation (1000 g, 10 minutes) or passive sedimentation at 4°C. The test sera results are determined as the highest dilution of serum that gives at least 50% haemolysis inhibition.

x) A negative serum should give haemolysis inhibition <50% at 1/4 dilution.

b) Indirect fluorescent antibody test

IFA test (11) is carried out using chicken embryo or RK-13 cell cultures in flat-bottomed wells of microtitre plates: cell suspension, 4 × 10^4 cells diluted in medium, is distributed into all wells and a confluent cell sheet is formed within 24 hours. The medium is discarded and 100 µl of viral suspension (with a multiplicity of infection of 0.05) is added to each well. After 2 hours, 100 µl of Minimal essential medium (MEM) containing 2% calf serum is added. After 48 hours of incubation, the plates are washed with PBS and fixed with acetone containing 50% ethanol for 30 minutes at –20°C, or paraformaldehyde (4% in PBS) at room temperature. The plates are then dried at 37°C for 15 minutes. The plates can be stored at −30°C or −70°C for 3 months. Sera are tested by IFA using anti-rabbit IgG conjugated to fluorescein isothiocyanate. The test results may be qualitative with sera diluted 1/20, or quantitative with serial dilutions of serum.

c) Enzyme-linked immunosorbent assay

A recently developed ELISA (10) uses a semi-purified myxomatosis virus (French hypervirulent T1 strain antigenically related to Lausanne strain) produced in RK-13. The virus is harvested as a suspension of cells 48 hours after infection, and is centrifuged. The cell pellet is homogenised in TL20 (20 mM Tris, pH 8.6, 150 mM NaCl, and 1 mM EDTA), disrupted in ground glass and centrifuged at 1200 × g for 10 minutes.

The supernatant fluid is laid down on an equal volume of a 36% sucrose cushion in TL20 and centrifuged at 200,000 g for 2 hours in an SW 41 rotor at 4°C. The pellet is homogenised in 4–12 ml TL20 and again laid down on a 36% sucrose cushion.

The new pellet is homogenised in 0.5–1 ml TL20 and quantified by the Bradford method (colorimetric reaction with Coomassie brilliant blue) (3) or spectrophotometry (viral proteins account for around three-fifths of total protein). It can be stored at −30°C before use.

i) Coat probind (Falcon) assay plates for 16 hours (overnight at 37°C) with 1 µg per well viral proteins in 100 µl PBS, pH 7.6, leaving one column blank (i.e. coat with PBS only). Note that different batches of antigen vary in activity, and should be titrated against known standards to select antigen with high optical densities (OD).

ii) After three washes in PBS, block free binding sites by incubation in 25 mg/ml gelatin in PBS for 1 hour at 37°C.

iii) Wash the plate three times in PBS–0.01% Tween 20, and add 100 µl serial twofold dilutions of serum in PBS–Tween. Include positive and negative serum standards in each plate.

iv) After 60 minutes' incubation at 37°C and three washes in PBS–Tween, add 100 µl of a dilution in PBS–Tween of goat anti-rabbit IgG serum (previously tested) conjugated to alkaline phosphatase for 1 hour at 37°C.

v) After four washes in PBS–Tween and one more in PBS alone add, as substrate, 100 µl of disodium p-nitrophenyl phosphate at a concentration of 1 mg/ml in 10% diethanolamine.

vi) After 12 minutes in the dark at room temperature, the enzymatic reaction is stopped by the addition of 50 µl of 2 N NaOH.

vii) Read OD in a spectrophotometer at a wavelength of 405 nm.
viii) Express the serum sample titre as the inverse of the highest dilution for which the OD value is bigger than three times the OD value of the negative serum standard at the same dilution.

The detection of specific MV antibodies by ELISA has been shown to be a highly sensitive and specific method for kinetic studies in experimental infection (3). Evaluation of the test has shown its great value for diagnostic application in wild rabbit populations (10).

For epidemiological surveys, the IFA test and the indirect ELISA can also be carried out using blood dried on blotting or filter paper: discs are cut (paper punch size) and two discs are placed in each well, to which is added 100 μl PBS to extract the serum. The extract product is about 1/20 diluted and can be used as a fresh sample for testing (11). Blood samples collected in anticoagulant-coated capillary tubes can be used for the ELISA. The sample is washed in the diluting solution to obtain the required dilution (17).

d) **Agar gel immunodiffusion test**

Agar gel immunodiffusion (AGID) (22) is qualitative and can detect antigen or antibody. Agar is prepared as described previously (Section B.1) using 6 ml per 10 cm Petri dish. Strips of filter paper containing the standard antigen and antiserum, and discs containing test sera are arranged on the surface of the agar (discs between the strips). The plates are incubated in a humid atmosphere at 37°C and read after 24–48 hours. Three precipitation lines should appear. If the test sera contain MV-specific antibody, at least one of the three lines is bent towards the antigen band; otherwise it remains straight. If sera contain MV antigen, at least one of the lines is bent towards the standard serum strip. The test can also be carried out in a more conventional manner using liquid reagents in wells cut in the agar.

C. **REQUIREMENTS FOR VACCINES AND DIAGNOSTIC BIOLOGICALS**

Two types of live vaccine are used for vaccination against myxomatosis: a heterologous vaccine prepared from Shope's fibroma virus (9, 13, 21), and a homologous vaccine prepared from an attenuated strain of MV (1, 12, 20, 23, 25). They are administered subcutaneously or intradermally.

A new recombinant MV expressing rabbit haemorrhagic disease virus (RHDV) capsid protein and conferring double protection against myxomatosis and RHDV (3) has been developed, but is not yet available commercially.

Guidelines for the production of veterinary vaccines are given in Chapter 1.1.8 Principles of veterinary vaccine production. The guidelines given here and in Chapter 1.1.8 are intended to be general in nature and may be supplemented by national and regional requirements.

A master seed virus (MSV) must be established and used according to a seed-lot system. A record must be kept of its origin, passage history and characteristics.

1. **Seed management**

   a) **Characteristics of the seed**

   The viruses employed are Shope’s fibroma virus or MV. The strains of Shope’s fibroma virus are usually the original Shope’s OA strain (1932), Boerlage’s strain or various closely related strains. The strains of MV are modified by passaging in embryonated chicken eggs, rabbit kidney cells at decreasing temperatures, or chicken embryo cells. The strains usually result from having been cloned several times.

   b) **Method of culture**

   Shope’s fibroma virus strains are maintained by passage in specific pathogen free (SPF) rabbits or in unvaccinated rabbits from a stock known to be free from myxomatosis. Skin on the backs of healthy adult rabbits is shaved, and multiple sites are inoculated with a 1% suspension of virulent material. Fibromas are fully developed within 8–10 days, at which time the rabbits are killed and the tumours are removed aseptically and homogenised with distilled water. The suspension is stored at −30°C or −70°C in 50% buffered glycerol, or as a 5% dilution in a protein solution (bovine albumin). The production of the Shope’s fibroma virus is also possible in rabbit dermal cell line.

   MV is grown on chicken embryo cell culture obtained from flocks free from specified pathogens, or on suitable cell lines (rabbit dermal cell line). Virus can also be cultivated on RK-13 cells.

   c) **Validation as a vaccine**

   i) **Identity**

   Specific antigenic characteristics of the Shope’s fibroma virus strains are verified by AGID using sera against fibroma and myxomatosis.
The identity of MV is confirmed by neutralisation tests in RK-13 cells, or in a suitable cell line using a monospecific antiserum (produced by vaccination of rabbits with the specific vaccine viral strain).

ii) **Purity**

The Master seed must be free from bacterial, fungal, mycoplasmal and viral contamination.

iii) **Safety**

Samples for safety testing are taken from a batch produced according to the manufacturing process. The dose to be used shall contain the maximum titre or potency established by the manufacturer (release titre).

Several tests are performed, at the Master Seed level, to demonstrate different aspects of safety. The safety of 10 times the normal dose must be demonstrated. Also, it is necessary to examine the dissemination of vaccine virus within the vaccinated animal, the ability of vaccine virus to spread from the vaccinated animal to in-contact animals and to test whether there is reversion-to-virulence of the vaccine virus, following serial passage in rabbits.

The pathogenicity of the Shope’s fibroma virus strains is tested by inoculating rabbits with serial dilutions of supernatant fluids obtained by centrifugation of tumour preparations. Macroscopic and histopathological features and the course of development of fibromas are tested in SPF rabbits periodically. (Numerous serial passages in rabbits may induce mutation to the inflammatory IA strain, which produces severe lesions that are more inflammatory than neoplastic.)

The residual pathogenicity of the MV strains is tested by intradermal inoculation into SPF rabbits or unvaccinated rabbits free from myxomatosis. These rabbits should not develop more than a local reaction with perhaps small secondary lesions on the head that disappear within a few days.

For both strains, the rectal temperature and the body weight should be recorded as additional parameters.

iv) **Efficacy**

Different trials must be undertaken from representative batches of final product containing the minimum titre or potency. The protective effect is demonstrated as follows:

A minimum of ten adult rabbits are inoculated with a dose of fibroma vaccine, and three rabbits serve as unvaccinated controls. After 14 days, all rabbits are inoculated, intradermally into the eyelids, with a pathogenic strain of MV (example: 0.1 ml inoculum containing $10^{3}\text{ID}_{50}$ [median infectious dose]). During the following 21 days, the controls will die from myxomatosis, and at least seven of the ten vaccinated rabbits must present no signs of generalised infection.

Similarly, myxoma vaccine is tested in ten rabbits with three controls. After 14 days, all the rabbits are challenged with a sufficient quantity of virulent strain (example: 0.1 ml of the Lausanne virus strain containing $10^{5}\text{ID}_{50}$). After 21 days, seven of ten vaccinated rabbits must have survived, while controls must have died from myxomatosis.

The manufacturer shall have established a minimum titre or potency taking into account loss in potency during the shelf life.

2. **Method of manufacture**

Shope’s fibroma virus is produced by multiple intradermal inoculations of seed virus into the skin on the back of a number of rabbits. The product of fibroma homogenate can be stored by freezing or used immediately. Production is also possible in rabbit dermal cell line. Only the second (and perhaps the third) viral passage can be used if modification of the virus is to be avoided. After clarification by centrifugation, the supernatant fluid is mixed with a stabiliser containing antibiotics and is distributed into ampoules or bottles for lyophilisation. Kaolin may be added as an adjuvant (40 mg/ml), in which case the vaccine is administered subcutaneously.

MV is produced in chicken embryo cells (derived from SPF eggs) or a suitable cell line, limiting the passage number to a maximum of five. Virus is harvested after 2–6 days. The viral suspension may be stored at –70°C. The vaccine is prepared by diluting in specified proportions the viral preparation with a stabiliser for lyophilisation. After homogenisation, the product is distributed into bottles for lyophilisation, the bottles being sealed under vacuum or in sterile nitrogen.

Each virus can also be produced in RK-13 cells.

3. **In-process control**

The Shope’s fibroma virus titre is measured by calculating the ID$_{50}$ after intradermal inoculation of serial dilutions of the clarified supernatant fluid into several sites (e.g. five) on up to six rabbits. A dilution of a standard
preparation of Shope’s fibroma virus is also inoculated into each rabbit to confirm the animal’s correct response to inoculation. The titration can also be performed in a rabbit cell line. In each case the titre should correlate with the required potency as defined by the test for efficacy, see Section C.1.c.

The identity of MV is checked in RK cells. Titration of each virus can also be done in RK-13 cells (TCID₅₀).

Testing for contaminating viruses is done by inoculating a confluent monolayer of Vero cells. Vaccine, adjusted to the equivalent of 20 doses/ml, is neutralised with an equal volume of monospecific hyperimmune serum for 30 minutes at 37°C. The mixture is filtered through a 0.22 µm membrane filter, and 1 ml volumes are inoculated into five 25 ml bottles of cell cultures. These are kept under observation for 7 days. After harvesting, the cells are suspended in medium and subjected to several freeze–thaw cycles, followed by centrifugation and filtration, and the material is inoculated into fresh cultures and observed for 7 days. There should be no evidence of CPE or haemadsorption to chicken RBCs.

4. Batch control

a) Sterility

Tests for sterility and freedom from contamination of biological materials may be found in Chapter 1.1.9.

b) Safety

After rehydration, ten doses of the lyophilised fibroma vaccine are injected subcutaneously into each of three susceptible rabbits, which are then observed for 21 days. Local reactions should be slight, with no generalisation and no effect on general health.

Myxoma vaccine is tested using ten doses injected intradermally into the ears of three susceptible rabbits, which are then observed for 21 days. The primary myxoma lesion should remain mild.

c) Potency

The batch potency is determined by measurement of virus content. Serial dilutions of the vaccine are inoculated into suitable cell cultures. One dose of vaccine shall contain not less than the minimum titre established in Section C.1.c.

If the vaccine strain is not adapted to cultures, an efficacy test in rabbits shall be carried out (see Section C.1.c).

d) Duration of immunity

Several groups of ten susceptible rabbits are vaccinated. One batch is tested by challenge infection (as in the batch potency test), at 1, 2, 3, etc., months post-vaccination for Shope’s fibroma virus, and at 1, 3, 6, and 9 months for MV. The duration of immunity is deduced from the time during which at least seven of the ten rabbits prove to be resistant to infection.

e) Stability

Titrations of vaccine virus are carried out at intervals until 3 months beyond the requested shelf life on at least three batches of vaccine.

5. Tests on the final product

a) Safety

See Section C.4.b.

b) Potency

See Section C.4.c.

REFERENCES


Chapter 2.6.1. — Myxomatosis


* * *
CHAPTER 2.6.2.

RABBIT HAEMORRHAGIC DISEASE

SUMMARY

Rabbit haemorrhagic disease (RHD) is a highly contagious and acute fatal disease of the European rabbit (Oryctolagus cuniculus), caused by a calicivirus. A similar disease, caused by a different calicivirus and termed European brown hare syndrome (EBHS), has been described in the hare (Lepus europaeus). The aetiological agent is a different calicivirus, antigenically related to the RHD virus (RHDV). RHD is characterised by high morbidity and high mortality (40–90%), and spreads very rapidly by direct and indirect transmission. Infection can occur by nasal, conjunctival or oral routes. Transmission of RHD is facilitated by the high stability of the virus in the environment. The incubation period varies from 1 to 3 days, and death usually occurs 12–36 hours after the onset of fever. The clinical manifestations have been described mainly in the acute infection (nervous and respiratory signs, apathy and anorexia). Clear and specific lesions, both gross and microscopic, are present. There is primary liver necrosis and a massive disseminated intravascular coagulopathy in all organs and tissues. The most severe lesions are in the liver, trachea and lungs. Petechiae are evident in almost all organs and are accompanied by poor blood coagulation.

Identification of the agent: The liver contains the highest viral titre and is the most suitable organ for viral identification. As no satisfactory growth conditions or sensitive cell substrates have been established, in-vitro isolation cannot be employed. The haemagglutination test using human Group O red blood cells was the first test applied for routine laboratory diagnosis of RHD. However, other tests (negative-staining electron microscopy, sandwich enzyme-linked immunosorbent assay [ELISA], immunohistological staining, polymerase chain reaction and Western blot) have shown a higher level of specificity and sensitivity.

Serological tests: Characterisation and titration of specific antibodies arising from natural infection or from immunisation are performed using the haemagglutination inhibition test or an indirect or competitive ELISA. The following reagents are prepared: antigen from infected rabbit liver, anti-RHDV serum from convalescent or hyperimmunised rabbits, and negative serum from rabbits fully sensitive to RHDV infection. Monoclonal antibodies have been produced in several laboratories. Some laboratories have produced a recombinant antigen, VP60 structural protein expressed in baculovirus, which can also be used as a diagnostic reagent.

Requirements for vaccines and diagnostic biologicals: Indirect control of the disease is achieved by vaccination using a killed vaccine prepared from clarified liver suspensions of experimentally infected rabbits and subsequently inactivated and adjuvanted. Vaccinated animals quickly produce solid protective immunity against RHDV infection (within 5–10 days) and experimental data indicate that protection lasts for a long period (over 1 year).

A. INTRODUCTION

Rabbit haemorrhagic disease (RHD) is a highly contagious and acute fatal disease of wild and domestic European rabbits (Oryctolagus cuniculus).

RHD was first reported in 1984 in the People’s Republic of China (26); RHD has been reported in over 40 countries in Asia, Central America, Europe and Oceania. Outbreaks have also been recorded in Mexico, Saudi Arabia and West and North Africa. In 2000 and 2001, three independent outbreaks were recorded in the United States of America (USA). At the end of 2004 it was reported in Uruguay and in 2005 again in the USA. RHD is still endemic in most parts of the world.
The causative agent of RHD is a calicivirus that is 32–35 nm in diameter and has a single major capsid polypeptide (60 kDa), a positively stranded RNA genome of 7437 kb and a sub-genomic RNA of 2.2 kb (8, 28, 29, 33). The RHD virus (RHDV) capsid protein (VP60) folds in two distinct domains held together by a hinge region: the N-terminal 200–250 residues constitute the inner domain and the C-terminal residues beyond 200–250 constitute the protruding domain. In the overall picture of the capsid, these domains form the inner shell and the outer shell, respectively, which is characterised by arch-like structures. This structure also correlates with the antigenic characteristics of RHDV, and the main antigenic determinants are located on the C-terminal end of the VP60 (3, 4, 35, 41).

Since 1991, a second type of virus particle was identified as the main component in approximately 5% of the RHDV-positive specimens, i.e. those taken from rabbits showing a protracted course of the disease (7). The characteristics of this particle are: i) a smooth surface and a diameter smaller than RHDV; ii) its protein is of 28–30 kDa; iii) it is reactive with RHD convalescent rabbit sera and with those anti-RHDV monoclonal antibodies (MAbs) reactive against the N-terminal end of the RHDV VP60; and iv) it is haemagglutination (HA) negative. This smaller viral particle only corresponds to the inner shell of RHDV, and two hypotheses have been proposed to explain its origin. Granzow et al. (19) assumed that it arose from a truncated RHDV genome or defective expression. However, Barbieri et al. (1) observed the following: i) a strict correlation between the higher prevalence of smooth RHDV (s-RHDV) particles in the organs and the appearance of specific anti-RHDV IgM at 3–4 days post-infection; ii) the presence of large amounts of s-RHDV only in the liver and spleen and not in the bloodstream, as occurs during the viraemic phase of acute RHD; and iii) the finding of fragments of the VP60 having different molecular weights (41–30 kDa) during transition from RHDV to s-RHDV. They concluded therefore that the genesis of the s-RHDV particle is due to a degenerative process that is probably the consequence of the physiological clearance of the RHDV-IgM immuno-complex formed in large amounts at the beginning of the humoral response. Apart from its origin, the identification of this second particle in the liver of a rabbit can be considered to be a marker of the subacute/chronic form of RHD that usually evolves between 4 and 8 days post-infection and is followed either by the death of the rabbit or, more often, by its recovery (1).

Most known RHD viral isolates appear to belong to one serotype. The complete sequence of genetically different RHD strains has been reported. Comparison reveals close overall homology in terms of genome sequence with few or no predicted changes in amino acid composition (differences between 2% and 5%). Nevertheless, isolates that exhibit temperature-dependant differences in haemagglutinating characteristics (2) have been described, and more recently a consistent genetic and antigenic RHDV variant has been identified simultaneously in Italy (3) and Germany (35). This RHDV variant, named RHDVa, presents amino acid changes in the surface-exposed E region (aa 344–434) that contains the main antigenic epitopes of calicivirus, three times higher than in all previously sequenced RHDV isolates. A group of related MAbs that protected against infection by RHDV were negative when tested by enzyme-linked immunosorbent assay (ELISA) against RHDV antibodies. However, rabbits experimentally vaccinated with the currently available RHDV vaccine were protected against challenge with RHDVa, even if with a lower efficiency (3, 35).

An epidemiological study carried out to compare the rate of spread of RHDV and RHDVa in Italy during the past few years (24) has shown that RHDVa is present in most parts of Italy and that it is rapidly replacing the RHDV ‘classical’ strain. Outside Italy, RHDVa was identified almost contemporaneously in Germany, but it also caused the first outbreaks of RHD in the USA during the spring of 2000, in Uruguay during the winter of 2004 and again in the USA in 2005. It has also been detected in France (2000) and Malta (2004), which suggests that RHDVa could be spreading in other European countries that have been experiencing the disease for many years. Taking account of the RHDV genetic sequences deposited at the NCBI databank, the presence of RHDVa in the People’s Republic of China is also evident.

Another virus, provisionally called rabbit calicivirus (RCV) and related to RHDV, has been identified in healthy rabbits (5, 6). It is significantly different from the previously characterised RHDV isolates in terms of pathogenicity, viral titre, tissue tropism, and primary sequence of the structural protein. It is avirulent, replicates in the intestine at a low titre and has about a 92% genomic similarity to RHDV. Results of cross-protection experiments indicate that RCV will not infect hares. In addition, the antigenic data and sequence comparisons have demonstrated that it is more closely related to RHD than to the European brown hare syndrome virus (EBHSV) (5).

As a result of the extensive use of serological tests on different rabbit populations, further evidence has been found showing that, in addition to RCV, one or more RHDV-like non-pathogenic viruses are present in wild rabbit populations over a large part of south-eastern Australia as well as in New Zealand (11, 31, 34).

Antibodies against RHD were detected in sera collected in Europe between 1975 and 1987, showing that RHDV-like viruses were already present, but had simply not been detected before the first signs of the disease. More recent serological data suggest that non-pathogenic strains may usually be present in wild European rabbit populations, because high antibody levels have been detected even in areas where RHD had never been recorded or suspected (27).
A study carried out in Britain showed that RNA particles related to RHDV were present in sera collected since 1955, confirming that RHDV-like viruses were present in Europe a long time before the first evidence of RHD (30). However, according to the authors’ data (30, 40), RHDV causes a highly prevalent persistent infection in seropositive rabbits in the absence of associated mortality and, since the responsible viral strain could not be phylogenetically distinguished from known pathogenic isolates, they suggest that “many – perhaps most – strains of RHDV may be propagated through both ‘pathogenic’ and ‘non-pathogenic’ modes of behaviour”.

RHDV is very stable and persists in the environment; the viral infectivity is not reduced by treatment with ether or chloroform and trypsin, by exposure to pH 3.0, or by heating to 50°C for 1 hour. The virus survives at least 225 days in an organ suspension kept at 4°C, at least 105 days in the dried state on cloth at room temperature, and at least 2 days at 60°C, both in organ suspension and in the dried state (36). Recent work suggests that RHDV in rabbit carcasses can survive for at least 3 months in the field, while virus exposed directly to environmental conditions is viable for a period of less than 1 month (22). RHDV also retains its infectivity at low temperatures, and remains quite stable during freezing and thawing. RHDV is inactivated by 1% sodium hydroxide and by other agents (e.g. bleach) that cause destruction of the viral proteins by increasing the pH to >12. Treatment with 1.0–1.4% formaldehyde or 0.2–0.5% beta-propiolactone at 4°C inactivates the virus but does not reduce its immunogenicity and is therefore indicated for the production of vaccines.

The European rabbit (Oryctolagus cuniculus) is the only species known to be affected by RHD. No other lagomorphs, such as the Volcano rabbit of Mexico (Romerolagus diazzi), the black-tail jackrabbit (Lepus californicus) and the cottontail (Sylvilagus floridanus) of North America, have been shown to be susceptible (20). Inoculation of tissue suspensions from infected rabbits into 28 different vertebrate species other than rabbits failed to produce disease and no replication of the virus was detected by reverse-transcription polymerase chain reaction (RT PCR) (18). A similar disease, termed European brown hare syndrome (EBHS), has been described to produce disease and no replication of the virus was detected by reverse-transcription polymerase chain reaction (18). A similar disease, termed European brown hare syndrome (EBHS), has been described in the hare (Lepus europaeus), but its aetiological agent, EBHSV, also a calicivirus, is distinct from RHDV while still sharing some antigenic similarity. Alignment of the RNA sequences of the EBHSV and RHDV genomes shows 71% nucleotide identity, and amino acid alignment shows 78% identity and 87% similarity (41). Cross infection does not occur by experimental infection of rabbits with EBHSV and hares with RHDV (23). Recent studies aimed at finding the susceptibility of cottontail rabbits to EBHSV revealed a diffuse seroprevalence of the virus in a wild population of cottontail rabbits and the possibility of inducing clinical disease and mortality in a low number of experimentally infected cottontails (Lavazza, unpublished data).

RHD is characterised by high morbidity and a mortality rate between 40% and 90%. Infection occurs in rabbits of all ages, but clinical disease is observed only in adults and animals older than 40–50 days. The pathogenic mechanism of resistance in young animals is still unclear (7). A difference in the cellular inflammatory response of susceptible adult and resistant young rabbits has been observed. The mechanism of resistance in young animals is still unclear (7). A difference in the cellular inflammatory response of susceptible adult and resistant young rabbits has been observed. The disease in hares lasts slightly longer and causes a lower mortality rate (around 50%) than RHD in rabbits; the peak of mortality in experimentally infected hares is commonly observed between 60 and 90 hours post-infection.

While the clinical evolution of the disease can be peracute, acute, subacute or chronic, clinical manifestations have been described mainly in the acute infection, as there are usually no clinical signs of disease in the peracute form, and the subacute form is characterised by similar but milder signs. The incubation period varies between 1 and 3 days; death may occur 12–36 hours after the onset of fever (>40°C). During an outbreak, a limited number of rabbits (5–10%) may show a chronic or subclinical form of the disease. These animals often die 1 or 2 weeks later, probably due to a liver dysfunction. Gross pathological lesions are variable and may be subtle. Liver necrosis and splenomegalia are the primary lesions. Gross findings include a pale swollen friable liver, enlarged spleen and the presence of clotted blood in blood vessels caused by disseminated intravascular coagulopathy (DIC). Such massive coagulopathy is usually the cause of haemorrhages in a variety of organs and sudden death (20, 39). In subacute and chronic disease, an icteric discoloration of the ears, conjunctiva and subcutis is clearly evident.

The clinical signs and the gross and microscopic lesions observed in hares affected by EBHSV are very similar to those described in rabbits with RHD. At necropsy, oedema and congestion of tracheal mucosa with foamy haemorrhagic contents, liver degeneration, enlargement of the spleen and generalised jaundice are the principal findings (7). The disease in hares lasts slightly longer and causes a lower mortality rate (around 50%) than RHD in rabbits; the peak of mortality in experimentally infected hares is commonly observed between 60 and 90 hours post-infection.

**B. DIAGNOSTIC TECHNIQUES**

1. **Identification of the agent**

The liver contains the highest viral titre (from 10^3 LD_{50} [50% lethal dose] to 10^6.5 LD_{50}) and is the organ of choice for viral identification for both RHDV and EBHSV. The amount of virus present in other parts of the body is directly
proportional to vascularisation; thus spleen and serum may serve as alternative diagnostic materials, albeit suboptimal. Higher levels of subviral particles have been reported in the spleen rather than the liver of animals that died from a subacute/chronic form of RHD (1). The initial treatment of the diagnostic samples is almost identical irrespective of the diagnostic method to be applied, with the exception of immunostaining techniques. An organ fragment is mechanically homogenised in 5–20% (w/v) phosphate buffered saline solution (PBS), pH 7.2–7.4, filtered through cheesecloth and clarified by centrifugation at 5000 g for 15 minutes. At this stage, the supernatant can be directly examined by the HA test or ELISA. If the sample is to be observed by electron microscopy (EM), it is advisable to perform a second centrifugation at 12,000 g for 15 minutes, before the final ultracentrifugation. For detection by PCR, viral RNA from the samples may be directly extracted from tissues or from the unclarified homogenate.

As no satisfactory growth conditions, in-vitro isolation of RHDV or EBHSV cannot be included among the diagnostic methods. Inoculation of tissue suspensions from infected rabbits failed to produce disease in 28 different vertebrate species other than rabbits; no replication of the virus was detected by reverse-transcription polymerase chain reaction (RT-PCR) in the inoculated animals (18). Rabbit inoculation therefore remains the only way of isolating, propagating and titrating the infectivity of the virus. However, experimental infection of rabbits is not a practical method for the routine diagnosis of RHD, but could be useful for the testing of samples that give equivocal test results (e.g. HA negative/ELSIA positive) or for the initial diagnosis of the disease in countries where RHD is not known to exist. Propagation of RHDV in rabbits is also useful for large-scale production of viral antigen for diagnostic reagents or to produce inactivated RHDV vaccines.

To perform successful experimental trials, the rabbits involved must be fully susceptible to the virus, i.e. they should be over 40–50 days and have no specific antibodies, even at low titres. RHD can be reproduced by using filtered and antibiotic-treated liver suspensions, inoculated either by the intramuscular, intravenous or oro-nasal route. When the disease is clinically evident, the signs and post-mortem lesions are similar to those described after natural infection. A rise in body temperature is registered between 18 and 24 hours post-infection, followed, in around 70–90% of cases, by death between 24 and 72 hours post-infection. A few individuals may survive until 6 days after infection. Animals that overcome the disease show only a transient hyperthermia, depression and anorexia, but present a striking seroconversion that can be detected easily 4–6 days post-infection.

a) Haemagglutination test

HA was the first test to be used for routine laboratory diagnosis of RHD (26). It should be performed with human Group O red blood cells (RBCs), freshly collected, stored overnight in Alsever’s solution, and washed in 0.85% PBS at pH 6.5 (range 6–7.2). HA is less evident or non-existent when RBCs of other species are used. Washed RBCs are suspended at 0.75% in PBS. A twofold dilution of the clarified supernatant of a 10% tissue homogenate of liver or spleen is incubated with an equal volume of washed RBCs in a sealed round-bottom microtitre plate at, preferably, 4°C. After 1 hour (range from 20 minutes to 2 hours) of incubation, agglutination at an end-point dilution of >1/160 is considered to be positive. Lower titres should be regarded as suspicious, and should be checked using other methods. Around 10% of samples found to be positive by ELISA or EM give negative results in HA (HA false-negative). Some RHD isolates may exhibit temperature-dependant differences in haemoagglutinating characteristics (2) and could show HA activity only when the test is performed at 4°C. Nevertheless, the HA false negativity is mainly detected in organs of rabbits showing a subacute/chronic form of the disease and it depends on the characteristics of the smooth, truncated s-RHDV particles.

Hare organs rarely give a significant titre when the RHDV HA protocol is used. To demonstrate HA activity in organs from EBHSV-infected rabbits, a modified procedure should be adopted: all steps are carried out at 4°C, the organ suspension is treated with an equal volume of chloroform, and RBCs are used at a pH not higher than 6.5 (7). Even using this method, only about 50% of the samples give positive results. This is because the disease of hares is often subacute or chronic and therefore the virus has the antigenic and structural characteristics typical of the s-RHDV particles (7).

Because of the practical difficulty of obtaining, keeping and the risk from working with human Group O blood cells, and because of the difficulty of obtaining consistent results, this test has been replaced by the virus-detection ELISA.

b) Electron microscopy

Negative-staining EM can be performed using the so-called ‘drop method’. A formvar/carbon-coated grid is placed on a drop of organ suspension (prepared as described in Section B.1), and left for 5 minutes. After removing excess fluid with the edge of a torn piece of filter paper, the grid is put to float on a drop of 2% sodium phosphotungstate (NaPT), pH 6.8, for 1.5 minutes. Excess stain is removed and the grid is finally observed at ×25,000 magnification.

Due to the lower sensitivity of the drop method, it is advisable to ultracentrifuge the sample in order to concentrate the viral particles. The pellet obtained after ultracentrifugation (at least 100,000 g for 30 minutes...
or, alternatively, using Beckman Airfuge at 21 psi for 5 minutes) is resuspended in PBS or distilled water, put on to a grid for a few minutes, and then stained as described. RHD virions are visible as uncoated particles, 32–35 nm in diameter, presenting an inner shell (25–27 nm in diameter), delineated by a rim from which radiate ten short regularly distributed peripheral projections. Smooth (s-RHDV) particles are identifiable by the complete loss of external portions, becoming perfectly hexagonal and smaller, with only the capsid rim visible (1, 7, 19).

For diagnostic purposes and especially when other methods give doubtful results, the best EM method is an immuno-EM technique (IEM). This method uses either a hyperimmune anti-RHDV serum, obtained from rabbit or other species, or specific MAbs, which are incubated with an equal volume of the sample for 1 hour at 37°C before ultracentrifugation. The immunological reaction induces the clumping of the viral particles into aggregates that are quickly and easily identified by EM. Immunogold methods can also be applied to better identify virions and viral proteins.

EBHSV can also be identified in diagnostic samples by EM examination. In addition, the IEM method using convalescent anti-EBHSV serum or specific anti-EBHS MAbs can be used to identify EBHSV. By using antisera that is specific for EBHSV and RHDV, it is possible to differentiate between the two viruses.

c) Enzyme-linked immunosorbent assay

Virus detection by ELISA relies on a ‘sandwich’ technique and several variations of this have been described. One procedure uses the reagents, solutions, times and temperature that are used in the competitive ELISA (C-ELISA) for serology (see Section B.2.b), except that the Tween 20 concentration is twofold (0.1% [v/v]). The microplate used should be of high adsorption capability (e.g. Nunc Maxisorp immunoplate). The liver homogenate is a 10% (w/v) suspension in standard PBS; 50 µl/well is the standard volume to use in each step. The ELISA buffer used for all steps is PBS with 1% yeast extract (or bovine serum albumin [BSA]), and 0.1% Tween 20, pH 7.4. All incubation steps are for 50–60 minutes at 37°C with gentle agitation. After all steps three washes of 3–5 minutes must be performed using PBS with 0.05% Tween 20. A positive and negative RHD rabbit liver homogenate must be used as controls. The horseradish peroxidase (HRPO) conjugate could be purified IgG from a specific polyclonal serum or MAbs (see Section B.2.b). Anti-RHDV MAbs have been produced in several laboratories and can be used instead of rabbit polyclonal sera. More recently, MAbs recognising specific epitopes expressed only by the RHDVa variant were also produced (Capucci, pers. data).

To better characterise the antigenicity of the RHD isolates by sandwich ELISA, it is advisable to test each sample in four replicates, and then to use four different HRPO conjugates, i.e. two MAbs recognising the same antigenic determinant present on the virus surface and expressed alternatively by the ‘classical’ strain or by the RHDVa variant, a polyclonal hyperimmune anti-RHDV serum (which could identify potential ‘new variant’ or correlated calicivirus, such as EBHSV) and a pool of MAbs recognising internal epitopes that can detect smooth, degraded s-RHDV particles as well as EBHSV. An alternative antigen-capture ELISA has been described using a sheep anti-RHDV as the capture antibody and an MAb for detection of RHDV (10).

• Test procedure (example)

For steps that are not specifically indicated see the procedure of the C-ELISA for serology (Section B.2.b).

i) Coat the plate with anti-RHDV hyperimmune serum and the negative RHDV serum. The latter serves as control for nonspecific reactions (false-positive samples). For each sample, four wells must be sensitised with the positive serum and four wells with the negative one.

ii) Dilute the liver extract to 1/5 and 1/30 (two replicates for each dilution) in ELISA buffer (see above), directly in the wells of the plate (e.g. add 45 µl of the buffer into all the wells of the plate, add 10 µl of the sample to the first two wells and then, after rocking, transfer 9 µl to the second wells). Treat the controls, both positive and negative, in the same way as the samples.

iii) After incubation and washing (see above), incubate with the HRPO conjugate.

iv) After a last series of washing, add the chromogenic substrate. Orthophenylene-diamine (OPD) must be used as peroxidase substrate for the final development of the reaction. Use 0.15 M citrate phosphate buffer, pH 5.0, with 0.5 mg/ml OPD and 0.02% H2O2. The reaction is stopped after 5 minutes by the addition of 50 µl of 1 M H2SO4.

v) Absorbance is read at 492 nm. Positive samples are those showing a difference in absorbance >0.3, between the wells coated with RHDV-positive serum and wells coated with the negative serum. Usually, at the dilution 1/30, positive samples taken from rabbits with the classical acute form of RHD give an absorbance value >0.8, while the absorbance value of the negative sample, at the dilution 1/5, ranges from 0.1 to 0.25.
For diagnosis of EBHSV, it is possible to use this RHDV-specific sandwich ELISA, but, due to the high antigenic difference existing between the two viruses, there is a risk of obtaining false-negative results. Therefore, the adoption of an EBHSV-specific sandwich ELISA technique using either a high-titre positive anti-EBHSV hare serum, or cross-reacting RHDV MAbs (4, 7), or specific EBHSV MAbs, instead of rabbit serum, is highly recommended (7).

d) Immunostaining

Tissue fixed in 10% buffered formalin and embedded in paraffin can be immunostained using an avidin–biotin complex (ABC) peroxidase method. The sections are first deparaffinised in xylene and alcohol, counter-stained with haematoxylin for 1 minute and rinsed in tap water. They are then put in a methanol bath containing 3% H2O2 and washed in PBS three times for 5 minutes each. To limit background interference due to nonspecific antibody binding, the samples are incubated with normal rabbit serum for 1 hour at room temperature prior to the addition of biotin. The slides are incubated overnight in a humid chamber at room temperature with biotinylated rabbit anti-RHDV serum or MAbs, are washed as before and incubated again for 30 minutes at 37°C with an ABC peroxidase. The slides are then washed three times. Aminoethylcarbazole is used as substrate. Finally, the slides are rinsed in tap water and mounted (37).

Intense nuclear staining and diffuse cytoplasmic staining of necrotic cells in the liver, mainly in the periportal areas, are characteristic and specific. Positive staining of macrophages and Kupffer’s cells is also observed, as well as hepatocellular reactions. Positive reactions can also be detected in the macrophages of the lungs, spleen and lymph nodes, and in renal mesangial cells (37).

Tissue cryosections fixed in methanol can be directly immunostained by incubation for 1 hour with fluorescein-conjugated rabbit anti-RHDV serum or MAbs. Specific fluorescence can be detected in the liver, spleen, and renal glomeruli.

e) Western blotting

When other tests such as HA or ELISA give doubtful results (low positivity) or the samples are suspected of containing s-RHDV particles, western blotting analysis is useful for determining the final diagnosis.

Homogenates are prepared as described previously, and virus particles are further concentrated (tenfold) by ultracentrifugation (100,000 g for 90 minutes) through a 20% (w/w) sucrose cushion.

Both the supernatant and the pellet can be examined to detect, respectively, the RHDV 6S subunits (4) and the denatured VP60 structural protein of RHDV or its proteolytic fragments, which can range in size from 50 to 28 kDa. A positive and negative control samples should be used on each occasion.

RHDV proteins could be detected with polyclonal antibodies or MAbs. If MAbs are used, they should recognise continuous epitopes. RHDV-specific MAbs recognising internal or buried epitopes could be used also to detect EBHSV. Rabbit anti-RHDV hyperimmune sera are less efficient than MAbs at recognising the same band patterns (5).

Sample proteins are denatured for 2 minutes at 100°C in the presence of 60 mM Tris, pH 6.8, 2% sodium dodecyl sulphate (SDS), 2% beta-mercaptoethanol, and 5% glycerol, separated on 10% SDS/PAGE (polyacrylamide gel electrophoresis), and then transferred by electroblotting to nitrocellulose or PVDF (polyvinylidene fluoride) membranes, in 25 mM Tris, 192 mM glycine pH 8.3 and 20% (v/v) methanol at 1.5 Å for 60 minutes with cooling or at 0.15 Å overnight. After transfer the membranes are saturated for 30–60 minutes in blocking buffer or PBS, pH 7.4 containing 2% bovine serum albumin (BSA), and subsequently incubated for 2 hours at room temperature with the appropriate serum dilution in PBS, pH 7.4, and 1% BSA. The filters are washed thoroughly with PBS and incubated for 1 hour at room temperature with anti-species alkaline phosphatase-labelled immunoglobulins at a dilution predetermined by titration. Finally, the filters are again washed and the chromogenic substrate (5-bromo-4-chloro-3-indolylphosphate nitro blue tetrazolium) is added.

Positive test samples and the positive control will produce a pattern consistent with reaction to proteins of molecular weights of, respectively, 60 kDa (the single structural protein of RHDV) or 41–28 kDa (the fragments of the VP60 associated with the transition from RHDV to s-RHDV), when examining the pellet, and 6 kDa (the subunits) when examining the supernatant.

Western blot analysis can also be used to identify EBHSV. The test procedure is identical. The pattern of protein bands, detected using either an anti-EBHSV polyclonal serum or cross-reacting anti-RHDV MAbs, is similar. However, the percentage of samples showing viral degradation is higher and therefore several fragments of lower molecular weight, originating from the VP60 structural protein, are often observed.
f) Nucleic acid recognition methods

The application of the RT-PCR to the detection of RHDV-specific nucleic acid has been described by several authors (18, 21). Owing to the low level of sequence variation among RHDV isolates and the high sensitivity of PCR, reverse transcription (RT)-PCR represents an ideal rapid diagnostic test for RHD. This method is carried out on organ specimens (optimally liver), urine, faeces and sera using different oligonucleotide primers derived from the capsid region of the RHDV genome (N-terminal portion). cDNA obtained from the RT reaction is usually PCR amplified as described by Guittre et al. (21). To reveal the PCR product, the amplified DNA reaction mixture is subjected to electrophoresis on agarose gel. If needed, specificity of the PCR product can be determined by sequencing or by Southern blot and hybridisation with a radioactively labelled internal probe. A similar RT-PCR method has been used to identify the nonpathogenic RCV (3). Several primers, specific for the RHDV RNA polymerase gene and complementary to the VP60 and ORF2 genes, are used and the amplified fragments are subjected to Southern blot analysis. RT-PCR represents an extremely sensitive method for the detection of RHDV, and is $10^4$-fold more sensitive than ELISA (21). It is not strictly necessary for routine diagnosis, but it is more sensitive, convenient and rapid than other tests.

Similarly an RT-PCR for the detection of EBHSV has been applied to the detection and characterisation of EBHSV strains (25, 32). An internally controlled multiplex real-time RT-PCR using TaqMan probes and external standards for absolute RNA quantification has been developed recently as a further diagnostic tool for the detection of RHDV. The test revealed a specificity of 100%, an analytical sensitivity of 10 copies/well and a linearity over a range from $10^1$ to $10^{10}$ copies. The method has been used to quantify RHDV RNA in experimental infection of vaccinated rabbits and in RHD convalescent rabbits (15, 16).

An in-situ hybridisation technique using either sense or antisense DNA probes has been developed for investigating the presence of RHDV in tissue samples (17). This method is highly sensitive and can be used for early diagnosis of RHD as it gives positive results as soon as 6–8 hours post-infection. However, it is expensive and difficult to carry out, and thus it is mainly indicated for research studies.

2. Serological tests

Infection by RHDV can be diagnosed through detection of a specific antibody response. As the humoral response has great importance in protecting animals from RHD, determination of the specific antibody titre after vaccination or in convalescent animals is predictive of the ability of rabbits to resist RHDV infection.

Three basic techniques are applied for the serological diagnosis of RHDV: haemagglutination inhibition (HI) (26), indirect ELISA (I-ELISA) and C-ELISA (7). Each of these methods has advantages and disadvantages. With respect to the availability of reagents and the technical complexity of carrying out the test, HI is the most convenient method, followed by the I-ELISA and C-ELISA, respectively. On the other hand, both ELISAs are quicker and easier than HI, particularly when a large number of samples are tested. The specificity of the C-ELISA is markedly higher than those achieved with the other two methods (7). An alternative C-ELISA method has been described (9). For improved serological interpretation and for correctly classifying the immunological status of rabbits, a combination of ELISA techniques that distinguish IgA, IgM and IgG antibody responses is also available.

Some other additional tests (Capucci, unpublished data, 12) could be used for particular investigations and when a higher level of sensitivity is needed to detect antibodies in non-target species or antibodies induced by cross-reacting RHDV-like agents (see Section A - Introduction). They are:

- **I-ELISA**: The antigen is linked to the solid phase by a RHDV-specific MAb (1H8). It has a slightly higher sensitivity than C-ELISA, making possible measurement of highly cross-reactive antibodies and it can detect antibodies with low avidity.

- **Solid-phase ELISA (SP-ELISA)**: The purified antigen is directly adsorbed to the solid phase and because of virus deformation, internal epitopes are exposed. Therefore it detects a wider spectrum of antibodies and has high sensitivity and low specificity.

- **Sandwich ELISA to detect IgM and IgG in liver or spleen samples already examined with the virological test**: Such a test is particularly useful in those animals that die from the ‘chronic’ form of the disease when detection of the virus may be difficult. In this case, a high level of RHDV-specific IgM and a low level, if any, of IgG are the unambiguous markers of positivity for RHD.

a) Haemagglutination inhibition

**Antigen**: The antigen is prepared using infected rabbit liver collected freshly at death. The liver is homogenised in 10% (w/v) PBS, pH 6.4, and clarified by two consecutive low speed centrifugations (500 g...
for 20 minutes and 6000 g for 30 minutes). The supernatant, drawn from the tube so as to avoid the superficial lipid layer, is filtered through a 0.22 µm pore size mesh, titrated by HA, and divided into aliquots, which are stored at –70°C.

**Serum samples:** Before testing, sera are inactivated by incubation at 56°C for 30 minutes. The sera are then treated with 25% kaolin (serum final dilution: 1/10) at 25°C for 20 minutes and centrifuged. This is followed by a second kaolin treatment, also at 25°C for 20 minutes, this time with 1/10 volume of approximately 50% packed human Group O RBCs. These are freshly collected, stored overnight in Alsever’s solution and washed in 0.85% PBS, pH 6.5. The sera are clarified by centrifugation.

- **Test procedure**
  i) Dispense 50 µl of serum into the first well of a round-bottom microtitre plate and make double dilutions into wells 2–8 using PBS with 0.05% BSA.
  ii) Add 25 µl of RHDV antigen containing 8 HA units to each well and incubate the plate at 25°C for 30–60 minutes.
  iii) Add 25 µl of human Group O RBCs at 2–3% concentration to each well and allow to settle at 25°C for 30–60 minutes.
  iv) Titrate the antigen with each test to ensure that 8 HA/25 µl were used, and include positive and negative serum controls.

The serum titre is the end-point dilution showing inhibition of HA. The positive threshold of serum titres is correlated to the titre of the negative control sera; it usually is in the range 1/20–1/80.

Because of the practical difficulty of obtaining, keeping and the risk from working with human Group O blood cells, and because of the difficulty in obtaining consistent results, this test is being superseded by the serological or antibody-detection ELISA.

b) **Competitive enzyme-linked immunosorbent assay**

**Antigen:** An international standard strain is not yet available; however, as only one serotype has been identified so far world-wide, reliable results can be obtained by different laboratories each using their own standard virus. Even the antibodies induced by the identified RHDV variants are recognised by the standard method described here. In addition, the test can also easily detect antibodies originating from infection of rabbits with the non-pathogenic RCV, due to its high genetic correlation with RHDV (5, 6).

The antigen can be prepared as described previously for HI (Section B.2.a), taking care to store it at −20°C in the presence of glycerol at 50% (v/v) to prevent freezing. If necessary, the virus can be inactivated before the addition of glycerol, using 1.0% binary ethylenimine (BEI) at 33°C for 24 hours. Antigen must be pretitrated in ELISA and then used as the limiting reagent: i.e. the dilution that corresponds to 60–70% of the plateau height (absorbance value at 492 nm in the range 1.1–1.3).

**Anti-RHDV serum:** specific polyclonal sera with high anti-RHDV titre can be obtained in different ways. Two possible and currently used methods are as follows:

i) Rabbits are infected with a RHDV-positive 10% liver extract diluted 1/100 in PBS to obtain convalescent sera (21–25 days) containing a high level of anti-RHDV IgG. Due to the high mortality rate associated with RHDV, it is necessary to infect at least 10–15 seronegative rabbits or to infect rabbits that are only partially protected (e.g. 4–8 rabbits infected from 3 to 7 days post-vaccination). Rabbits that survive the infection must be bled 21–25 days post-infection to obtain the convalescent sera. Alternatively, convalescent rabbits can be re-infected 3–4 months post-infection and bled 10–15 days later to obtain RHDV hyperimmune sera.

ii) RHDV is purified from the livers of experimentally infected rabbits that died from an acute form of the disease (between 28 and 40 hours post-infection), using one of the methods that has been published (4, 7, 8, 28, 33). Then the purified RHDV can be used to immunise sheep or goats according to classical protocols using oil adjuvants. The same procedure can also be used to inoculate rabbits if the purified virus is inactivated before inoculation.

Anti-RHDV MAbs may be used instead of rabbit polyclonal sera. Purification of rabbit IgG and conjugation to HRPO can be done following the standard protocols. The conjugated antibody is titrated in a sandwich ELISA in the presence and absence of RHDV antigen (negative rabbit liver). It is then used at the highest dilution showing maximum (plateau high) absorbance (if the serum had a good anti-RHDV titre, the value of the HRPO conjugate should range from 1/1000 to 1/3000).

**Control sera:** Negative serum is taken from rabbits fully susceptible to RHDV infection. Positive serum is either a convalescent serum diluted 1/100 in a negative serum or a serum taken from a vaccinated animal.
Test procedure (example)

i) The rabbit anti-RHDV serum diluted to a predetermined titre, e.g. 1/5000 in 0.05 M carbonate/bicarbonate buffer, pH 9.6, should be adsorbed to an ELISA microplate of high adsorption capability (e.g. Nunc Maxisorb Immunoplate) at 4°C overnight.

ii) Wash the plate three times for 3–5 minutes each time, in PBS, pH 7.4, with 0.05% Tween 20 (PBST). When the plates are not immediately used, they can be stored, closed in a plastic bag, for 1 month at −20°C.

iii) Distribute 25 µl/well PBST with 1% yeast extract (PBSTY) or 1% BSA (PBST-BSA) to all the wells needed on the plate (see below). Add 7 µl of the first serum sample to the first two wells (A1 and B1), 7 µl of the second serum to the second two wells (C1 and D1), and continue with the third (E1 and F1) and the fourth (G1 and H1) sera, thus completing the first column. If qualitative data (positive/negative) are needed, repeat the operation in the second column with sera samples from 5 to 8, and in the third column with sera samples from 9 to 12, and so on. If the titre of the serum needs to be determined, the serum must be diluted further. Agitate the plate and then use an eight-channel micropipette to transfer 7 µl from the wells in column 1 to the wells in column 2. This corresponds to a four-fold dilution of the sera. This last operation can be repeated once (titre 1/160), twice (titre 1/640), or four times (titre 1/10,240). Either in the case of testing sera for qualitative data (single dilution), or for getting the final titre (several dilutions), complete each plate leaving 12 wells free for the control sera. Add 7 µl of positive sera to wells G7 and H7, and 7 µl of negative sera to wells G10 and H10, then dilute them once and twice (1/40–1/160).

iv) Add 25 µl/well antigen suspended in PBSTY to all the wells on the plate, at a dilution that is double the decided dilution, as described above in the antigen section (see the first part of this ELISA method description).

v) Incubate the plate at 37°C on a rocking platform for 50–60 minutes.

vi) Wash the plate as described in step ii.

vii) Add 50 µl/well rabbit IgG anti-RHDV conjugated with HRPO at the decided dilution, as described above in the ‘anti-RHDV serum’ section (see the first part of this ELISA test description).

viii) Incubate the plate at 37°C on a rocking platform for 50–60 minutes, and wash as described in step ii adding a fourth wash of 3 minutes duration.

ix) Use 50 µl/well OPD as hydrogen donor under the following conditions: 0.5 mg/ml OPD in 0.15 M phosphate/citrate buffer, pH 5, and 0.02% H2O2. Stop the reaction after 5 minutes by addition of 50 µl/well 1 M H2SO4.

x) Read the plate on a spectrophotometer using a 492 nm filter.

The serum is considered to be negative when the absorbance value of the first dilution (1/10) decreases by less than 15% of the reference value (dilution 1/10 of the negative control serum), while it is positive when the absorbance value decreases by 25% or more. When the absorbance value of the 1/10 dilution decreases by between 15% and 25% of the reference value, the sera is considered to be doubtful.

The serum titre corresponds to the dilution giving an absorbance value equal to 50% (±10) of the average value of the three negative serum dilutions.

A wide range of titres will be found, depending on the origin of the sample. Positive sera range from 1/640 to 1/10,240 in convalescent rabbits, from 1/80 to 1/640 in vaccinated rabbits and from 1/10 to 1/160 in ‘nonpathogenic’ infection. Knowing the origin of the sample allows a choice to be made between testing one or more dilutions. Testing only the first dilution gives a positive or negative result. The titre is established by testing all dilutions, up to the sixth one.

Due to the significant antigenic differences existing between RHDV and EBHSV (8, 30), the serological techniques described above, which use RHDV as antigen, are not recommended for the serological diagnosis of EBHS. However, a direct ELISA method could be employed for the detection of positive and negative EBHSV hare sera; in fact, the adsorption of RHDV on to the solid phase of an ELISA microplate exposes cross-reactive antigenic determinants. Alternatively, a specific C-ELISA for EBHSV can be arranged in a similar way, using specific reagent (antigen and antisera) prepared as described above for RHDV.

c) Isotype enzyme-linked immunosorbent assays (isoELISAs)

These ELISAs enable the detection and titration of isotypes IgA, IgM and IgG (6). The isotype titres are critical for the interpretation of field serology in four main areas: cross-reactive antibodies, natural resilience of young rabbits, maternal antibodies, antibodies in previously infected rabbits (12).
To detect RHVD-specific IgG, one RHVD-specific MAb is adsorbed to the Maxisorp plate at a concentration of 2 µg/ml by the method described above for the polyclonal serum in the C-ELISA (see above Section B.2.b, test procedure step i). Virus is added to the plates at a concentration double that used in the C-ELISA and after incubation and washing, sera are added and serially diluted four-fold starting from 1/40. An MAb anti-rabbit IgG HRPO conjugate is used to detect IgG bound to the virus. The final step for the isoELISAs for IgG, IgM and IgA is the addition of OPD and H₂SO₄ as for the C-ELISA. To detect IgM and IgA isotypes the phases of the ELISA reaction are inverted in order to avoid competition with IgG, which is usually the predominant isotype. MAb anti-rabbit IgM or anti-rabbit IgA is adsorbed to the wells and then the sera are diluted as described above. Incubation with the antigen follows and then HRPO-conjugated MAb is used to detect the RHVD bound to the plate. Sera are considered to be positive if the OD₄₉₂ (optical density) value at the 1/40 dilution is more than 0.2 OD units (two standard deviations) above the value of the negative serum used as a control. The titre of each serum is taken as the last dilution giving a positive value. Because isoELISA tests do not follow identical methodology, equivalent titres do not imply that isotypes are present in the same amounts.

C. REQUIREMENTS FOR VACCINES AND DIAGNOSTIC BIOLOGICALS

In countries where RHD is endemic, indirect control of the disease is achieved by vaccination using the appropriate type of vaccine – one that is prepared from clarified liver suspension of experimentally infected rabbits, and that is subsequently inactivated and adjuvanted. The methods of inactivation (formaldehyde, beta-propiolactone or other substances) and the adjuvants used (incomplete mineral oil or aluminium hydroxide), can vary according to the protocol used by the different manufacturers.

In the last ten years several studies were carried out on the expression of RHVD capsid protein in Escherichia coli, in vaccinia virus, and in attenuated myxomavirus (MV). Moreover, it has been shown by various authors that a recombinant capsid protein, VP60, expressed in the baculovirus/Sf9 cell expression system, self assembled into virus-like particles (VLPs) that are structurally and antigenically identical to RHVD virions. While the fusion protein expressed in E. coli is highly insoluble and of low immunogenicity, active immunisation can be achieved with VLPs obtained in the baculovirus system or by using recombinant vaccinia, MV and canarypox, administered either intramuscularly or orally. In particular rabbits vaccinated with recombinant MV expressing the RHVD capsid protein were protected against lethal RHVD and MV challenges. The resulting recombinant virus was also capable of spreading horizontally and promoting protection of contact animals, thus providing the opportunity to immunise a wild rabbit population (38). Similarly, the immunogenicity of VLPs administered by the oral route as an alternative to parenteral immunisation offers an economical and practical way to administer a vaccine for mass immunisation of wild animals.

More recently, the VP60 structural protein has been expressed in transgenic plants, either with a new plum pox virus (PPV)-based vector (PPV-NK), or in transgenic potato plants under the control of a cauliflower mosaic virus 35S promoter or a modified 35S promoter. In both cases the immunisation of rabbits with extracts of Nicotiana clevelandii plants infected with the PPV-NK VP60 chimera and with leaf extracts from potatoes carrying this modified 35S promoter, respectively, induced an efficient immune response that protected animals against a lethal challenge with RHVD. However, at the present time, recombinant vaccines are not yet registered and commercially available.

In France, a vaccine (Dercunimix®, Merial) has recently been commercialised that is a combination of a traditional inactivated liver-derived RHD vaccine and a live attenuated Myxovirus vaccine, and which can be administered by the intradermal route.

The usual programme is to administer the inactivated vaccine twice with an interval of at least 2 weeks. Normally, a 1-ml dose is inoculated subcutaneously in the neck region. In those units with no history of disease, where the anamnesis for RHD is negative, it is advisable to vaccinate only the breeding stock, the first injection should be given at 2–3 months of age. Annual revaccination is strongly recommended to ensure a good level of protection, although experimental data indicate that protection usually lasts for a long time (over 1 year). The vaccination of meat animals is not necessary if disease has not occurred on the farm. Following an outbreak of RHD, even if strict hygiene and sanitary measures are adopted, including cleaning and disinfection, safe disposal of carcasses and an interval before restocking, it is strongly recommended to vaccinate meat animals at the age of 40 days, because the incidence of re-infection is very high. Only after several production cycles is it advisable to stop vaccination of meat animals. In order to verify the persistence of infective RHD inside the unit, a variable number of rabbits, starting with a small sentinel group, should not be vaccinated.

Vaccinated animals quickly produce strong immunity against RHVD infection, therefore vaccination is considered to be effective in protecting non-exposed rabbits and its primary use is in rabbitries after an outbreak of the disease has been diagnosed; once RHD has been confirmed in some sick or dead rabbits, the remaining healthy animals are immediately vaccinated.
Chapter 2.6.2. — Rabbit haemorrhagic disease

The administration of immune serum is also effective in producing a rapid, but short-lived, protection against RHDV infection.

Vaccine should be stored at 2–8°C and it should not be frozen, or exposed to bright light or high temperatures.

1. Seed management

a) Characteristics of the seed

The source of seed virus for the production of inactivated tissue vaccines is infected liver homogenates obtained by serial passages in rabbits that have been inoculated with a partially purified RHD viral suspension. The latter is obtained by centrifuging the 1/5 liver suspension (w/v) in PBS at 10,000 g for 20 minutes at 4°C. The resulting supernatant is treated with 8% (v/v) polyethylene glycol (PEG 6000) overnight at 4°C. The pellet is resuspended at a dilution of 1/10 in PBS, and subsequently centrifuged at 10,000 g for 20 minutes at 4°C. The supernatant is ultracentrifuged at 80,000 g for 2 hours at 4°C through a 20% cushion of sucrose. The pellet is resuspended in PBS (1/100 of the starting volume). This viral suspension is then characterised by negative-stain EM examination, determination of reactivity in ELISA, and capability of HA at room temperature with slow elution (HA titre against RBCs of human Group O higher than 1/1280). Seed virus is titrated before use and should contain at least 10^5 LD₅₀. It should be stored frozen (−70°C) or freeze-dried.

b) Method of culture

At present, RHDV replication can be obtained exclusively in susceptible animals. The rabbits used for inoculation are selected from colonies shown to be susceptible to the disease by periodic serological testing. The animals (at least 4 months old) must be kept in strict quarantine on arrival, in a separate area and reared under satisfactory health conditions (see Laboratory animal facilities in Chapter 1.1.2 Biosafety and biosecurity in the veterinary microbiology laboratory and animal facilities). Seed virus propagation and production of vaccine batches relies on the same protocol of experimental infection, involving intramuscular injection of a dose of at least 100 LD₅₀.

c) Validation as a vaccine

The seed virus used for vaccine production must be shown to be free from other viruses, bacteria, mycoplasma and fungi. Seed virus is controlled by direct inoculation into susceptible rabbits followed by evaluation of the clinical signs in the course of the experimental infection. Suitable seed virus should cause the death of 70–80% of the rabbits within 24–72 hours post-inoculation, with the internal organ lesions characteristic of RHD. To validate the test, gross and histopathological examination of all rabbits should be performed to exclude intercurrent diseases.

2. Method of manufacture

Following inoculation of susceptible rabbits, the liver and spleen of those rabbits that die between 24 and 72 hours post-inoculation are collected. The organs are minced in 1/10 (w/v) sterile PBS, pH 7.2–7.4, and the mixture is homogenised for 10 minutes in a blender in a refrigerated environment. The mixture is then treated with 2% chloroform (18 hours at 4°C), followed by centrifugation at 6000 g for 1 hour at 4°C. The supernatant is collected by high pressure continuous pumping and is subsequently inactivated. The viral suspension is assayed by HA test and ELISA (see Section C.3.) and, once the number of HA units from the initial titration is known, more sterile PBS is added in sufficient volume to provide, after inactivation and adsorption on adjuvant, a concentration of 640–1280 HA units/dose in the commercial product. Various agents have proved effective at abolishing viral infectivity. The most frequently used are formaldehyde and beta-propiolactone, which can be used at different concentrations and temperatures, for variable periods of time and also in combination. During inactivation, it is advisable to continuously agitate the fluid. Aluminium hydroxide, Freund’s incomplete adjuvant or another oil emulsion is then incorporated into the vaccine as adjuvant. A preservative, thiomersal (merthiolate), is finally added at a dilution of 1/10,000 (v/v) before distribution into bottles.

3. In-process control

Antigen content: The RHD titre is determined before inactivation by calculating the HA titre, which should be higher than 1/1280, and the ELISA reactivity. Both values are again determined after inactivation and adsorption on adjuvant. Negative-staining EM confirms the identity of RHD.

Sterility: The organs are tested for the presence of viable bacteria, viruses, fungi or mycoplasma according to the protocol used for testing master seed virus. PBS solution and aluminium hydroxide gel are sterilised by autoclaving; oil emulsion is sterilised by heating at 160°C for 1 hour.
**Inactivation:** Before incorporation of the adjuvant, the inactivating agent and the inactivation process must be shown to inactivate the vaccine virus under the conditions of manufacture. Thus, a test is carried out on each batch of the bulk harvest as well as on the final product. Five rabbits are inoculated with a 2-ml dose of the suspension and five unvaccinated rabbits are kept as controls. After 10 days, adequate inactivation and absence of undesirable side-effects are demonstrated by the absence of clinical signs of disease and by similar weight increments in the two groups. At the end of the trials, the animals are slaughtered and liver extracts are tested by HA, ELISA and EM.

4. **Batch control**

Sterility, safety and potency tests should be carried out on each batch of final vaccine; tests for duration of immunity should be carried out once using a typical batch of vaccine, and stability tests should be carried out on three batches.

a) **Sterility**

Each batch of vaccine must be tested for the presence of viable bacteria, viruses, fungi or mycoplasma according to the same protocol recommended for testing master seed virus.

b) **Safety**

Ten rabbits should be inoculated by the recommended routes with three times the vaccinal dose. The rabbits are observed for 3 weeks. No abnormal local or systemic reaction should develop.

c) **Potency**

Ten seronegative adult rabbits that are at least 4 months old are vaccinated with one full dose of vaccine given by the recommended route. Two other groups of five animals each are vaccinated with 1/4 and 1/16 of the full dose, respectively. A fourth group of ten unvaccinated rabbits is maintained as controls. All animals are challenged at 4 weeks post-vaccination by intramuscular inoculation of a dose of RHDV containing at least 100 LD$_{50}$ or presenting a HA titre higher than 1/2560. No vaccinated rabbits should show signs of infection, while the mortality rate among control animals should be higher than 70%. The antibody response of each vaccinated animal is then determined with reference to titrated standard antisera; the mean antibody level should not be significantly less than the level recorded in the protection test performed using as vaccine the inactivated seed virus.

d) **Duration of immunity**

The data reported in the literature indicate a long-term duration of immunity induced by a single vaccination (up to 15 months). However, it is advisable to carry out the following test: 20 rabbits vaccinated once are divided into four groups and are serologically tested at monthly intervals over a period of 1 year. Each group is inoculated with virulent RHDV at 3, 6, 9 months or 1 year post-vaccination (see Section C.4.c). Challenge infection should produce increasing seroconversion, which is directly related to the time that has elapsed since vaccination. The absence of clinical signs of disease and mortality supports the efficacy of the vaccine.

e) **Stability**

Evidence should be provided to show that the vaccine passes the batch potency test at 3 months beyond the suggested shelf life.

f) **Preservatives**

A suitable preservative is normally required for vaccine in multidose containers (see Section C.2). Its persistence throughout shelf life should be checked.

g) **Precautions (hazards)**

When oil-emulsion vaccines are prepared, vaccinators should be warned against the risk and consequences of accidental self-injection, which must be treated urgently as a ‘grease-gun’ injury.

5. **Tests on the final product**

The tests for safety, potency and sterility of the final product must be performed after bottling and packaging. Thus, it is important that these two last manufacturing steps be performed following standardised good manufacturing procedures. The tests are conducted by removing samples from a statistically determined number of randomly taken multidose containers (20 or 100 doses) of vaccine.
a) **Safety**
See Section C.4.b.

b) **Potency**
See Section C.4.c.

c) **Sterility**
See Section C.4.a.

**REFERENCES**


* * *

NB: There is an OIE Reference Laboratory for Rabbit haemorrhagic disease (see Table in Part 3 of this Terrestrial Manual or consult the OIE Web site for the most up-to-date list: www.oie.int).
SECTION 2.7.
OVIDAE AND CAPRIDAE

CHAPTER 2.7.1.
BORDER DISEASE

SUMMARY

Border disease (BD) is a viral disease of sheep and goats first reported in sheep in 1959 from the border region of England and Wales, and since recorded world-wide. Distribution of the virus is world-wide. Prevalence rates vary in sheep from 5% to 50% between countries and from region to region within countries. Clinical signs include barren ewes, abortions, stillbirths and the birth of small weak lambs. Affected lambs can show tremor, abnormal body conformation and hairy fleeces (so-called ‘hairy-shaker’ or ‘fuzzy’ lambs) and the disease has been referred to as ‘hairy shaker disease’. Vertical transmission plays an important role in the epidemiology of the disease. Infection of fetuses can result in the birth of persistently infected (PI) lambs. These PI lambs are viraemic, antibody negative and constantly excrete virus. The virus spreads from sheep to sheep, with PI animals being the most potent source of infection. Infection in goats is less common with abortion being the main presenting sign. In many regions the commonest cause of BD is the pestivirus border disease virus (BDV), but in some parts of the world, bovine viral diarrhoea virus (BVDV) may be a more common cause of BD. The source of BVDV for sheep often is close contact with cattle.

It is important to identify the viraemic PI animals so that they will not be used for breeding or trading purposes. Serological testing is insufficient. It is generally considered that serologically positive, nonviraemic sheep are ‘safe’, as latent infections are not known to occur in recovered animals.

Identification of the agent: BD virus (BDV) is a Pestivirus in the family Flaviviridae and is closely related to classical swine fever virus and BVDV. Nearly all isolates of BDV are noncytopathogenic in cell culture. There are no defined serotypes but virus isolates exhibit considerable diversity. Three distinct antigenic groups, plus two further separate genotypes, have been identified.

Apparently healthy PI sheep resulting from congenital infection can be identified by isolation and immunostaining of noncytopathogenic virus from blood or sera in laboratory cell cultures. Rapid direct methods to identify PI sheep include detection of viral antigen or viral RNA in leukocytes and immunohistochemical demonstration of viral antigen in skin biopsies. The demonstration of virus is less reliable in lambs younger than 2 months that have received colostral antibody. Acute infection is usually subclinical and viraemia is transient and difficult to detect. From dead animals, the isolation of virus from tissues of aborted or stillborn lambs is difficult, but tissues from PI sheep contain high levels of virus, which can be easily detected by isolation and direct methods.

Serological tests: Acute infection with BDV is best confirmed by demonstrating seroconversion using paired or sequential samples from several animals in the group. The enzyme-linked immunosorbent assay and virus neutralisation test are the most commonly used antibody detection methods.

Requirements for vaccines and diagnostic biologicals: There is no standard vaccine for BDV, but a commercial killed whole-virus vaccine has been produced. Ideally, such a vaccine should be
suitable for administration to females before breeding for prevention of transplacental infection. The use of BVDV vaccines has been advocated, but the antigenic diversity of BD viruses must be considered.

BD viruses have contaminated several modified live veterinary vaccines produced in sheep cells or containing sheep serum. This potential hazard should be recognised by manufacturers of biological products.

A. INTRODUCTION

Border disease virus (BDV) is a Pestivirus of the family Flaviviridae and is closely related to classical swine fever virus (CSFV) and bovine viral diarrhea virus (BVDV). Pestivirus taxonomy is quite problematic at present. There are four recognised species, namely – CSFV, BVDV types 1 and 2 and BDV (20). While CSF viruses are predominantly restricted to pigs, examples of the other three species have all been recovered from sheep, with the majority of isolates being BD viruses (29). Nearly all virus isolates of BDV are noncytopathogenic, although occasional cytopathic viruses have been isolated (24). BDV spreads naturally among sheep by the oro-nasal route and by vertical transmission. It is principally a cause of congenital disease in sheep and goats, but can also cause acute and persistent infections. Infection is less common in goats, in which persistent infection is rare as abortion is the main presenting sign. Sheep can become infected with BVDV from cattle (6), and in some countries, BVDV can be a more common cause of BD than BDV. Pigs may also be infected by pestiviruses other than CSFV and antibodies to BDV in pigs may interfere with tests for the diagnosis of CSF (15). Several new BD viruses from sheep, goats and Pyrenean chamois (Rupicapra pyrenaica pyrenaica) have been described recently. Phylogenetic analysis using computer-assisted nucleotide sequence analysis suggests that genetic variability among BD viruses is greater than within each of the other pestivirus species. Four distinguishable genogroups of BDV have been described as well as putative novel pestivirus genotypes from Tunisian sheep and a goat (2, 28). The chamois BD virus is similar to isolates from sheep in the Iberian Peninsula (22). This chapter describes BDV infection in sheep.

a) Acute infections

Healthy newborn and adult sheep exposed to BDV experience only mild or inapparent disease. Slight fever and a mild leukopenia are associated with a short-lived viraemia detectable between days 4 and 11 post-infection, after which virus neutralising antibody appears in the serum (19).

Acute infections are best diagnosed serologically using paired sera from a representative number of sheep. Occasional BD isolates have been shown to produce high fever, profound and prolonged leukopenia, anorexia, conjunctivitis, nasal discharge, dyspnoea and diarrhoea, and 50% mortality in young lambs. One such isolate was recovered from a severe epidemic of BD among dairy sheep in 1984 (7). A second such isolate was a BDV contaminant of a live CSFV vaccine (31).

b) Fetal infection

The main clinical signs of BD are seen following the infection of pregnant ewes. While the initial maternal infection is subclinical or mild, the consequences for the fetus are serious. Fetal death may occur at any stage of pregnancy, but is more common in fetuses infected early in gestation. Small dead fetuses may be resorbed or their abortion may pass unnoticed as the ewes continue to feed well and show no sign of discomfort. As lambing time approaches, the abortion of larger fetuses, stillbirths and the premature births of small, weak lambs will be seen. Confirmation that an abortion or stillbirth is due to BDV is often difficult to establish, but virus may be isolated from fetal tissues in some cases. In aborted fetuses, it is also possible to detect virus by immunohistochemistry of brain, thyroid and other tissues (21). Samples of fetal fluids or serum should be tested for BDV antibody.

During lambing, an excessive number of barren ewes will become apparent, but it is the diseased live lambs that present the main clinical features characteristic of BD. The clinical signs exhibited by BD lambs are very variable and depend on the breed of sheep, the virulence of the virus and the time at which infection was introduced into the flock. Affected lambs are usually small and weak, many being unable to stand. Nervous signs and fleece changes are often apparent. The nervous signs of BD are its most characteristic feature. The tremor can vary from violent rhythmic contractions of the muscles of the hindlegs and back, to barely detectable fine trembling of the head, ears, and tail. Fleece abnormalities are most obvious in smooth-coated breeds, which develop hairy fleeces, especially on the neck and back. Abnormal brown or black pigmentation of the fleece may also be seen in BD-affected lambs. Blood samples to be tested for the presence of BDV and/or antibody should be collected into anticoagulant from suspect lambs before they have received colostrum. Once lambs have ingested colostrum, it is difficult to detect virus until they are 2 months old and maternal antibody levels have waned. However, during this period, it may be
possible to detect viral antigen in skin biopsies, by immunohistochemistry, in washed leukocytes by enzyme-linked immunosorbent assay (ELISA) or by reverse-transcription polymerase chain reaction (RT-PCR).

With careful nursing, a proportion of BD lambs can be reared, although deaths may occur at any age. The nervous signs gradually decline and can disappear by 3–6 months of age. Weakness, and swaying of the hind-quarters, together with fine trembling of the head, may reappear at times of stress. Affected lambs often grow slowly and under normal field conditions many will die before or around weaning time. In cases where losses at lambing time have been low and no lambs with obvious signs of BD have been born, this can be the first presenting sign of disease.

Some fetal infections occurring around mid-gestation can result in lambs with severe nervous signs, locomotor disturbances and abnormal skeletons. Such lambs have lesions of cerebellar hypoplasia and dysplasia, hydranencephaly and porencephaly resulting from necrosing inflammation. The severe destructive lesions appear to be immune mediated, and lambs with such lesions frequently have high titres of serum antibody to BDV. Most lambs infected in late gestation are normal and healthy and are born free from virus but with BDV antibody. Some such lambs can be weak and may die in early life (1).

c) Persistent viraemia

When fetuses survive an infection that occurs before the onset of immune competence, they are born with a persistent viraemia. The ovine fetus can first respond to an antigenic stimulus between approximately 60 and 85 days of its 150-day gestation period. In fetuses infected before the onset of immune competence, viral replication is uncontrolled and 50% fetal death is common. In lambs surviving infection in early gestation, virus is widespread in all organs. Such lambs appear to be tolerant of the virus and have a persistent infection, usually for life. A precolostral blood sample will be virus positive and antibody negative. Typically, there is no inflammatory reaction and the most characteristic pathological changes are in the central nervous system (CNS) and skin. Throughout the CNS, there is a deficiency of myelin, and this causes the nervous signs. In the skin, primary wool follicles increase in size and the number of secondary wool follicles decreases, causing the hairy or coarse fleece.

Persistently viraemic sheep can be diagnosed by virus isolation/detection in a blood sample. Viraemia is readily detectable at any time except within the first 2 months of life, when virus is masked by colostral antibody; however, the virus may be detected in washed leukocytes during this period, and in animals older than 4 years, some of which develop low levels of anti-BDV antibody (13). Although virus detection in blood during an acute infection is difficult, persistent viraemia should be confirmed by retesting animals after an interval of at least 3 weeks.

Some viraemic sheep survive to sexual maturity and are used for breeding. Lambs born to these infected dams are always persistently viraemic. Persistently viraemic sheep are a continual source of infectious virus to other animals and their identification is a major factor in any control programme. Sheep being traded should be screened for the absence of BDV viraemia.

Usually persistently infected (PI) rams have poor quality, highly infective semen and reduced fertility. All rams used for breeding should be screened for persistent BDV infection on a blood sample. Semen samples can also be screened for virus, but virus isolation is much less satisfactory than from blood due to the toxicity of semen for cell cultures. RT-PCR for detecting pestivirus nucleic acid may be justifiable on semen from some rams.

d) Late-onset disease in persistently viraemic sheep

Some PI sheep housed apart from other animals spontaneously develop intractable diarrhoea, wasting, excessive ocular and nasal discharges, sometimes with respiratory distress. At necropsy such sheep have gross thickening of the distal ileum, caecum and colon resulting from focal hyperplastic enteropathy. Cytopathic BDV can be recovered from the gut of these lambs. With no obvious outside source of cytopathic virus, it is most likely that such virus originates from the lamb's own virus pool. Other PI sheep in the group do not develop the disease. This syndrome, which has been produced experimentally and recognised in occasional field outbreaks of BD, has several similarities with bovine mucosal disease (13).

B. DIAGNOSTIC TECHNIQUES

1. Identification of the agent (the prescribed test for international trade)

There is no designated OIE reference laboratory for BDV, but the reference laboratories for BVDV or CSFV will be able to provide advice (see Table given in Part 3 of this Terrestrial Manual). One of the most sensitive proven
methods for identifying BDV remains virus isolation. Direct immunofluorescence or other immunohistochemical techniques on frozen tissue sections as well as antigen-detecting ELISA and conventional and real-time RT-PCR are also valuable methods for identifying BDV-infected animals.

a) Virus isolation

It is essential that laboratories undertaking virus isolation have a guaranteed supply of pestivirus-free susceptible cells and fetal bovine serum (FBS), or equivalent, that contain no anti-pestivirus activity and no contaminating virus. It is important that a laboratory quality assurance programme be in place.

The virus can be isolated in a number of primary or secondary ovine cell cultures (e.g. kidney, testes, lung). Ovine cell lines for BDV growth are rare. Semicontinuous cell lines derived from fetal lamb muscle (FLM), whole embryo (19) or sheep choroid plexus can be useful, but different lines vary considerably in their susceptibility to the virus. Ovine cells have been used successfully for the isolation and growth of BD viruses and BVDV types 1 and 2 from sheep. In regions where sheep may become infected with BVD viruses from cattle, a virus isolation system using both ovine and bovine cells could be optimal. Several bovine cell cultures may be suggested, including testicular, embryonic tracheal or turbinate cells, or a susceptible continuous kidney cell line. However, bovine cells are insensitive for the primary isolation and growth of some BD viruses, so reliance on bovine cells alone is inadvisable.

From live animals, serum can be tested for the presence of infectious virus, but the most sensitive way to confirm pestivirus viraemia is to wash leukocytes repeatedly (at least three times) in culture medium before co-cultivating them with susceptible cells for 5–7 days. Cells are frozen and thawed once and an aliquot passed onto further susceptible cells grown on cover-slips, chamber slides or plastic plates. The cells are stained, 3–4 days later, for the presence of pestivirus using an immunofluorescence or immunoperoxidase test. Tissues should be collected from dead animals in virus transport medium (10% [w/v]). In the laboratory, the tissues are ground, centrifuged to remove debris, and the supernatant passed through 0.45 µm filters. Spleen, thyroid, thymus, kidney, brain, lymph nodes and gut lesions are the best organs for virus isolation.

Semen can be examined for the presence of BDV, but raw semen is strongly cytotoxic and must be diluted, usually at least 1/10 in culture medium. As the major threat of BDV-infected semen is from PI rams, blood is a more reliable clinical sample than semen for identifying such animals. There are many variations in virus isolation procedures. All should be optimised for maximum sensitivity using a standard reference virus preparation and, whenever possible, recent BDV field isolates. A practical sensitive isolation procedure is outlined below:

i) Cultures with subconfluent or newly confluent monolayers of susceptible ovine cells are washed at least twice with Hanks balanced salt solution to remove growth medium before being inoculated with approximately 0.2 ml of sample, which is allowed to adsorb for 2 hours at 37°C.

ii) Cultures are washed with at least 2 ml medium. This is discarded and an appropriate volume of culture maintenance medium is added.

iii) Cultures are incubated for 5–7 days at 37°C. They are examined microscopically on a daily basis and evidence of cytopathic effect (CPE) is recorded.

iv) Cultures are frozen at −70°C and then thawed for passage, as before, on to fresh cultures.

v) 3–4 days later, cells growing on glass are fixed in cold acetone for 15 minutes while cells growing on plastic are fixed as described in the virus neutralisation test section below. Fixed cells are stained using an indirect or direct immunofluorescence method. Essential controls must include known negative cells and cells growing standard cytopathic and noncytopathic BDV strains.

vi) The cells are examined under a UV microscope for the diffuse cytoplasmic fluorescence that is characteristic of pestiviruses.

Immunoperoxidase staining can also be used on cover-slips, chamber slides as well as microtitre plates (see method under virus neutralisation [VN] test below). Frozen and thawed cultures can also be tested in an antigen detection ELISA system that employs monoclonal antibodies (MAbs) against epitopes on the conserved nonstructural NS 2-3 protein. Staining for noncytopathic pestiviruses will usually detect virus at the end of the first passage, but in order to detect slow-growing viruses in poorly permissive cells two passages are desirable.

b) Immunohistochemistry

Viral antigen demonstration is possible in most of the tissues of PI animals (4, 21). This should be done on acetone-fixed frozen tissue sections (cryostat sections) or paraffin wax embedded samples using appropriate antibodies. Panpestivirus-specific antibodies with NS2-3 specificity are suitable. Tissues with a
high amount of viral antigen are brain, thyroid gland and oral mucosa. Skin biopsies have been shown to be useful for in-vivo diagnosis of persistent BDV infection.

c) Enzyme-linked immunosorbent assay for antigen detection

The first ELISA for pestivirus antigen detection was described for detecting viraemic sheep. This has now been modified into a double MAb capture ELISA for use in sheep and cattle. Two capture MAbs are bound to wells in microtitre plates, and two other MAbs, conjugated to peroxidase, serve as detector MAbs (9). The test is most commonly employed to identify PI viraemic sheep using washed, detergent-lysed blood leukocytes. The sensitivity is close to that of virus isolation and it is a practical method for screening high numbers of blood samples. As with virus isolation, high levels of colostral antibody can mask persistent viraemia. The ELISA is more effective than virus isolation in the presence of antibody, but may give false-negative results in viraemic lambs younger than 2 months old. The ELISA is usually not sensitive enough to detect acute BDV infections on blood samples. As well as for testing leukocytes, the antigen ELISA can also be used on tissue suspensions, especially spleen, from suspected PI sheep and, as an alternative to immunofluorescence and immunoperoxidase methods, on cell cultures. Several pestivirus ELISA methods have been published and commercial kits are now available for detecting BDV. ELISAs employing MAbs recognising epitopes on the conserved non-structural NS2-3 should recognise all strains of BDV. ELISAs relying on MAbs recognising epitopes on structural proteins, such as Ems, that are used for BVDV detection in cattle, are unsuitable for the diagnosis of BDV viraemia in sheep.

d) Nucleic acid detection methods

The complete genomic sequences of three BD viruses have been determined and compared with those of other pestiviruses (3, 17). Phylogenetic analysis shows BD viruses to be more closely related to CSFV than to BVDV (2, 23, 28, 29). RT-PCR for diagnosing pestivirus infection is now used widely. Various formats are described. Basic RT-PCR protocols involve the following stages:

i) Total RNA is isolated by phenol-chloroform, TRIZOL, guanidine isothiocyanate (GITC), or a commercially available spin column or magnetic bead separation methods. NOTE: many of these chemicals are highly toxic; adhere to the manufacturers’ safety procedures.

ii) RT-PCR is performed. This is a two-stage reaction that consists of:
   a) Reverse transcription to produce single-stranded cDNA from viral RNA;
   b) Subsequent PCR amplification of the cDNA to produce readily detectable amounts of double-stranded DNA.

This process may be done as two separate reactions, each done in a separate PCR tube (two-step RT-PCR), or as two stages in a single PCR tube (one-step RT-PCR). In a two-step format, either random hexamers or specific primers may be used to prime the RT stage; in the one-step format only specific primers may be used.

iii) Specific product is detected by one of the following methods:
   a) Use of BD-specific primers in the RT-PCR, with visualisation by agarose gel electrophoresis, ethidium bromide staining and UV transillumination to demonstrate the correct sized amplicon. NOTE: ethidium bromide is highly toxic; adhere to manufacturer’s recommendations for handling. NOTE: UV transillumination must be carried out taking appropriate precautions to minimise skin exposure.

   b) Nested PCR, using pan-pestivirus primers (usually directed to the 5’UTR region) in a primary PCR, followed by specific BD virus primers in a secondary (nested) PCR. Such assays typically employ approximately 25 cycles in the primary PCR and 30–35 cycles in the nested PCR. Amplicons are detected by visualisation by agarose gel electrophoresis as above. These assays increase specificity and sensitivity but are more susceptible to contamination.

   c) Real-time RT-PCR, using specific BD primers and/or fluorophore-labelled oligonucleotide probe to detect BD. This method has advantages in specificity and prevention of contamination. It is also possible to carry out a nested form of real-time RT-PCR.

Oligonucleotide primer/probe design is critical to the success of these assays, due to the genetic variability of BDV isolates. Panpestivirus primers are valuable for detecting and typing all species of Pestivirus (18, 27), and can be combined with sequencing if required for specificity or epidemiological investigation. Specific primers for the specific recognition of BD viruses have also been described (11, 30, 33). Using a closed one-tube RT-PCR with fluorescent probes reduces the potential for cross-contamination of diagnostic samples (12, 33). The development of a real-time RT-PCR allows the rapid simultaneous detection and typing of ovine pestiviruses (33). Important applications of RT-PCR methods include the detection of viral RNA in fetal tissues and in cell culture constituents or vaccines (26); it may also prove valuable for detecting virus when BDV-specific antibodies are present. Validation of the RT-PCR is in
process. The precautions to be taken with RT-PCR have been covered in Chapter 1.1.5 Validation and quality control of polymerase chain reaction methods used for the diagnosis of infectious diseases.

2. Serological tests

Antibody to BDV is usually detected in sheep sera using VN or an ELISA. The less sensitive agar gel immunodiffusion (AGID) test may also be used. Control positive and negative reference sera must be included in every test. These should give results within predetermined limits for the test to be considered valid. Single sera can be tested to determine the prevalence of BDV in a flock, region or country. For diagnosis, however, acute and convalescent sera are the best samples for confirming acute BDV infection. Repeat sera from one animal should always be tested alongside each other on the same plate.

a) Virus neutralisation test

A standard cytopathic strain of BDV (e.g. Moredun strain) can be used for the VN test with semicontinuous cells such as FLM. An outline protocol is given below.

i) Test and control sera are heat-inactivated for 30 minutes at 56°C.

ii) From a starting serum dilution of 1/4, serial twofold dilutions of the test sera are made in cell culture growth media in a cell culture-grade flat-bottomed 96-well microtitre plate. For each sample two or four wells are used at each dilution depending on the accuracy required. The range of dilution can also vary. It is common to screen sera initially at a dilution of 1/4 and titrate out positive sera. To screen sera a minimum of four wells is required. The standard working volume is 25 µl: 25 µl of the diluted serum is added to each well; 25 µl of media is added to each of the lower two control wells and 25 µl of medium containing 100 TCID₅₀ (50% tissue culture infective dose) of virus is added to each of the two upper test wells. Control positive and negative sera and a virus titration are included in every test.

iii) Plates are sealed with nontoxic plate sealers or lids, and incubated at 37°C for 1 hour.

iv) 100 µl of a cell suspension with a count of 2 × 10⁵ cells/ml is added to every well. The FBS or equivalent serum used for cell growth must be free from antibody to BDV.

v) The plate is sealed or incubated in a humid chamber in 5% CO₂ for 4 days at 37°C.

vi) The wells are examined microscopically for CPE. In the control wells of the test sera, cell degeneration will be due to toxicity. Further dilution of toxic sera can be attempted, but it may not be possible to obtain reliable results with occasional sera. The VN titre for each serum is the dilution at which the virus is neutralised in 50% of the wells. This can be calculated by the Spearman–Kärber method. A seronegative animal will show no neutralisation at the lowest dilution (i.e. 1/4).

The choice of test virus is difficult due to antigenic diversity among pestiviruses (8, 14) Standard strains of cytopathic BVD viruses and bovine cells can be used. Oregon C24V results correlate better with Moredun BDV than results with the NADL strain. No single strain is ideal. A local strain that gives the highest antibody titre with a range of positive sheep sera should be used. The VN test can also be used with noncytopathic viruses when the following immunoperoxidase staining system is used after step v above:

i) The culture medium is removed and the cells are washed gently with warm phosphate buffered saline (PBS), air-dried and cooled to 4°C.

ii) The cells are fixed by quickly adding to all wells 95% acetone (in water) previously cooled to –20°C. The plates are held at –20°C for 30 minutes and should not be stacked or allowed to warm as etching of the plastic may occur.

iii) The acetone is removed and the plates are dried quickly in a cool environment.

iv) 50 µl of BDV antiserum is added to all wells at a predetermined dilution in PBS with 1% Tween 80 (PBST). The plates are incubated at 37°C for 30 minutes in a humid atmosphere.

v) The plates are emptied and washed three times with PBST.

vi) The wells are drained and an appropriate anti-species serum conjugated to peroxidase at a predetermined dilution is added, and the plates are left for 30 minutes at 37°C in a humid atmosphere.

vii) The plates are emptied and washed three times with PBST.

viii) The plates are drained well and 50 µl of activated substrate, e.g. 3-amin9-ethyl carbazole (AEC) is added. AEC stock solution is: AEC (0.1 g) dissolved in dimethyl formamide (15 ml). For use add stock (0.3 ml) to membrane-filtered 0.05 M acetate buffer, pH 5.0, (4.7 ml), and then add 30% H₂O₂ (5 µl). NOTE. This solution is toxic and should be handled with adequate precautions.
ix) The plates are incubated at room temperature and known virus-positive control wells are monitored for development of specific red-brown cytoplasmic staining. When staining is complete the substrate is removed carefully and the wells are washed thoroughly with tap water. Leaving the tap water in the wells, the plates are examined microscopically for virus-containing wells.

x) The VN titre is calculated as above using the Spearman–Kärber method.

xi) Alternatively, the test can be performed using direct fluorescein-isothiocyanate conjugate staining.

Occasionally there may be a need to determine whether antibody in a flock is against a virus belonging to a particular Pestivirus serogroup. A differential VN test can be used in which sera are titrated out against representative viruses from each of the four Pestivirus groups, i.e. BDV, BVDV types 1 and 2, and CSFV. Maximum titre will identify the infecting serotype and the spectrum of cross-reactivity with the other serotypes will also be revealed.

b) Enzyme-linked immunosorbent assay

An MAb-capture ELISA for measuring BDV antibodies has been described. Two panpestivirus MAbs that detect different epitopes on the immunodominant nonstructural protein NS 2/3 are used to capture detergent-lysed cell-culture grown antigen. The results correlate qualitatively with the VN test (10).

Antigen is prepared as follows: Use eight 225 cm² flasks of newly confluent FLM cells; four flasks will be controls and four will be infected. Wash the flasks and infect four with a 0.01–0.1 m.o.i. (multiplicity of infection) of Moredun cytopathic BDV. Allow the virus to adsorb for 2 hours at 37°C. Add maintenance media containing 2% FBS (free from BDV antibody), and incubate cultures for 4–5 days until CPE is obvious. Pool four control flask supernatants and separately pool four infected flask supernatants. Centrifuge at 3000 g for 15 minutes to pellet cells. Discard the supernatants. Retain the cell pellets. Wash the flasks with 50 ml of PBS and repeat the centrifugation step as above. Pool all the control cell pellets in 8 ml PBS containing 1% Nonidet P40 and return 2 ml to each control flask to lyse the remaining attached cells. Repeat for infected cells. Keep the flasks at 4°C for at least 2 hours agitating the small volume of fluid on the cells vigorously every 30 minutes to ensure total cell detachment. Centrifuge the control and infected antigen at 12,000 g for 5 minutes to remove the cell debris. Supernatant antigens are stored at –70°C in small aliquots.

• Test procedure
  i) The two MAbs are diluted to a predetermined dilution in 0.05 M bicarbonate buffer, pH 9.6. All wells of a suitable ELISA-grade microtitre plate (e.g. Nunc maxisorb, Greiner 129b) are coated overnight at +4°C.
  ii) After washing three times in PBST, a blocking solution of PBST containing 10% horse serum (PBSTH) is added to all wells, which are incubated at 37°C for 1 hour.
  iii) The antigen is diluted to a predetermined dilution in PBSTH and alternate rows of wells are coated with virus and control antigens for 1 hour at 37°C. The plates are then washed three times in PBST before addition of test sera.
  iv) Test sera are diluted 1/50 in PBSTH and added to duplicate virus and duplicate control wells for 1 hour at 37°C. The plates are then washed three times in PBST.
  v) Anti-ovine IgG peroxidase conjugate is diluted to a predetermined dilution in PBSTH and added to all wells for 1 hour at 37°C. The plates are washed three times in PBST.
  vi) A suitable activated enzyme substrate, such as ortho-phenylene diamine (OPD) or tetramethyl blue (TMB), is added noting the manufacturer’s toxicity warning. After colour development, the reaction is stopped with sulphuric acid and the absorbance read on an ELISA plate reader. The mean value of the two control wells is subtracted from the mean value of the two virus wells to give the corrected absorbance for each serum. Results are expressed as corrected absorbance with reference to the corrected absorbance of known positive and negative sera. Alternatively, ELISA titres can be extrapolated from a standard curve of a dilution series of a known positive reference serum.

If antigens of sufficient potency can be produced the MAb capture stage can be omitted. In this case alternate rows of wells are coated with virus and control antigen diluted to a predetermined dilution in 0.05 M bicarbonate buffer, pH 9.6, overnight at +4°C. The plates are washed and blocked as in step ii above. After washing, diluted test sera are added and the test proceeds from step iv as above.

c) Agar gel immunodiffusion test

The AGID test was first used to demonstrate an immunological relationship between BD, BVD and CSF viruses.

The Oregon C24V strain of BVDV grown on calf testis cells has been used to detect antibody in sheep. Suitable antigen can be prepared using medium harvested from cells showing early CPE. Concentration of
the medium approximately 100-fold by dialysis against polyethylene glycol (PEG) is required. Alternatively, PEG 6000 can be added to sonicated virus/cell suspensions at the rate of 8% (w/v). After constant stirring overnight at 4°C, the precipitate is removed by centrifugation at 1800 g for 1 hour. The supernatant is decanted thoroughly and the precipitate resuspended to 1% of the original virus/cell culture volume in distilled water. The resuspended precipitate is centrifuged at 286,000 g for 2 hours and the supernatant withdrawn for use as antigen. The precipitate is discarded.

**C. REQUIREMENTS FOR VACCINES AND DIAGNOSTIC BIOLOGICALS**

To be useful, a BDV vaccine should be effective when administered to female sheep before breeding to prevent transplacental infection. Experimental and commercial inactivated whole virus BDV vaccines have been produced in Europe (5, 25).

Pestivirus contaminants of modified live virus vaccines have been found to be a cause of serious disease following their use in pigs, cattle, sheep and goats. Contaminated vaccines have included those used for the control of Aujesky’s disease, CSF, rotavirus, coronavirus, rinderpest, sheep pox and contagious pustular dermatitis. The insidious ability of pestiviruses to cross the placenta, and thus establish PI animals, gives them the potential to contaminate vaccines through cells, serum used as medium supplement, or seed stock virus. As nearly all isolates of pestiviruses are noncytopathic, they will remain undetected unless specific tests are carried out.

1. **Seed management**

a) **Characterisation of the seed**

An ideal vaccine should contain a strain or strains of virus that give protection against all sheep pestiviruses. Recent evidence is that three antigenically distinguishable groups of pestiviruses infect sheep. One group is represented by the Moredun reference strain of BDV; the second group contains viruses similar to the majority of cattle BVDV strains (BVDV type 1); and the third group contains the less common BVDV (type 2) strains (32). More recently, ovine pestivirus isolates have been divided on the basis of phylogenetic and antigenic analysis into BDV-1, BDV-2 and BDV-3 genotypes (2). Phylogenetic analysis alone suggests that a BDV Italian caprine isolate and the chamois/Iberian sheep isolates represent two further genotypes (28). Further cross-neutralisation studies are required to determine the significance of these findings. Nevertheless it would appear that any BDV vaccine should contain at least a representative of the BDV and BVDV (type 1) groups. Characterisation of the biologically cloned vaccine viruses should include typing with MAbs and genotyping (16).

b) **Culture**

A variety of ruminant cell cultures can be used. Optimal yields depend on the cell type and isolate used. A commercial BDV vaccine containing two strains of virus is prepared on ovine cell lines (5). Cells must be produced according to a seed-lot system from a master cell seed (MCS) that has been shown to be free from all contaminating microorganisms. Vaccine should only be produced in cells fewer than 20 passages from the MCS. Control cells from every passage should be checked for pestivirus contamination.

c) **Validation as a vaccine**

All vaccines should pass standard tests for safety and efficacy. Safety testing of inactivated BDV vaccines should include monitoring of all vaccine components for contaminating pestiviruses.

Efficacy tests of BDV vaccines should demonstrate their ability to prevent transplacental spread of virus. Effective challenge of vaccinated pregnant ewes at 50–60 days gestation has been achieved by intranasal installation of virus or by mixing with PI sheep (5).

2. **Method of manufacture**

Inactivated vaccines have been prepared using conventional laboratory techniques with stationary or rolled cell cultures. Inactivants have included formalin and beta-propiolactone. Adjuvants have included aluminium hydroxide and oil (5, 25).

3. **In-process control**

Cultures should be inspected daily to ensure they are free from gross bacterial contamination and that any CPE observed is appropriate to the cytopathic virus being grown. No CPE should be observed in cultures being used to grow noncytopathic strains of virus.
4. Batch control
   a) Sterility
      Tests for sterility and freedom from contamination of biological materials may be found in Chapter 1.1.9.
   b) Safety
      Samples from inactivated vaccines should be tested rigorously for viable virus. Samples of the product should be passaged several times in sensitive cell cultures to ensure absence of live BDV. This *in-vitro* monitoring can be augmented by injecting two BDV-seronegative sheep with 20 doses of unformulated antigen as part of a standard safety test. Presence of live virus will result in the development of a more convincing serological response than will occur with inactivated virus alone. The sheep sera can also be examined for antibody to other prescribed agents.
   c) Potency
      Vaccine potency is also best tested in seronegative sheep in which the development and level of antibody is measured. An indirect measure of potency is given by the level of virus infectivity prior to inactivation. The antigen content following inactivation can be assayed by MAb-capture ELISA and related to the results of established *in-vivo* potency results. As recommended for potency testing of BVDV vaccine in cattle it should be demonstrated that the vaccine can prevent transplacental transmission of BDV in pregnant sheep.
   d) Duration of immunity
      No information is available on duration of immunity following vaccination. Inactivated vaccines are unlikely to provide sustained levels of immunity and it is likely that after an initial course of two or three injections annual booster doses may be required. Insufficient information is available to determine any correlation between vaccinal antibody titres in the dam and fetal protection.
   e) Stability
      There is little information on the stability of BDV vaccines. Inactivated vaccines could be expected to have at least a 1 year shelf life when protected from light and stored at 4°C.
   f) Preservatives
      Preservatives may be added to multidose vaccine containers subject to the approval of the Control Authority.
   g) Precautions (hazards)
      BDV is not considered to be a hazard to human health. Standard good microbiological practice should be used when handling the virus.

5. Tests on the final product
   a) Safety
      *In-vitro* test only.
   b) Potency
      *In-vitro* antigen content test.

REFERENCES


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CHAPTER 2.7.2.

CAPRINE AND OVINE BRUCELLOSIS
(excluding Brucella ovis)

SUMMARY

Brucella melitensis (biovars 1, 2 or 3) is the main causative agent of caprine and ovine brucellosis. Sporadic cases caused by B. abortus have been observed, but cases of natural infection are rare in sheep and goats. Brucella melitensis is endemic in the Mediterranean region, but infection is widespread world-wide. North America (except Mexico) is believed to be free from the agent, as are Northern and Central Europe, South-East Asia, Australia and New Zealand.

Clinically, the disease is characterised by one or more of the following signs: abortion, retained placenta, orchitis, epididymitis and, rarely, arthritis, with excretion of the organisms in uterine discharges and in milk. Diagnosis depends on the isolation of Brucella from abortion material, udder secretions or from tissues removed at post-mortem. Presumptive diagnosis can be made by assessing specific cell-mediated or serological responses to Brucella antigens.

Brucella melitensis is highly pathogenic for humans, causing one of the most serious zoonoses in the world, and all infected tissues, cultures and potentially contaminated materials should be handled at containment level 3.

Identification of the agent: Presumptive evidence of Brucella is provided by the demonstration, by modified acid-fast staining of organisms typical of Brucella in abortion material or vaginal discharge, especially if supported by serological tests. The polymerase chain reaction (PCR) methods provide additional means of detection. Whenever possible, Brucella spp. should be isolated using selective or non-selective media by culture from uterine discharges, aborted fetuses, udder secretions or selected tissues, such as lymph nodes, spleen, uterus, testes and epididymes. Species and biovars should be identified by phage lysis, and by cultural, biochemical and serological criteria. PCR has been introduced as a complementary identification method based on specific genomic sequences.

Serological and allergic skin tests: The Brucella-buffered antigen test and the complement fixation test (CFT) tests are usually recommended for screening flocks and individual animals. The serum agglutination test is not considered to be reliable for use in small ruminants. The indirect enzyme-linked immunosorbent assay (ELISA) can also be used for screening purposes. For pooled samples, there are no useful tests such as the milk ring test for cattle. The brucellin allergic skin test can be used as a screening or complementary test in unvaccinated flocks, provided that a purified, lipopolysaccharide (LPS)-free, standardised antigen preparation is used. Results must then be interpreted in relation to the clinical signs, history, and results of serological or cultural examination.

Requirements for vaccines and diagnostic biologicals: Brucella melitensis strain Rev.1 remains the reference vaccine to immunise sheep and goats at risk of infection from B. melitensis and is the vaccine with which any other vaccines should be compared. Production of Brucella antigens or Rev.1 vaccine is based on a seed-lot system. Seed cultures to be used for antigens for serological and allergic skin tests and for vaccines should originate from reference centres. They must conform to minimal standards for viability, smoothness, residual infectivity and immunogenicity, purity, identity and safety, if applicable. Brucellin preparations for the intradermal test must be free of smooth lipopolysaccharide and must not produce nonspecific inflammatory
Brucellosis in sheep and goats (excluding *Brucella ovis* infection) is primarily caused by one of the three biovars of *B. melitensis*. Sporadic infections caused by *B. abortus* or *B. suis* have been observed in sheep and goats, but such cases are rare. Pathologically and epidemiologically, *B. melitensis* infection in sheep and goats is very similar to *B. abortus* infection in cattle (see Chapter 2.4.3 Bovine brucellosis). In most circumstances, the primary route of transmission of *Brucella* is the placenta, fetal fluids and vaginal discharges expelled by infected ewes and goats when they abort or have a full-term parturition. Shedding of *Brucella* is also common in udder secretions and semen, and *Brucella* may be isolated from various tissues, such as lymph nodes from the head, spleen and organs associated with reproduction (uterus, epididymides and testes), and from arthritic lesions (2).

*Brucella melitensis* infection in domestic and wild susceptible species (see Chapter 2.4.3) is not rare when these species are reared in close contact with sheep and goats in enzootic areas. The manifestations of brucellosis in these animals are similar to those in cattle or sheep and goats.

The World Health Organization (WHO) laboratory biosafety manual classifies *Brucella* (and particularly *B. melitensis*) in Risk group III. Brucellosis is readily transmissible to humans, causing acute febrile illness – undulant fever – which may progress to a more chronic form and can also produce serious complications affecting the musculo–skeletal, cardiovascular, and central nervous systems. Infection is often due to occupational exposure and is essentially acquired by the oral, respiratory, or conjunctival routes, but ingestion of dairy products constitutes the main risk to the general public. There is an occupational risk to veterinarians, abattoir workers and farmers who handle infected animals and aborted fetuses or placentas. Brucellosis is one of the most easily acquired laboratory infections, and strict safety precautions should be observed when handling cultures and heavily infected samples, such as products of abortion. Specific recommendations have been made for the safety precautions to be observed with *Brucella*-infected materials (for further details see Chapter 1.1.2 Biosafety and biosecurity in the veterinary microbiology laboratory and animal facilities, and refs 2, 42, 95 and 96 of Chapter 2.4.3). Laboratory manipulation of live cultures or contaminated material from infected animals is hazardous, as is handling large volumes of *Brucella*, and must be done under containment level 3 or higher conditions, as outlined in Chapter 1.1.2, to minimise occupational exposure.

Genetic and immunological evidence indicates that all members of the *Brucella* genus are closely related and it has been proposed (but not yet accepted by the Taxonomy Subcommittee) that the genus contains a single species of which the classical species (*B. abortus, B. melitensis*, etc.) would be mere biovars (for a review see Chapter 2.4.3, ref. 53). Nevertheless, in 2005, based on relevant differences in host preference and epidemiology displayed by the major variants, as well as molecular evidence of genomic variation, the International Committee on Systematics of Prokaryotes, Subcommittee on the Taxonomy of *Brucella* took a clear position to return to pre-1986 *Brucella* taxonomy; the consequences of this imply the re-approval of the six *Brucella* nomenspecies with recognised biovars. The classical names related to the six *Brucella* nomenspecies are published in the Approved Lists of Bacterial Names, 1980, and the designated type strains are attached to these published names: *Brucella abortus, B. melitensis, B. suis, B. neotomae, B. ovis* and *B. canis* (http://www.the-icsp.org/subcoms/ Brucella.htm). The first three of these are subdivided into biovars based on cultural and serological properties (see Tables 1 and 2 in Chapter 2.4.3). The classification, microbiological and serological properties of the genus *Brucella* and related species and biovars are given in the Chapter 2.4.3 Bovine brucellosis.

### A. INTRODUCTION

### B. DIAGNOSTIC TECHNIQUES

1. **Identification of the agent**

Refer to Chapter 2.4.3 Bovine brucellosis for the detailed agent identification procedure for *Brucella*.

2. **Serological tests**

In situations where bacteriological examination is not practicable, diagnosis of *Brucella* infection must often be based on serological methods (2, 18). In routine tests, anti-*Brucella* antibodies are detected in serum. The most widely used serum-testing procedures for the diagnosis of smooth *Brucella* infections in sheep and goats are the buffered *Brucella* antigen tests (BBAT), i.e. the card and Brucella-buffered antigen tests, which are essentially the same, and the complement fixation test (CFT). The bulk milk ring test, which has been very useful in cattle, is ineffective in small ruminants.
In small ruminants, the *Brucella*-buffered antigen test and the CFT are the most widely used methods (17) and are the only tests prescribed for international trade. The *Brucella*-buffered antigen test is not completely specific, but is adequate as a screening test for detecting infected flocks or for guaranteeing the absence of infection in brucellosis-free flocks. However, due to the relative lack of sensitivity of both tests, discrepancies between results obtained using the *Brucella*-buffered antigen test and the CFT are not rare in infected sheep and goats (6). The results of the two tests should therefore be considered simultaneously to increase the likelihood of detecting infected individuals and to improve control of the disease in areas where it has not been completely eradicated (1, 4, 6). When, for practical or economic reasons, the CFT cannot be used simultaneously with the *Brucella*-buffered antigen test in eradication programmes, it is recommended to improve the sensitivity of the *Brucella*-buffered antigen test by using 75 µl of serum and 25 µl of antigen in place of an equal volume of each. This simple modification increases *Brucella*-buffered antigen test sensitivity and minimises the discrepancies between *Brucella*-buffered antigen test and CFT results (6). Because antibodies induced after Rev.1 vaccination cannot be differentiated in both tests from those induced by *B. melitensis* infection, *Brucella*-buffered antigen test and CFT results should be carefully interpreted according to the vaccination status in the flock. In addition, both tests are not specific enough to discriminate serological reactions due to *B. melitensis* from the false-positive reactions (FPSR) due to cross-reacting bacteria such as *Yersinia enterocolitica* O:9.

Good diagnostic results have been obtained in sheep and goats with indirect or competitive enzyme-linked immunosorbent assays (ELISAs) using various antigens, but generally the ELISAs that use antigens with a high content of smooth lipopolysaccharide (sLPS) are the most useful. These tests provide similar (competitive ELISA – C-ELISA) or better (indirect ELISA – I-ELISA) sensitivity than both *Brucella*-buffered antigen test and CFT, but like the classical tests, both ELISAs are generally unable to differentiate *B. melitensis* infected animals from those recently vaccinated with the Rev.1 vaccine (19) or infected with cross-reacting bacteria. However, there have been preliminary studies on a C-ELISA that can differentiate vaccine antibody from that produced by infection. I- and C-ELISAs with a highly immunogenic periplasmic protein from *B. abortus* (20) and *B. melitensis* (11) have been applied in sheep and reported to be promising in differentiating Rev.1 vaccinated from *B. melitensis* infected animals (10, 13). Some of these ELISAs have potential advantages in sensitivity and/or specificity with respect to both *Brucella*-buffered antigen test and CFT, but the standardisation of reagents is still required (15).

- **Reference sera**

  The primary reference serum for standardising *Brucella*-buffered antigen test and CFT in sheep and goats is the OIE International Standard Serum (OIEISS; see Chapter 2.4.3 Bovine brucellosis).

- **Production of cells**

  Please refer to Chapter 2.4.3 Bovine brucellosis. *Brucella abortus* biovar 1 strains 99 or 1119 are the only strains recommended for the preparation of *Brucella*-buffered antigen test and CFT in sheep and goats.

  a) **Brucella-buffered antigen test (a prescribed test for international trade)**

     Please refer to Chapter 2.4.3 Bovine brucellosis.

     - **Antigen production**

       Please refer to Chapter 2.4.3 Bovine brucellosis. Note that RB antigen made with *B. abortus* is usually used to test for *B. melitensis*. The standardisation of RB antigen, as it is prescribed in Chapter 2.4.3, provides a sufficient sensitivity to the *Brucella*-buffered antigen test for international trade purposes. Moreover, it helps assure an adequate specificity in free areas where FPSR occur because of cross-reacting bacteria such as *Yersinia enterocolitica* O:9. However this standardisation is probably the main cause of the reduced sensitivity of some RB antigen batches and of the discrepancies with the CFT (6). Therefore, when *Brucella*-buffered antigen test is used in eradication programmes in endemic areas, it could be advisable to adjust the RB antigen titre so that it is positive at a 1/45 OIEISS dilution and negative at a 1/55 dilution, without affecting the specificity of the test. The discrepancies with the CFT can also be minimised by using 75 µl of serum and 25 µl of antigen in place of an equal volume of each as mentioned in the standard test procedure.

     - **Test procedure**

       Please refer to Chapter 2.4.3 Bovine brucellosis.

  b) **Complement fixation test (a prescribed test for international trade)**

     - **Antigen production**

       Please refer to Chapter 2.4.3 Bovine brucellosis. Note that CF antigen made with *B. abortus* is usually used to test for *B. melitensis*. 

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c) Enzyme-linked immunosorbent assays

Several variations of the I-ELISA have been described employing different antigen preparations, antiglobulin-enzyme conjugates, and substrate/chromogens. Several commercial I-ELISAs are available but to date there is no internationally recognised procedure for standardising those tests for use for international trade purpose in small ruminant brucellosis.

The test method is described in Chapter 2.4.3 Bovine brucellosis

3. Other tests

a) Fluorescence polarisation assay (an alternative test for international trade)

The fluorescence polarisation assay (FPA) is a simple technique for measuring antigen/antibody interaction and may be performed in a laboratory setting or in the field. It is an homogeneous assay in which analytes are not separated and it is therefore very rapid.

The mechanism of the assay is based on random rotation of molecules in solution. Molecular size is the main factor influencing the rate of rotation, which is inversely related. Thus a small molecule rotates faster than a large molecule. If a molecule is labelled with a fluorochrome, the time of rotation through an angle of 68.5° can be determined by measuring polarised light intensity in vertical and horizontal planes. A large molecule emits more light in a single plane (more polarised) than a small molecule rotating faster and emitting more depolarised light.

For most FPAs, an antigen of small molecular weight, less than 50 kD, is labelled with a fluorochrome and added to serum or other fluid to be tested for the presence of antibody. If antibody is present, attachment to the labelled antigen will cause its rotational rate to decrease and this decrease can be measured.

For the diagnosis of brucellosis, a small molecular weight fragment (average 22 kD) of the O-polysaccharide (OPS) of \( B. \) \( \text{abortus} \) sLPS is labelled with fluorescein isothiocyanate (FITC) and used as the antigen. This antigen is added to diluted serum or whole blood and a measure of the antibody content is obtained in about 2 minutes using a fluorescence polarisation analyser.

The FPA procedure for the diagnosis of \( Brucella \) is described in detail in Chapter 2.4.3 Bovine brucellosis,

b) Brucellin skin test (an alternative test for international trade)

An alternative diagnostic test is the brucellin skin test, which can be used for screening unvaccinated flocks, provided that a purified (free of sLPS) and standardised antigen preparation (e.g. brucellin INRA) is used.

The brucellin skin test has a high sensitivity for the diagnosis of \( B. \) \( \text{melitensis} \) infection in small ruminants and, in absence of vaccination, is considered one of the most specific diagnostic tests (2, 4, 15, 17). This test is of particular value for the interpretation of FPSR due to infection with cross-reacting bacteria (FPSR affected animals are always negative in the skin test), especially in brucellosis-free areas.

Despite its high sensitivity not all infected animals show positive skin test responses to brucellin and, moreover, Rev.1 vaccinated animals can react in this test for years (15). Therefore this test cannot be recommended either as the sole diagnostic test or for the purposes of international trade.

To obtain suitable results it is essential to use standardised brucellin preparations that do not contain sLPS, as this antigen may provoke antibody-mediated inflammatory reactions or induce antibodies that interfere with subsequent serological screening. One such preparation is brucellin INRA, which is prepared from a rough strain of \( B. \) \( \text{melitensis} \) that is commercially available¹.

Test procedure

i) A volume of 0.1 ml of brucellin is injected intradermally into the lower eyelid.

ii) The test is read after 48 hours.

¹ Brucellergène OCB®, Synbiotics Europe, 2 rue Alexander Fleming, 69007 Lyon, France.
iii) Any visible or palpable reaction of hypersensitivity, such as an oedematous reaction leading to an elevation of the skin or thickening of the eyelid (≥ 2 mm), should be interpreted as a positive reaction.

Although in the absence of vaccination the brucellin intradermal test is one of the most specific tests in brucellosis, diagnosis should not be made exclusively on the basis of positive intradermal reactions and should be supported by adequate serological tests. The intradermal inoculation of brucellin might induce a temporary anergy in the cellular immune response. Therefore an interval of 6 weeks is generally recommended between two tests repeated on the same animal.

**C. REQUIREMENTS FOR VACCINES AND DIAGNOSTIC BIOLOGICALS**

**C1. Brucellin**

Please refer to Chapter 2.4.3 Bovine brucellosis.

**C2. Vaccines**

*Brucella melitensis* strain Rev.1 vaccine

The most widely used vaccine for the prevention of brucellosis in sheep and goats is the *Brucella melitensis* Rev.1 vaccine, which remains the reference vaccine with which any other vaccines should be compared. The Rev.1 vaccine is used as a freeze-dried suspension of live *B. melitensis* biovar 1 Rev.1 strain for the immunisation of sheep and goats. It should be normally given to lambs and kids aged between 3 and 6 months as a single subcutaneous or conjunctival inoculation. The standard dose is between $0.5 \times 10^9$ and $2.0 \times 10^9$ viable organisms. The subcutaneous vaccination induces strong interferences in serological tests and should not be recommended in combined eradication programmes (14, 19). However, when this vaccine is administered conjunctivally, it produces a similar protection without inducing a persistent antibody response, thus facilitating the application of eradication programmes combined with vaccination (14, 19). Care must be taken when using Rev.1 vaccine to avoid the risk of contaminating the environment or causing human infection. In many developing countries and endemic areas, vaccination of the whole population has to be considered as the best option for the control of the disease (5). However, Rev.1 vaccine is known to often cause abortion and excretion in milk when animals are vaccinated during pregnancy, either with a full or reduced dose (5). These side-effects are considerably reduced when adult animals are vaccinated conjunctivally (full dose) before mating or during the last month of pregnancy. Therefore, when mass vaccination is the only means of controlling the disease, a vaccination campaign should be recommended using the standard dose of Rev.1 administered by the conjunctival route when the animals are not pregnant or during the lambing season (5).

The subcutaneous vaccination of young animals and the vaccination of adult animals, even at reduced doses, may lead to long-term persistence of vaccinal antibodies in a significant proportion of vaccinated animals that creates serious interferences in the serological diagnosis of brucellosis. As indicated above, conjunctival vaccination minimises these problems and thus it is the recommended method for combined eradication programmes. Therefore, the serological diagnosis of brucellosis should take into account the vaccinal state of the herd and the overall frequency distribution of antibody titres detected in the group of animals tested.

1. **Seed management**

a) **Characteristics of the seed**

*Brucella melitensis* biovar 1 strain Rev.1 original seed for vaccine production can be obtained commercially. A European reference Rev.1 strain that possesses the characteristics of the Rev.1 original seed has been recently produced by the European Pharmacopoeia.

Production of *Brucella* live vaccines is based on the seed-lot system described above (Section B.2) for *Brucella*-buffered antigen test and CFT antigens. Strains should be cultured in a suitable medium. Strain Rev.1 must conform to the characteristics of *B. melitensis* biovar 1, except that it should grow more slowly. Additionally, when incubated in air (atmospheres containing CO₂ alter the results) at 37°C, it should grow on agar containing streptomycin (2.5 µg/ml), and it should be inhibited by the addition to a suitable culture medium of sodium benzylpenicillin (3 µg [5 International Units (IU)]/ml), thionin (20 µg/ml) or basic fuchsin (20 µg/ml). Recently, polymerase chain reaction and molecular techniques have been used to further

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2 Obtainable from Institut National de la Recherche Agronomique (INRA), Laboratoire de Pathologie Infectieuse et Immunologie, 37380 Nouzilly, France.

3 Obtainable from the European Pharmacopoeia, BP 907, 67029 Strasbourg Cedex 1, France.
characterise the vaccine (3, 12). It must also conform to the characteristics of residual virulence and immunogenicity in mice of the original seed.

b) Method of culture

Serum–dextrose agar, and trypticase–soy agar, to which 5% serum or 0.1% yeast extract may be added, are among the solid media that have been found to be satisfactory for propagating the Rev.1 strain (2, 21). Rev.1 strain does not grow well on potato agar.

For vaccine production, Rev.1 may be grown under conditions similar to those described for S99 and S1119-3 (see Chapter 2.4.3), except that Rev.1 generally needs 3–5 days to grow, the phenol saline is replaced by a freeze-drying stabiliser, and the organisms are not killed but are stored at 4°C while quality control examinations are carried out as described below. Moreover, the specific requirements for Rev.1 vaccine production recommend that: each seed lot (i.e. the culture used to inoculate medium for vaccine production) should be no more than three passages removed from an original seed culture and that the harvest of a vaccine lot should be no more than three passages from a seed lot or an original seed. The original seed culture should always be checked for the absence of dissociation before use. The recommended method for preparing seed material is given in ref. 2. The following freeze-drying stabiliser (sterilised by filtration) is of proven value: enzymatic digest of casein (2.5 g); sucrose (5 g); sodium glutamate (1 g); distilled water (100 ml).

c) Validation as a vaccine

Numerous independent studies have confirmed the value of B. melitensis strain Rev.1 as a vaccine for protecting sheep and goats from brucellosis. Its virulence is unchanged after passage through pregnant sheep and goats. Abortions may result when the Rev.1 vaccine is inoculated into pregnant ewes or goats. The vaccine-induced abortions are not avoided using reduced doses, and doses as low as 10^6, used either subcutaneously or conjunctivally, have been demonstrated to induce abortions and milk excretion of the vaccine strain (5).

A Rev.1 vaccine is efficient if it possesses the characteristics of the Rev.1 original strain, i.e. those of B. melitensis biovar 1 reference strain 16M (ATCC No. 23456), except those specific for the strain Rev.1 (2, 17), and if it proves to be satisfactory with respect to immunogenicity and residual virulence in the mouse model (8) (see below).

2. Method of manufacture (2, 21)

For production of B. melitensis strain Rev.1 vaccine, the procedures described above for antigens (2) can be used except that the cells are collected in a freeze-drying stabiliser and deposited by centrifugation. The yield from one fermenter run or the pooled cells from a batch of Roux flask cultures inoculated on the same occasion from the same seed lot constitutes a single harvest. More than one single harvest may be pooled to form the final bulk that is used to fill the final containers of a batch of vaccine. Before pooling, each single harvest must be checked for purity, cell concentration, dissociation and identity. The volume of the final bulk is adjusted by adding sufficient stabiliser so that a dose contains an appropriate number of viable organisms. After adjusting the cell concentration of the final bulk, tests for identity, dissociation and absence of contaminating organisms are conducted (see below).

3. In-process control

In-process checks should be made on the growth of Rev.1 vaccine from either solid or liquid medium to verify identity and to ensure purity and freedom from dissociation to rough forms during preparation of seed lots, single harvests, final bulks and the final (filling) lots. At least 99% of cells in seed lots and 95% of cells in final lots should be in the smooth phase.

Cell concentration should be estimated on the bulks and precisely determined on final lots. Immunogenicity and the residual virulence (50% persistence time or 50% recovery time) should also be determined on seed lots and final lots.

4. Batch control

With freeze-dried vaccine, the control tests should be conducted on the vaccine reconstituted in the form in which it will be used.

a) Sterility (or absence of extraneous microorganisms)

The Rev.1 vaccine should be checked for bacterial and fungal contamination as prescribed in Chapter 1.1.9 Tests for sterility and freedom from contamination of biological materials.
b) Safety

The Rev.1 vaccine is a virulent product per se, and it should keep a minimal virulence to be efficacious (see Section C2.4.c in Chapter 2.4.3).

c) Potency

A Rev.1 vaccine is efficient if it possesses the characteristics of the Rev.1 original strain, i.e. if it is satisfactory with respect to immunogenicity, and residual virulence (9). Batches should also be checked for the number of viable organisms.

- Identity
  
The reconstituted Rev.1 vaccine should not contain extraneous microorganisms. Brucella melitensis present in the vaccine is identified by suitable morphological, serological and biochemical tests and by culture: when incubated in air at 37°C, Rev.1 strain is inhibited by addition to the suitable culture medium of 3 µg (5 IU) per ml of sodium benzyl-penicillin, thionin (20 µg/ml) or basic fuchsin (20 µg/ml); the strain grows on agar containing 2.5 µg per ml of streptomycin.

- Smoothness (determination of dissociation phase)
  
Please refer to Chapter 2.4.3 Bovine brucellosis.

Sometimes slight and difficult to observe differences, can be seen in the size of Rev 1 colonies. The small colonies (1–1.2 mm in diameter) are typical for Rev.1, but larger Rev. 1 colonies can appear depending on the medium used, the amount of residual moisture in the incubator atmosphere, and the presence or absence of CO₂. The frequency of variation in colony size occurs normally at a ratio of 1 large to 10³ small colonies. Both Rev.1 variants are of the S (smooth) type. To avoid an increase in this colony size variation along successive passages, it is important to always select small colonies for preparation of seed lots.

- Enumeration of live bacteria
  
Please refer to Chapter 2.4.3 Bovine brucellosis.

- Residual virulence (50% persistence time or 50% recovery time) (7, 16)
  
The same technical procedures indicated for 50% recovery time (RT₅₀) calculation of S19 vaccine (see Chapter 2.4.3) have to be applied for Rev.1, except that B. abortus S19 seed lot or batch to be tested (test vaccine) and the S19 original seed culture (used as a reference strain), respectively, are replaced by the corresponding B. melitensis Rev.1 seed lot or batch to be tested (test vaccine) and the B. melitensis Rev.1 original seed culture as the reference strain. For the reference original Rev.1 strain, RT₅₀ and confidence limits are around 7.9 ± 1.2 weeks. A given Rev.1 vaccine seed lot or batch should keep similar residual virulence to be acceptable.

If this test has been done with good results on a representative batch of the test vaccine, it does not have to be repeated routinely on other vaccine lots prepared from the same seed lot and with the same manufacturing process.

- Immunogenicity in mice
  
The same technical procedures indicated for immunogenicity calculation of S19 vaccine (see Chapter 2.4.3) have to be applied for Rev.1, except that B. abortus S19 seed lot or batch to be tested (test vaccine) and the B. melitensis Rev.1 seed lot or batch to be tested (test vaccine) and the B. melitensis Rev.1 original seed culture as the reference strain.

Conditions of the control experiment are satisfactory when: i) the response in unvaccinated mice (mean of Y) is at least of 4.5; ii) the response in mice vaccinated with the reference Rev.1 vaccine is lower than 2.5; and iii) the standard deviation calculated on each lot of six mice is lower than 0.8.

If this test has been done with good results on a representative batch of the test vaccine, it does not have to be repeated routinely on other vaccine lots prepared from the same seed lot and with the same manufacturing process.

d) Duration of immunity

It is accepted that subcutaneous or conjunctival vaccination with standard doses of Rev.1 confers a solid and durable immunity in sheep and goats. However, growing field evidence shows that the immunity conferred declines with time, and revaccination could be advisable in endemic areas.
Chapter 2.7.2. — Caprine and ovine brucellosis (excluding Brucella ovis)

The use of reduced doses of Rev.1 produces a less efficient immunity, while side-effects, such as antibody responses or induction of abortion, are not fully avoided.

e) Stability

Strain Rev.1 vaccine prepared from seed stock from appropriate sources is stable in characteristics provided that the in-process and batch control requirements described above are fulfilled, and shows no tendency to reversion to virulence. The lyophilised vaccine shows a gradual loss of viable count, but should retain its potency for the recommended shelf life. Allowance for this phenomenon is normally made by ensuring that the viable count immediately following lyophilisation is well in excess of the minimum requirement. Maintenance of a cold chain during distribution of the vaccine will ensure its viability.

f) Preservatives

Antimicrobial preservatives must not be used in live Rev.1 vaccine. For preparation of the freeze-dried vaccine, a stabiliser as described in Section C2.4.f of Chapter 2.4.3 is recommended.

g) Precautions (hazards)

Brucella melitensis Rev.1, although an attenuated strain, is still capable of causing disease in humans. The cell cultures and suspensions must be handled under appropriate conditions of biohazard containment (see Chapter 1.1.2). Reconstitution and subsequent handling of the reconstituted vaccine should be done with care to avoid accidental injection or eye or skin contamination. Vaccine residues and injection equipment should be decontaminated with a suitable disinfectant (phenolic, iodophor or aldehyde formulation) at a recommended concentration. Medical advice should be sought in the event of accidental exposure. The efficacy of the antibiotic treatment of infections caused by Rev.1 in humans has not been adequately established. If Rev.1 contamination occurs, a combined treatment with doxycycline plus rifampicin could be recommended.

5. Tests on the final product

a) Safety

See Section C2.4.b of Chapter 2.4.3.

b) Potency

For the freeze-dried vaccine, the potency must be determined on the final product. The tests are as described in Section C2.4.c of Chapter 2.4.3.

In order to assess the vaccine efficiency, a representative sample of previously seronegative animals vaccinated with each new vaccine batch should be bled 15–20 days after vaccination and the serum samples submitted to Brucella-buffered antigen test. If adequate and independently of the vaccination route used, more than 80% of vaccinated animals should be Brucella-buffered antigen test positive.

REFERENCES


| * | * |

**NB:** There are OIE Reference Laboratories for Caprine and ovine brucellosis (excluding *Brucella ovis*) (see Table in Part 3 of this Terrestrial Manual or consult the OIE Web site for the most up-to-date list: www.oie.int).
CAPRINE ARTHRITIS-ENCEPHALITIS & MAEDI-VISNA

SUMMARY

Maedi-visna (MV) and caprine arthritis-encephalitis (CAE) are persistent lentivirus infections of sheep and goats and are often grouped together as the small ruminant lentiviruses (SRLVs). Maedi-visna is also known as ovine progressive pneumonia (OPP). Phylogenetic analyses comparing nucleotide sequences of MV virus (MVV) and CAE virus (CAEV) has demonstrated that these are closely related lentiviruses. One source of CAEV and MVV transmission is colostrum and milk. The source of horizontal transmission in the absence of lactation remains unknown; however, faeces and lung fluids are known to harbour infectious virus. Ovine lentiviruses have been identified in most of the sheep-rearing countries of the world, with the notable exceptions of Australia and New Zealand. The distribution of CAEV is highest in industrialised countries, and seems to have coincided with the international movement of European breeds of dairy goats. Clinical and subclinical MV and CAE are associated with progressive, mononuclear cell inflammatory lesions in the lungs, joints, udder and central nervous system. Indurative mastitis is common in both species, and its economic significance may be underestimated. Labourted breathing associated with emaciation caused by progressive pneumonitis is the predominant features in clinically affected sheep, whereas polyarthritis is the main clinical sign in goats. However, most lentivirus-infected sheep and goats are largely asymptomatic, but remain persistent carriers of virus and are capable of transmitting infection via colostrum or milk and respiratory secretions. The most practical and reliable approach to confirming a diagnosis of MV or CAE is a combination of serology and clinical evaluation. Although serology represents the most cost-effective method of diagnosing persistently infected, clinically normal animals, it should be understood that testing errors occur. The frequency of error depends on several factors including but not limited to: 1) the assay format, 2) the homology between the strain of virus used in the assay and the strains of virus present in the testing populations, and 3) the viral antigen used in the assay.

Identification of the agent: Virus isolation can be attempted from live clinical or subclinical cases by co-cultivating peripheral blood or milk leukocytes with appropriate ovine or caprine cell cultures, such as choroid plexus (MVV) or synovial membrane (CAEV) cells. Virus isolation is very specific but has variable sensitivities. Following necropsy, virus isolation is most readily accomplished by establishing explant cultures of affected tissues, e.g. lung, choroid plexus, synovial membrane or udder. Also, alveolar macrophages may be obtained from the lung at post-mortem and co-cultivated with susceptible cells. The cytopathic effects are characteristic, consisting of the appearance of refractile stellate cells and syncytia. The presence of MVV or CAEV can be confirmed by immunoblotting methods and electron microscopy.

Nucleic acid recognition methods: Many standard and a few quantitative polymerase chain reaction (PCR) assays for detecting MV and CAE provirus have been described and are now being used routinely in many laboratories for the rapid detection, quantitation, and identification of the small ruminant lentivirus strains. Cloning and/or sequencing of PCR products is the most direct method to confirm specificity of PCR products.

Serological tests: Most infected sheep and goats possess detectable specific antibodies that can be assayed by a number of different serological tests. The two most commonly used are the agar gel immunodiffusion test and the enzyme-linked immunosorbent assay (ELISA). Western blot analysis and radio-immunoprecipitation are also performed, but only in specialised laboratories. A milk antibody assay may be appropriate in dairy goat herds. The time required for seroconversion
following infection can be relatively prolonged and unpredictable, being measured in months rather than in weeks. However, after seroconversion, the antibody response usually persists and antibody-positive sheep and goats are regarded as virus carriers.

Requirements for vaccines and diagnostic biologicals: There are no biological products available.

A. INTRODUCTION

Maedi-visna (MV), of sheep and caprine arthritis/encephalitis (CAE) of goats are persistent virus infections caused by closely related lentiviruses (34). Maedi-visna is also known as ovine progressive pneumonia (OPP). Sheep can be experimentally infected with CAE and goats can be experimentally infected with MV (4). In addition, phylogenetic analyses comparing nucleotide sequences of MV virus (MVV) and CAE virus (CAEV) show clear indications of the existence and epidemiological importance of cross-species transmission between sheep and goats without demonstrating clearly that one virus has emerged from the other (17, 23, 28, 37, 40, 41, 45, 47). MV and CAE are characterised by lifelong persistence of the causal agent in host monocytes and macrophages, and a variable length of time between infection and induction of a serologically detectable antiviral antibody response. Most infected sheep and goats do not exhibit clinical disease, but remain persistently infected and are capable of transmitting virus (2, 5, 7).

Maedi-visna is an Icelandic name that describes two of the clinical syndromes recognised in MV virus (MVV)-infected sheep. ‘Maedi’ means ‘laboured breathing’ and describes the disease associated with a progressive interstitial pneumonitis, and ‘visna’ means ‘shrinkage’ or ‘wasting’, the signs associated with a paralysing meningoencephalitis. Whereas progressive lung disease is the primary finding with MVV infection, chronic polyarthritis, with synovitis and bursitis is the primary clinical outcome of CAE virus (CAEV) infection. Encephalitis occurs primarily in kids aged between 2 and 6 months following CAEV infection, but careful differential diagnoses need to be conducted to rule out other syndromes or infections in kids. Indurative mastitis occurs in both syndromes. The lungs of sheep affected by MV do not collapse when removed from the thorax and often retain the impression of the ribs. The lungs and lymph nodes increase in weight (up to 2–3 times the normal weight). The lesions are uniformly distributed throughout the lungs, which are uniformly discoloured or mottled grey-brown in colour and of a firm texture. Udders affected by MV are diffusely indurated and associated lymph nodes may be enlarged.

When MV or CAE is the suspected cause of clinical disease, confirmation of the diagnosis can be achieved by a combination of clinical evaluation, serology and, when necessary, histological examination of appropriate tissues collected at necropsy. Important tissues to examine include lung for progressive interstitial pneumonitis, brain and spinal cord for meningoencephalitis, udder for indurative mastitis, affected joints and synovium for arthritis, and kidney for vasculitis (6, 9, 10, 26, 30, 31). The nature of the inflammatory reaction in each site is similar, consisting of an interstitial, mononuclear cell reaction, sometimes with large aggregates of lymphoid cells and follicle formation.

B. DIAGNOSTIC TECHNIQUES

1. Identification of the agent

Isolation and characterisation of MVV or CAEV would not normally be attempted for routine diagnostic purposes. Due to the persistent nature of these infections, the establishment of a positive antibody status is sufficient for the identification of virus carriers. However, due to a late seroconversion after infection, negative serology may occur in recently infected animals.

There are two approaches to the isolation of MVV and CAEV: one for use with the live animal, and the second for use with necropsy tissues.

a) Isolation from the live animal

- Maedi-visna virus

The MV provirus DNA is carried in circulating monocytes and tissue macrophages. Virus isolation from the live animal therefore requires the establishment of leukocyte preparations, with aseptic precautions, from peripheral blood or milk during lactation, culturing them together with indicator cells. Sheep choroid plexus (SCP) cells are commonly used for this purpose. These indicator cells can be prepared as primary explant cultures from fetal or newborn virus-free lambs, and their number can be multiplied over three to four passages for storage in liquid nitrogen. The recovered SCP cells are suitable for co-cultivation for up to 10 or 15 passages. Although the cells continue to grow well thereafter, their susceptibility to MVV may become reduced.
Leukocyte preparations can be made from peripheral blood as buffy coats by the centrifugation at 1000 g of heparinised, ethylene diamine tetra-acetic acid (EDTA) or citrated samples for 15 minutes. The cells are aspirated, suspended in Hanks’ balanced salt solution (HBSS), and further purified by centrifugation at 400 g on to a suitable cushion (e.g. Ficoll Paque [Pharmacia]) for 40 minutes. The interface cells are spin-washed once or twice in HBSS at 100 g for 10 minutes, and the final cell pellet is resuspended in medium to a concentration of approximately 10^6 cells/ml; cells are generally cultured for 10–12 days in Teflon bags and are then added to a washed monolayer of slightly subconfluent SCP cells in a flask with an area of 25 cm^2.

Leukocytes can be similarly deposited from milk by centrifugation, when they are spin-washed, resuspended and finally added to SCP monolayer cultures.

These cultures are maintained at 37°C in a 5% CO₂ atmosphere, changing the medium and passing as necessary. They are examined for evidence of a cytopathic effect (CPE), which is characterised by the appearance of refractile stellate cells with dendritic processes accompanied by the formation of syncytia. The cultures should be maintained for several weeks before being discarded as uninfected. Once a CPE is suspected, cover-slip cultures should be prepared. These are fixed, and evidence of viral antigen is sought by immunolabelling, usually by means of indirect fluorescent antibody or by the use of indirect immunoperoxidase methods. In addition, the cells of any suspect monolayers are deposited by centrifugation, and preparations are made for the identification of any characteristic lentivirus particles by transmission electron microscopy. Reverse transcriptase in the supernatant of the cell culture is indicative of the presence of retroviruses.

• **Caprine arthritis/encephalitis virus**

The same principles that apply to the isolation of MVV also apply to the isolation of CAEV. CAEV was originally isolated by explantation of synovial membrane from an arthritic goat (6). With live CAEV-infected goats, peripheral blood, milk, and possibly joint fluid aspirate represent the most suitable specimens from which leukocyte preparations can be established. Goat synovial membrane (GSM) cells are suitable indicator cells. If a CPE is suspected, tests for detection of viral antigen should be carried out, as described above.

b) **Isolation from necropsy tissues**

• **Caprine arthritis/encephalitis virus and Maedi-visna virus**

Samples of suspect tissues, collected as fresh as possible, such as lung, synovial membranes, udder, etc., are collected aseptically into sterile HBSS or cell culture medium and minced finely in a Petri dish using scalpel blades. Individual fragments are collected by Pasteur pipette and transferred to flasks of 25 cm^2, approximately 20–30 fragments per flask, and a drop of growth medium is placed carefully on each. The flasks are then incubated at 37°C in a humid 5% CO₂ atmosphere, and left undisturbed for a few days to allow the individual explants to adhere to the plastic. Fresh medium can be added with care, after which rafts of cells will gradually grow out from the fragments. When there is sufficient cell out-growth, the cultures are trypsin dispersed to allow the development of cell monolayers. These can be examined for CPE, and any suspected virus growth is confirmed in the same way as for the co-cultivations.

Adherent macrophage cultures are easy to establish from lung-rinse material (post-mortem broncho-alveolar lavage) and can be tested for virus production by serology, electron microscopy, or reverse transcriptase assay within 1–2 weeks. Virus isolations can be done by co-cultivation of macrophages and SCP or GSM cells as described for leukocytes above.

c) **Nucleic acid recognition methods**

Most virus disease diagnostic laboratories will be equipped for the basic cell culture procedures described above. Many laboratories can now also perform nucleic acid recognition methods for the detection, quantitation, and identification of MV and CAE proviral DNA using the standard polymerase chain reaction (PCR) followed by Southern blotting, in situ hybridisation, or cloning and/or sequencing of the PCR product (3, 19, 22). Standard PCR techniques for the detection of MV and CAE proviral DNA in cells and tissues are routinely used in many laboratories and are generally used as supplemental tests for determining infection status of those animals that cannot be definitively diagnosed by serology (13, 24). Real-time or quantitative PCR techniques are beginning to be used in a few laboratories and these tests, in addition to determining infection status, also quantify the amount of MV or CAE provirus in an animal (3, 19). Furthermore, molecular techniques such as PCR, cloning and sequencing also provide knowledge on a country’s or region’s specific MV and CAE strains, which may influence which serological assay and corresponding MV or CAE antigen to use. Phylogenetic analyses of MV and CAE proviral DNAs from SRLV strains throughout the world have suggested that in some areas, MV may have naturally infected goats, and CAE may have naturally infected sheep (40, 41). In the future, molecular diagnostic tests along with phylogenetic analyses of MV and CAE provirus may be used to track transmission.
An important issue in the use of PCR is specificity. Because of the possibility of amplifying unrelated sequences from the host's genomic DNA (false positives), the amplified product should be checked by either hybridisation, restriction endonuclease digestion patterns, or sequencing. Sequencing provides the best proof of specificity in the validation of PCR-based tests and is recommended by the OIE. Sensitivity of PCR-based tests can be improved by the use of nested PCR, but specificity of the nested PCR test should be checked using hybridisation, restriction endonuclease digestion patterns, or sequencing methods.

2. Serological tests

Ovine and caprine lentivirus infections are persistent, so antibody detection is a valuable serological tool for identifying virus carriers. The close antigenic relationship between MVV and CAEV does not extend to detection of heterologous antibody in some serological assays (25).

The assays most commonly used to serologically diagnose the presence of a small ruminant lentivirus infection are agar gel immunodiffusion (AGID) and the enzyme-linked immunosorbent assay (ELISA). AGID was first developed and reported in 1973 (44) and the ELISA was first developed and reported in 1982 (20). The AGID is specific, reproducible and simple to perform, but experience is required for reading the results. The ELISA is economical, quantitative and can be automated, thus making it useful for screening large numbers of sera. The sensitivity and specificity of both the AGID assay and ELISA depend upon the virus strain used in the assay, viral antigen preparation, and the standard of comparison assay. Western blot analysis and/or radio-immunoprecipitation are the standards of comparison used to access sensitivity and specificity of new AGID tests and ELISAs.

a) Agar gel immunodiffusion (a prescribed test for international trade)

There are two MV and CAE viral antigens of major importance in routine serology, a viral surface envelope glycoprotein commonly referred to as SU or gp135, and a nucleocapsid protein referred to as CA or p28. These are both conserved in an antigen preparation consisting of medium harvested from infected cell cultures and concentrated approximately 50-fold by dialysis against polyethylene glycol. As an example the WLC-1 strain of MV virus is commonly used in the AGID assay in the United States (8)1 and a Canadian MV field strain is used for AGID tests in Canada (43).

It is important to recognise that the sensitivity of the AGID test for detecting anti-CAEV antibody is dependent on both the virus strain and the viral antigen used (1, 25). It was demonstrated that an AGID test with CAEV gp135 afforded greater sensitivity than an AGID test with CAEV p28 (1). Also, it was shown that when compared with radio-immunoprecipitation, the sensitivity of the AGID test for anti-CAEV antibody was 35% greater using CAE virus antigen than using MV virus antigen (25). The most likely explanation for this difference in sensitivity between the CAE and MV virus antigen for the detection of anti-CAEV antibody is that although the radio-immunoprecipitation assay requires only the binding of a single epitope by antibody to obtain a positive result, precipitation in an agar gel requires multiple epitope–antibody interactions. Although the MV and CAE viruses have 73–74.4% nucleotide sequence identity in the envelope gene (17), this amount of identity may not be sufficient to produce sufficient antibody to CAE and MV mutually common epitopes resulting in undetectable antibody/antigen precipitin lines using MV virus antigen. When the appropriate antigen is used, the AGID test performance is high. When compared with immunoprecipitation, the AGID for the detection of anti-CAEV antibody, if CAEV antigen was used, had 92% sensitivity and 100% specificity (25). In addition, the AGID for detection of anti-MVV antibody, if MVV antigen was used, had 99.3 and 99.4% sensitivity and specificity, respectively (16).

In adult persistently MVV-infected sheep and CAEV-infected goats, the predominant immunoprecipitating antibody response is directed against gp135 antigen (18, 26). An anti-p28 response is usually present at a lower titre than the anti-gp135 response in persistently infected adult small ruminants using immunoprecipitation. In some CAEV-infected goats there is evidence to suggest that an anti-gp135 antibody response is produced, in the absence of an anti-p28 response and vice versa, in a proportion of individuals (11, 36). Hence, for validation of a test, standard sera producing both anti-gp135 and anti-p28 precipitin lines are required.

The gel medium is 0.7–1% agarose in 0.05 M Tris buffer, pH 7.2, with 8.0% NaCl. The test is conveniently performed in plastic Petri dishes, or in 10 cm² plastic trays. The pattern and size of the wells will determine the number of sera tested per plate. Various well patterns can be adopted, but a hexagonal arrangement with a central well is usual: for example, a pattern with alternating large (5 mm in diameter) and small (3 mm in diameter) peripheral wells, 2 mm apart and 2 mm from a central antigen well that is 3 mm in diameter. The large peripheral wells are used for test sera and the small ones for standard sera. A weak positive control must be included in each test. The plates are incubated overnight at 20-25°C in a humid chamber.

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1 This virus has been distributed by Dr Howard Lehmkuhl, National Animal Disease Center, United States Department of Agriculture, P.O. Box 70, Ames, Iowa, USA.
and then examined for precipitin lines. Plates may be incubated at 2-8°C for another 24 hours to enhance the precipitin lines.

An important consideration is the need for experienced personnel to interpret the AGID. Interpretation of AGID results is dependent on the antigen used. Examples of AGIDs with different antigen preparations and a guide for interpretation of the results can be found in ref. 2.

b) Enzyme-linked immunosorbsent assay (a prescribed test for international trade)

Currently, there are over 30 different ELISAs reported for the detection of anti-MVV or anti-CAEV antibodies in the sera of sheep or goats, respectively (13). Most of these ELISAs are indirect ELISAs (I-ELISA) although there are three reported competitive ELISAs (C-ELISA) using monoclonal antibodies (14–16, 21). Half of I-ELISAs use purified whole virus preparations for antigen whereas the other half use recombinant protein and/or synthetic peptide antigens. A few of the I-ELISAs have shown high sensitivity and specificity against a standard of comparison, western blot analysis or radio-immunoprecipitation (27, 38, 39). When compared with radio-immunoprecipitation, one C-ELISA has shown high sensitivity and specificity both in sheep and goats in the USA suggesting that this one test can be used for both MVV and CAEV US surveillance (15, 16). Although ELISAs have been used for several years in some European countries (33) in control and eradication schemes of MVV in sheep (29) and CAEV in goats, AGID remains the most frequently used test.

Whole-virus antigen preparations are produced by differential centrifugation of supernatants from infected cell cultures and detergent treatment of purified virus, and are coated on microplates (12, 42, 46). Whole-virus preparations should contain both gp135 and p28. Recombinant antigens or synthetic peptides are usually produced from whole or partial segments of the gag or envelope genes and may be used in combination (27, 35, 38, 39). Thus, recombinant gag or envelope gene products fused with glutathione S-transferase fusion protein antigen that have been produced in Escherichia coli provide a consistent source of antigen for international distribution and standardisation.

The ELISA technique is also applicable to colostrum or milk, and some studies have evaluated paired serum and milk samples. Because colostrum and milk are sources of CAEV transmission, the testing of milk samples for anti-CAEV or anti-MVV antibody would not provide timely information for the prevention of transmission, especially to offspring from the immediate gestation (24).

The ELISA is performed at room temperature (~25°C) and is easy to perform in laboratories that have the necessary equipment (microplate reader) and reagents. It is convenient for large-scale screening, as it is a reliable and quantitative technique for demonstrating small ruminant lentiviruses (SRLVs) antibodies in sheep and goats. It requires relatively pure antigen. One disadvantage of several ELISAs is that many have not been validated against a standard of comparison such as western blot analysis or radio-immunoprecipitation. The OIE recommends the validation of 1000 negatives and 300 positives using a standard of comparison such as western blot analysis or radio-immunoprecipitation, and to date, only one ELISA has met these testing standards (46).

For I-ELISA, wells of the microplate are coated with antigen. Diluted serum samples are added to the wells and react to antigens bound to the solid support. Unbound material is removed by washing after a suitable incubation period. Conjugate (e.g. horseradish-peroxidaselabelled anti-ruminant Ig) reacts with specific antibodies bound to the antigen. Unreacted conjugate is removed by washing after a suitable incubation period. Enzyme substrate is added. The rate of conversion of substrate is proportional to the amount of bound antibodies. The reaction is terminated after a suitable time and the amount of colour development is measured spectrophotometrically. A disadvantage of the I-ELISA is that test sera typically need to be diluted 1/50 or greater in order to lower the number of false positives.

Specific MAbs have been used in a C-ELISA for SRLVs to capture gp135 or p28 as antigen (14–16, 21, 32). C-ELISA overcomes the problem of antigen purity, as the specificity of this test depends on the MAb epitope. For C-ELISA, sample sera containing anti-SRLV antibodies inhibit binding of enzyme–labelled MAb to SRLV antigen coated on the plastic wells. Binding of the enzyme-labelled MAb conjugate is detected by the addition of enzyme substrate and quantified by subsequent colour product development. Strong colour development indicates little or no blockage of enzyme-labelled MAb binding and therefore the absence of SRLV antibodies in sample sera. In contrast, weak colour development due to the inhibition of the enzyme-labelled MAb binding to the antigen on the solid phase indicates the presence of SRLV antibodies in sample sera. The format of the C-ELISA requires that serum antibodies must bind to or bind in close proximity to the specific MAb epitope.

- Materials and reagents

Microtitre plates with 96 flat-bottomed wells, freshly coated or previously coated with SRLV antigen; microplate reader (equipped with 405, 450, 490 and 620 nm filters); 37°C humidified incubator; 1- 8- and 12-channel pipettes with disposable plastic tips; microplate shaker (optional); fridge; freezer.
Indirect ELISA: test procedure

i) Dilute the serum samples, including control sera, to the appropriate dilution (e.g. 1/20) and distribute 0.1–0.2 ml per well (in duplicate if biphasic ELISA). Control sera are positive and negative sera provided by the manufacturer and an internal positive reference serum from the laboratory in order to compare the titres between different tests.

ii) Cover the plate with a lid and incubate at room temperature or 37°C for 30–90 minutes. Empty the contents and wash three times in washing solution at room temperature.

iii) Add the appropriate dilution of freshly prepared conjugate to the wells (0.1 ml per well). Cover each plate and incubate as in step ii. Wash again three times.

iv) Add 0.1 ml of freshly prepared or ready-to-use chromogen substrate solution to each well (e.g. ABTS in citrate phosphate buffer, pH 5.0, and 30% H₂O₂ solution [0.1 µl/ml]).

v) Shake the plate; after incubation, stop the reaction by adding stopping solution to each well (e.g. 0.1 ml sulphuric acid).

vi) Read the absorbance of each well with the microplate reader at 405 nm (ABTS) or 450–620 nm (TMB). The absorbance values will be used to calculate the results.

Interpretation of the results

For commercial kits, interpretations and validation criteria are provided with the kit.

For example: calculate the mean absorbance (Ab) of the sample serum and of the positive (Abpos) and negative (Abneg) control sera, and for each serum, calculate the percentage:

\[
\frac{Ab - Ab_{neg}}{Ab_{pos} - Ab_{neg}} \times 100
\]

Interpret the results as follows:

- Ab <30% negative serum
- Ab 30–40% doubtful serum
- Ab >40% positive serum

Competitive ELISA: test procedure

i) Add 0.05 ml of undiluted serum and positive/negative controls to antigen-coated plate.

ii) Incubate for 1 hour at room temperature.

iii) Empty the plate and wash the plate three with diluted wash solution.

iv) Add 0.05 ml of diluted antibody-peroxidase conjugate to each well. Mix well and incubate for 30 minutes at room temperature.

v) After the 30-minute incubation, empty the plate and repeat the washing procedure described in step iii.

vi) Add 0.05 ml of substrate solution (ex: TMB) to each well. Mix and cover plate with aluminium foil. Incubate for 20 minutes at room temperature. Do not empty wells.

vii) Add 0.05 ml of stop solution to each well. Mix. Do not empty wells.

viii) Immediately after adding the stop solution, the plate should be read on a plate reader (620, 630 or 650 nm).

Interpretation of results

Example: Calculation: 100 – \([\text{Sample Ab} \times 100]/(\text{Mean negative control Ab})\] = % inhibition.

For goats, if a test sample causes >33.2% inhibition, it is positive; if a test sample causes <33.2% inhibition, it is negative. For sheep, if a test sample causes >20.9% inhibition, it is positive; if a test sample causes <20.9% inhibition, it is negative.

C. REQUIREMENTS FOR VACCINES AND DIAGNOSTIC BIOLOGICALS

There are no biological products available. MAbs recognising conformational epitopes of the CAEV gp135 have been described (32).
REFERENCES


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**NB:** There are OIE Reference Laboratories for Caprine arthritis/encephalitis & Maedi-visna (see Table in Part 3 of this *Terrestrial Manual* or consult the OIE Web site for the most up-to-date list: www.oie.int).
CHAPTER 2.7.5.

CONTAGIOUS AGALACTIA

SUMMARY
Contagious agalactia is a serious disease syndrome of sheep and goats that is characterised by mastitis, arthritis, keratoconjunctivitis and, occasionally, abortion. Mycoplasma agalactiae is the main cause of the disease in sheep and goats, but M. capricolum subsp. capricolum (Mcc), M. mycoides subsp. capri (Mmc) (formerly named M. mycoides subsp. mycoides LC [LC = large colonies]) and M. putrefaciens produce a clinically similar disease, more often in goats, which may be accompanied by pneumonia. Antibodies to Mmc and Mcc have been detected in South American camelids (alpacas, llamas and vicunas), but no mycoplasmas have yet been isolated.

Identification of the agent: Definitive diagnosis requires the isolation of the causative mycoplasmas from the affected animals, which are then identified by biochemical, serological and, increasingly, molecular tests such as the polymerase chain reaction. Samples of choice include milk, conjunctival and ear swabs, and joint fluid. All four mycoplasmas grow relatively well in most mycoplasma media although M. agalactiae shows a preference for organic acids such as pyruvate as substrates.

Serological tests: Detection of antibodies in serum by complement fixation test or enzyme-linked immunosorbent assay (ELISA) provides rapid diagnosis of disease, but may not be very sensitive in chronically affected herds and flocks. Indirect ELISAs have been used routinely in control programmes for screening herds for M. agalactiae. Confirmation of infection by isolation and identification is usually necessary in areas believed to be free of contagious agalactia. Serological tests are not widely available for M. putrefaciens.

Requirements for vaccines and diagnostic biologicals: Commercial vaccines for M. agalactiae, inactivated with formalin, are widely used in southern Europe, but are not considered to be very efficacious. Under experimental conditions, M. agalactiae vaccines inactivated with saponin or phenol have been shown to be more protective than formalised preparations. Live vaccines for M. agalactiae are used in Turkey, where they are reported to be more protective than inactivated vaccines. A commercial vaccine containing M. agalactiae, Mmc and Mcc is available. Autogenous vaccines for Mmc and, occasionally, for Mcc are believed to be used in some countries. No vaccines exist for M. putrefaciens, as the disease it causes is not considered to be sufficiently serious or widespread.

A. INTRODUCTION
Contagious agalactia is a disease of sheep and goats that is characterised by mastitis, arthritis and keratoconjunctivitis, and has been known for nearly 200 years. It occurs in Europe, western Asia, the United States of America (USA) and North Africa, and is mainly caused by Mycoplasma agalactiae (3). In recent years, M. capricolum subsp. capricolum (Mcc) and M. mycoides subsp. capri (formerly M. mycoides subsp. mycoides LC [LC = large colonies]) have also been isolated in many countries from sheep and goats with mastitis and arthritis. The clinical signs of these infections are sufficiently similar to be considered indistinguishable from contagious agalactia. In addition, M. putrefaciens also causes mastitis and arthritis in goats, which is very similar to that caused by M. agalactiae, Mmc and Mcc (25). Furthermore, the consensus of the working group on

1 International Committee on Systematics of Prokaryotes Subcommittee on the Taxonomy of Mollicutes has proposed the merging of these two subspecies into the single subsp: M. mycoides subsp. capri; a decision is pending.
contagious agalactia of the EC COST\(^2\) Action 826 on ruminant mycoplasmoses, which met in Toulouse, France, in 1999, was that all four mycoplasmas should be considered as causal agents of contagious agalactia.

Clinically, the disease caused by *M. agalactiae* is recognised by elevated temperature, inappetence and alteration in the consistency of the milk in lactating ewes with decline and subsequent failure of milk production, often within 2–3 days, as a result of interstitial mastitis (3); lameness and keratoconjunctivitis affects about 5–10% of infected animals. Fever is common in acute cases and may be accompanied by nervous signs, but both signs are rare in the more frequently observed subacute and chronic infections. Pregnant animals may abort. *Mycoplasma agalactiae* may occasionally be found in lung lesions (14), but pneumonia is not a consistent finding. Bacteraemia is common, particularly for *Mmc* and *Mcc* and could account for the isolation of the organism from sites where it is only transiently present.

*Mastitis, arthritis, pleurisy, pneumonia, and keratoconjunctivitis* may all result from infection with *Mmc*, which has one of the widest geographical distribution of ruminant mycoplasmas, being found on all continents where small ruminants are kept and wherever contagious agalactia and caprine pleuropneumonia are reported (6, 18); however the lack of diagnostic facilities for mycoplasma diseases in many countries means that it is probably under reported. *Mmc* is mostly confined to goats but has occasionally been isolated from sheep with reproductive disease and cattle with arthritis or respiratory disease. Cases usually occur sporadically, but the disease may persist and spread slowly within a herd. After parturition, the opportunity for spread in milking animals increases, and kids ingesting infected colostrum and milk become infected. The resulting septicaemia, with arthritis and pneumonia, causes high mortality in kids (3, 6).

*Mcc* is widely distributed and highly pathogenic, particularly in North Africa but the frequency of occurrence is low (3). Goats are more commonly affected than sheep, and clinical signs of fever, septicaemia, mastitis, and severe arthritis may be followed rapidly by death (3, 4). Pneumonia may be seen at necropsy. The severe joint lesions seen in experimental infections with this organism are accompanied by intense periarticular subcutaneous oedema affecting tissues some distance from the joint (4).

*Mycoplasma putrefaciens* is common in milking goat herds in western France where it can be isolated from animals with and without clinical signs (15). It has also been associated with a large outbreak of mastitis and agalactia leading to severe arthritis in goats accompanied by abortion and death without pyrexia in California, USA (3). *Mycoplasma putrefaciens* was the major finding in an outbreak of polyarthritis in kids in Spain (25).

Antibodies to *Mmc* and *Mcc*, but not *M. agalactiae*, have been detected in South American camelids, including llamas, alpacas and vicunas, but as yet no mycoplasmas have been isolated (17). These camelids are affected by a range of mycoplasma-like diseases, including polyarthritis and pneumonia, so it is likely that mycoplasmas including *Mmc* and *Mcc* may be found in the future.

### B. DIAGNOSTIC TECHNIQUES

1. **Identification of the agents**

   a) **Selection of samples**

   Preferred samples from living animals include: nasal swabs and secretions; milk from mastitic females or from apparently healthy females where there is a high rate of mortality/morbidity in kids; joint fluid from arthritic cases; conjunctival swabs from cases of ocular disease; and blood for antibody detection from affected and non-affected animals (19). The ear canal has also been shown to be a source of pathogenic mycoplasmas, although in practice the presence of nonpathogenic mycoplasmas at this site may make confirmation difficult (19). Mycoplasmas may be isolated from the blood during the acute stage of the disease when there is mycoplasmaemia. From dead animals, samples should include: udder and associated lymph nodes, joint fluid, lung tissue (at the interface between diseased and healthy tissue) and pleural/pericardial fluid. Samples should be dispatched quickly to a diagnostic laboratory in a moist and cool condition. All four causative mycoplasmas are relatively easy to isolate from internal organs, joints and milk and grow well in most mycoplasma media, producing medium to large colonies in 3–4 days.

   b) **Mycoplasma medium**

   The usual techniques used in the isolation of mycoplasmas apply to all four causative organisms (19). Many media have been reported to grow the causative mycoplasmas. Improved growth rates of *M. agalactiae* have been seen in media containing organic acids such as pyruvate and isopropanol (12). The formulation of PRM medium (12) is as follows:

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2 European Cooperation in the field of Scientific and Technical Research.
Heat inactivated porcine serum 100 ml/litre, special peptone 20 g/litre, yeast extract 5 g/litre, glycerol 5 g/litre, sodium chloride 5 g/litre, HEPES 9 g/litre, fresh yeast extract 100 ml/litre, sodium pyruvate 5 g/litre, 12.5 ml of 0.2% phenol red and ampicillin (200,000 International Units/ml). Make up to 1 litre in distilled water and sterilise by filtration. Adjust the pH of the broth medium to 7.6. Prepare solid medium by adding 10 g of LabM agar No. 1 (Bury UK, or agar of equivalent quality) and dispense into sterile Petri dishes.

Thallium acetate (250 mg/litre), which is toxic and inhibitory to some mycoplasmas but not those causing contagious agalactia, may be a necessary component of the transport medium to reduce bacterial contamination from clinical samples, but should be omitted once the mycoplasmas begin to grow in vitro. A satisfactory alternative to thallium acetate may be colistine sulphate (37.5 mg/litre).

- **Test procedure**
  i) Make tenfold dilutions (10⁻¹–10⁶) of the liquid sample (milk, synovial fluid, conjunctival and ear swabs) or tissue homogenate in appropriate broth medium.
  ii) Spread a few drops of each sample on the agar medium and dispense a 10% (v/v) inoculum into broth medium.
  iii) Streak swabs directly on to agar medium.
  iv) Incubate inoculated broths (optimally with gentle shaking) and agar media at 37°C in humidified atmosphere with 5% carbon dioxide.
  v) Examine broths daily for signs of growth (indicated by a fine cloudiness or opalescence) or changes in pH indicated by a colour change and examine agar media under × 35 magnification for typical ‘fried egg’ colonies.
  vi) If no mycoplasma growth is seen after 7 days, subculture a 10% (v/v) inoculum of broth into fresh broth and spread about 50 µl of this on to agar media.
  vii) Repeat as for step v. If no mycoplasmas are seen after 21 days’ incubation, consider the results to be negative.
  viii) If bacterial contamination results (seen as excessive turbidity), filter sterilise by passing 1 ml of contaminated broth through a 0.45 µm filter into fresh broth medium.

Clinical samples frequently contain more than one mycoplasma species so clone purification of colonies is often considered necessary before performing biochemical and serological identification, in particular the growth and film inhibition tests (GIT and FIT, respectively). However, cloning is a lengthy procedure taking at least 2 weeks. The immunofluorescence test (5), dot immunobinding tests (21) and, more recently, polymerase chain reaction (PCR) tests (see Section B.1.e) do not require cloning as these tests can detect the pathogenic mycoplasmas in mixed cultures, saving a great deal of time.

c) **Biochemical tests**

The first test that should be performed on the cloned isolates is sensitivity to digitonin, which separates mycoplasmas from acholeplasmas; the latter are ubiquitous contaminants that can overgrow the mycoplasmas of interest. Growth in liquid medium containing glucose (1%), arginine (0.2%), and phenolphthalein diphosphate (0.01%), on solid medium containing horse serum or egg yolk for the demonstration of film and spots, and on casein agar or coagulated serum agar to test for proteolysis, are among the most useful tests for differentiating the four mycoplasmas (22). These biochemical characteristics, however have been increasingly found to be variable for the individual mycoplasmas and have little diagnostic value. The most impressive biochemical characteristic that differentiates *M. putrefaciens* from all other mycoplasmas is the odour of putrefaction it produces in broth culture. Other features that may be helpful include: film and spot production seen on the surface of the broth and solid media caused by *M. agalactiae* and to a lesser extent by *M. putrefaciens*; and the proteolytic activity of Mcc and MmmLC on casein and coagulated serum.

A rapid and highly convenient biochemical test that exploits the C8-esterase activity of *M. agalactiae* has been reported (11). The mycoplasma forms red colonies on agar media within 1 hour of adding the chromogenic substrate, SLPA-octanoate (a newly synthesised ester formed from a C8 fatty acid and a phenolic chromophore). This activity is shared with *M. bovis*, although this mycoplasma is rarely found in small ruminants. Isolates need not be cloned as *M. agalactiae* can be detected easily in mixed cultures. If necessary PCRs can be used to distinguish rapidly *M. agalactiae* from *M. bovis* (see Section B.1.e).

d) **Serological identification**

Identification of isolates using specific antisera is usually carried out with the GIT, FIT (23) or the indirect fluorescent antibody (IFA) test (5). A recently developed dot immunobinding test, which is carried out in microtitre plates, offers many improvements over the other serological tests such as rapidity and higher throughputs (21) but requires subjective judgements of staining intensity. For *M. agalactiae*, film inhibition
may often be more reliable as growth inhibition is not seen with all isolates; it can also be used for serodiagnosis. Film production by the mycoplasma may be enhanced by the incorporation of 10% egg yolk suspension into the solid medium.

- **Test procedure**
  
i) Inoculate at least two dilutions of 48-hour cloned broth cultures ($10^{-1}$ and $10^{-2}$) on to predried agar media by allowing 50 µl of the cultures to run down the tilted plates using the ‘running drop’ technique (23). Remove any excess liquid with a pipette.

  ii) Allow the plates to dry. It is possible to apply two or three well separated running drops to each 90 mm diameter plate.

  iii) Apply predried filter paper discs containing 30 µl of specific antiserum to the culture; ensure good separation of discs (at least 30 mm).

  iv) Incubate the plates as for mycoplasma culture and examine daily by eye against a light background.

- **Interpretation of the results**

A zone of inhibition over 2 mm, measured from the paper disc to the edge of mycoplasma growth is considered to be significant. Partial inhibition can occur with weak antiserum or where there are mixed cultures. Stronger reactions can be obtained if about 60 µl of antiserum is added to 6 mm diameter wells made in the agar with a cork borer or similar device (23).

In the IFA test, specific antisera are applied to colonies on solid medium. Homologous antiserum remains attached after washing and is demonstrated by adding fluorescein-conjugated antiglobulin, washing, and viewing the colonies with an epifluorescence microscope (5). Controls should include known positive and known negative control organisms, and a negative control serum. However like the immunobinding tests subjective judgements are required to assess staining intensity.

Antisera for these serological tests have traditionally been prepared against the type strains of the various *Mycoplasma* species, and most field isolates have been readily identified using these antisera. As more strains have been examined, however, some have been found to react poorly with these antisera, while reacting well with antisera to other representative strains of the species. Intraspecies variation in antigenic composition has not been reported for *M. putrefaciens*, but occurs to some degree with *M. agalactiae* and with *Mcc* strains. Thus, diagnostic laboratories may need to have several antisera to enable all strains of the species to be identified.

e) **Nucleic acid recognition methods**

PCR assays are routinely used in many laboratories and are extremely sensitive. They can provide a rapid early warning system when carried out on clinical samples, enabling a full investigation to take place when results are positive. However negative results should not be considered definitive. Several PCRs specific for *M. agalactiae* have been developed and show similar levels of sensitivity, although they are based on different gene sequences (1, 7, 26, 28). They can be used directly on nasal, conjunctival, synovial and tissue samples; they have been used on milk samples where they have been reported to be more sensitive than culture (28), although occasionally undefined inhibitors may interfere with the test. PCRs can also be used, more reliably, on mycoplasmas growing in culture; a 24 hour enrichment of the mycoplasma in the appropriate medium greatly facilitates PCR detection even in the presence of bacterial contamination (18). A newly described PCR based method called denaturing gradient gel electrophoresis (DGGE) that uses mycoplasma-specific primers is capable of identifying the majority of small ruminant mycoplasmas including all the causative agents of contagious agalactia by their migration pattern (16). A positive PCR result, particularly in an area previously free of contagious agalactia, should be confirmed by isolation and identification of the mycoplasma using standard procedures.

Individual PCRs have been reported for *Mmc* and *Mcc* (2) and *M. putrefaciens* (20) respectively. In addition a multiplex test has been described which can detect simultaneously *M. agalactiae*, *Mcc* and *Mmc* (9).

- **Test procedure**

The following primers based on the uvrC gene have been shown to be specific for *M. agalactiae* (26). PCRs may need to be optimised in each laboratory. Positive and negative control DNA should be run in each assay.

- MAGAUVRc1-L: CTC-AAA-AAT-ACA-TCA-ACA-AGC
- MAGAUVRc1-R: CTT-CAA-CTG-ATG-CAT-CAT-AA
i) Extract DNA from *Mycoplasma* isolates or clinical material using the appropriate method (4).

ii) Carry out PCR methods in 50 µl reaction mixtures containing: 1 µl of sample DNA, 20 pmol of each primer (see above), 1 mM each dNTP, 10 mM Tris/HCl, pH 8.3, 1.5 mM MgCl₂, 50 mM KCl and 1.25 mM U Taq DNA polymerase.

iii) Subject the mixture to 35 amplification cycles in a thermal cycler with the following parameters: 30 seconds at 94°C, 30 seconds at 50°C annealing temperature and 1 minute at 72°C.

iv) Analyse the PCR products by electrophoresis on a 0.7% agarose at 110 V for 2 hours and visualise by staining with ethidium bromide. A 1.7 kb fragment indicates the presence of *M. agalactiae*.

2. Serological tests

a) Complement fixation

A standard complement fixation test (CFT) for *M. agalactiae* has also been applied to other mycoplasmas involved in the contagious agalactia syndrome (3). Antigens are prepared from washed organisms, standardised by opacity, and lysed, either ultrasonically or by using sodium lauryl sulphate followed by dialysis. Sera are inactivated at 60°C for 1 hour, and the test is carried out in microtitre plates with overnight fixation in the cold or at 37°C for 3 hours. The haemolytic system is added, and the test is read after complete lysis is shown by the antigen control. A positive result is complete fixation at a serum dilution of 1/40 or greater for the following mycoplasmas: *M. agalactiae*, *Mcc*, and *Mmc*. The CFT is regarded as a herd test and at least ten sera are tested from each herd, preferably from acute and convalescent cases.

Some sera from healthy flocks react in the CFT using *M. agalactiae* up to a serum dilution of 1/20, but rarely react with the other two antigens. However, in flocks infected with *M. agalactiae*, sera giving a homologous reaction at 1/80 may cross-react at up to 1/40, the positive threshold, with the other two antigens. It is often difficult to perform the CFT if the quality of the test sera is poor; where possible, the enzyme-linked immunosorbent assay (ELISA) is preferred.

b) Enzyme-linked immunosorbent assay

ELISAs using sonicated or Tween-20-treated antigens have been reported to be more sensitive than the CFT for the detection of antibody to *M. agalactiae* (3). Problems of nonspecificity have been overcome by the use of monoclonal or protein G conjugates in the ELISA (13). The use of these conjugates enables the testing of sera from a wide range of mammalian species, including camels. A number of commercial ELISA kits are now available and these are being used for large-scale surveys in France and the United Kingdom (3, 17). In a ring trial of serological tests for *M. agalactiae* organised in 1998 under the auspices of the EC COST Action 826 on ruminant mycoplasmoses, commercial ELISAs performed better than ‘home-made’ kits.

ELISAs are not widely available for the other three causative mycoplasmas although ‘home-made’ assays are carried out by some laboratories.

c) Immunoblotting test

Immunoblotting tests have been reported as the most sensitive and specific test for *M. mycoides* subsp. mycoides SC, the cause of contagious bovine pleuropneumonia (see Chapter 2.4.9). Immunoblotting tests have also been described for *M. agalactiae* (17, 29). Strong bands at approximately 80 and 55 kDa were seen with sera with antibodies to *M. agalactiae*, while sera from healthy flocks show no bands or very faint bands of different sizes. Diluting the sera to 1/50 improves the discrimination between positive and negative sera (17).

C. REQUIREMENTS FOR VACCINES AND DIAGNOSTIC BIOLOGICALS

Vaccines for the prevention of contagious agalactia due to *M. agalactiae* are used widely in the Mediterranean countries of Europe and in western Asia. No single vaccine has been universally adopted, and no standard methods of preparation and evaluation have been applied.

1. Vaccines for *Mycoplasma agalactiae* infection

a) Inactivated vaccines for *Mycoplasma agalactiae* infection

In Europe, where live vaccines for *M. agalactiae* are not acceptable, attention has focused on the use of killed organisms, mostly using formalin and an adjuvant such as aluminium hydroxide in an oil emulsion. The titres of the preparations, before inactivation, are very high (10⁸–10¹⁰ colony-forming units per ml) and
are derived from laboratory strains. Some products are available commercially including a trivalent preparation containing \textit{M. agalactiae}, \textit{Mcc} and \textit{Mmc} but there are few data on their efficacy. A formalin-inactivated oil emulsion vaccine was shown to be immunogenic and protective in a small trial in lactating sheep and also prevented transmission of \textit{M. agalactiae} (8).

It is possible that in some instances the apparent lack of protection given by vaccines could be the result of animals being infected with one of the other four mycoplasmas involved in the contagious agalactia syndrome (10). A multivalent formalin inactivated vaccine incorporating all four causative mycoplasmas and adjuvanted with saponin and aluminium hydroxide appears beneficial in preliminary trials (24).

More recently vaccines inactivated with phenol or with saponin have given superior protection against experimental infections compared with formalin, sodium hypochlorite or heat-inactivated vaccines (30).

b) Live attenuated vaccines for \textit{Mycoplasma agalactiae} infection

Live attenuated vaccines against \textit{M. agalactiae} have been used in Turkey for many years and have been reported to provide better protection in ewes and their lambs than inactivated vaccines (18). However they can produce a transient infection with shedding of mycoplasma. Live vaccines should not be used in lactating animals and should be part of a regional plan in which all flocks from which animals are likely to come into contact be vaccinated at the same time.

2. Vaccines for \textit{Mycoplasma mycoides} subsp. \textit{capri} infection

There is little recent published information on the availability of vaccines for \textit{Mmc} although it is believed that inactivated vaccines are widely used in many Mediterranean countries and in Asia suggesting that their production and use is localised (3). Saponised vaccines have been reported in India which provoke a strong antibody response and show some protection (27).

3. \textit{Mycoplasma capricolum} subsp. \textit{capricolum} and \textit{M. putrefaciens}

Although infections with \textit{Mcc} and \textit{M. putrefaciens} can be severe, their prevalence is relatively low and, as might be expected, little or no work appears to have been carried out on preventive vaccination for these infections.

REFERENCES


Chapter 2.7.5. — Contagious agalactia


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**NB:** There is an OIE Reference Laboratory for contagious agalactia (see Table in Part 3 of this *Terrestrial Manual* or consult the OIE Web site for the most up-to-date list: www.oie.int).
CHAPTER 2.7.6.

CONTAGIOUS CAPRINE PLEUROPNEUMONIA

SUMMARY

Contagious caprine pleuropneumonia (CCPP) is a severe disease of goats caused by Mycoplasma capricolum subspecies capripneumoniae (Mccp). This organism is closely related to three other mycoplasmas: M. mycoides subs. mycoides large colonies (LC), M. mycoides subs. capri, and M. capricolum subs. capricolum. Unlike true CCPP, which is confined to the thoracic cavity, the disease caused by the latter three mycoplasmas is accompanied by prominent lesions in other organs and/or parts of the body besides the thoracic cavity.

Typical cases of CCPP are characterised by extreme fever (41–43°C), high morbidity and mortality rates in susceptible herds affecting all ages and both sexes, and abortions in pregnant goats. It appears to be transmitted by an infective aerosol. After approximately 2–3 days of high fever, respiratory signs become apparent: respiration is accelerated and painful, and in some cases is accompanied by a grunt. Coughing is frequent, violent and productive. In the terminal stages, animals are unable to move – they stand with their front legs wide apart, the neck is stiff and extended, and sometimes saliva continuously drips from the mouth. Post-mortem examination reveals fibrinous pleuropneumonia with massive lung hepatisation and pleurisy, accompanied by accumulation of straw-coloured pleural fluid.

The disease has been shown recently to affect wild ruminants such as the wild goats (Capra aegagru), Nubian ibex (Capra ibex Nubian) and Laristan mouflon (Ovis orientalis laristanica) and Gerenuk (Litocranius walleri). Clinical disease and seropositivity have been reported in sheep in contact with affected goats, but the role of sheep as reservoirs of infection is unclear.

Identification of the agent: Definitive diagnosis requires culture of the causative organism from lung tissue samples and/or pleural fluid taken at post-mortem. After cloning and purification, isolates can be identified by several biochemical, immunological and molecular tests. Isolating the causative agent is a difficult task. Recently polymerase chain reaction based tests have been described and shown to be specific, sensitive and can be applied directly to clinical material, such as lung and pleural fluid.

Serological tests: Serological tests have been applied for the diagnosis of CCPP in outbreaks in Eritrea and Turkey. Such tests are best used on a herd basis rather than for diagnosis in individual animals. The complement fixation test remains the most widely used serological test for CCPP, although the latex agglutination test is being increasingly used in the diagnostic laboratories as well as a pen side test; it can used to test whole blood as well as serum. Indirect hemagglutination is also used. A specific competitive enzyme-linked immunosorbent assay has been developed, but is not widely available. As with the other serological tests, it does not detect all reactors, but its specificity and suitability for large-scale testing make it an appropriate test for epidemiological investigations.

Requirements for vaccines and diagnostic biologicals: Vaccine against CCPP caused by Mccp is available commercially.

A. INTRODUCTION

Contagious caprine pleuropneumonia (CCPP) is a severe disease of goats occurring in many countries in Africa and Asia where the total goat population is more than 500 million (1). The first description of the disease was in 1873 in Algeria. Shortly after, in 1881, the disease was introduced in the ‘Cape Colony’ of South Africa by a shipment of Angora goats (15, 16). The disease was eradicated using a policy of slaughter of the infected goats.
coupled with a traditional vaccination procedure for the in-contact goats. Classical, acute CCPP is caused by *Mycoplasma capricolum* subsp. *capripneumoniae* (*Mccp*) (23), originally known as the F38 biotype. This organism was first isolated and shown to cause CCPP in Kenya (24–27); it has subsequently been isolated in the Sudan, Tunisia, Oman, Turkey, Chad, Uganda, Ethiopia, Niger, Tanzania, Eritrea and the United Arab Emirates. CCPP was first reported in mainland Europe in 2004, when outbreaks were confirmed in Thrace, Turkey, with losses of up to 25% of kids and adults in some herds (35). However, the exact distribution of the disease is not known and it may be much more widespread than the zone represented by the countries where *Mccp* has been isolated as CCPP is often confused with other respiratory infections and also because the isolation of the causative organism is difficult.

In CCPP outbreaks in mixed goat and sheep herds, sheep may also be affected, as verified by isolation of *Mccp* (5) or detection of antibodies from clinically affected sheep (19). *Mccp* has also been isolated from healthy sheep (21) and the role of sheep as a reservoir for the disease has to be considered.

Recently CCPP was confirmed in wild ruminants kept in a wildlife preservation reserve in Qatar. The disease affected wild goats (*Capra aegagrus*), Nubian Ibex (*Capra ibex nubiana*), Laristan mouflon (*Ovis orientalis laristanica*) and Gerenuk (*Litocranius walleri*) with significant morbidity and mortality in these species (2). Disease indistinguishable from naturally occurring CCPP has been experimentally reproduced with *Mccp* by several groups of workers.

**B. DIAGNOSTIC TECHNIQUES**

The diagnosis of outbreaks of respiratory disease in goats, and of CCPP in particular, is complicated, especially where it is endemic. It must be differentiated from other similar clinico-pathological syndromes such as: peste des petits ruminants, to which sheep are also susceptible; pasteurellosis, which can be differentiated on the basis of distribution of gross lung lesions; and what has been called ‘mastitis, arthritis, keratitis, pneumonia and septicaemia syndrome or more often as contagious agalactia syndrome (47). As the longer name implies, the pneumonia is accompanied by prominent lesions in other organs. The disease caused by *Mccp* is readily contagious and fatal to susceptible goats of all ages and both sexes, rarely affects sheep and does not affect cattle.

1. Identification of the agent

a) **Microscopy of lung exudates, impression smears or sections**

*Mccp* is characterised histopathologically by an interstitial pneumonia with interstitial, intralobular oedema of the lung (18, 34). It shows a branching filamentous morphology *in vivo* that can be observed by dark-field microscopy in exudates or tissue suspensions from lesions or pleural fluid. Alternatively, smears made from cut lung lesions can be stained by the method of May–Grünwald–Giesma and examined by light microscopy. The other caprine mycoplasmas show a short filamentous or coccobacillary morphology. Neither of these techniques provides a definitive diagnosis.

b) **Nucleic acid recognition methods**

Two polymerase chain reaction (PCR) assays for the specific identification of *Mccp* have now been published. The first one (3) is based on the amplification of the 16S rRNA gene of the mycoides cluster. The PCR product is then analysed by restriction enzyme cleavage for the identification of the *Mccp* amplicon. The second one (50) is based on a specific amplification. These PCR techniques can be used directly on clinical materials such as lung tissue and pleural fluid (6). Due to the difficulty in isolating *Mccp*, PCR is the technique of choice for the diagnosis of CCPP. However, isolation of *Mccp* remains the confirmatory test. All mycoplasmas of the mycoides cluster can be assigned a precise phylogenetic position by using a multilocus sequence typing approach which may be used for identification purposes (30).

c) **Gel precipitin tests to detect antigen in tissue specimens**

*Mccp* releases an antigenic polysaccharide (41) to which a specific monoclonal antibody (MAb) (WM-25) has been produced (42, 43). This MAb immunoprecipitates in agar gel with the polysaccharide produced by *Mccp*, and is used to identify the causative agent in cases of CCPP, particularly when specimens are no longer suitable for culture because of deterioration during transit.

d) **Isolation of mycoplasmas**

i) **Selection of samples**

The necropsy samples of choice are lung lesions, particularly from the interface between consolidated and unconsolidated areas, pleural fluid, and mediastinal lymph nodes. If microbiological examination cannot be performed immediately, samples or whole lungs can be stored at –20°C for considerable
periods (months) with little apparent loss of mycoplasma viability. During transport, samples should always be kept as cool as possible, as mycoplasma viability diminishes rapidly with increasing temperature. Lung samples can be dispatched to other laboratories in frozen form.

ii) **Treatment of samples**

Swabs are suspended in 2–3 ml of culture medium. Tissue samples are best minced using scissors, and then shaken vigorously, or pulverised in medium\(^1\) using 1 g of tissue to 9 ml of medium. Tissues should not be ground. The suspension is usually prepared with a mycoplasma medium, but if parallel bacteriological examination is to be carried out, a high quality bacteriological medium, such as nutrient broth, may be used to provide a suspension suitable for both forms of examination. Pleural fluid, or a tissue suspension or swab, is serially diluted through at least three tenfold steps (to a nominal \(10^{-4}\)) in the selected mycoplasma medium. Dilutions should also be plated on to solid medium.

iii) **Mycoplasma media**

The medium used by MacOwan & Minette to culture \(\text{Mccp}\) organisms (25), is termed ‘viande foie goat’ (VFG), and includes goat-meat liver broth and goat serum. Alternative suitable media are WJ (17), modified Hayflick’s, and modified Newing’s tryptose broth (19) Media enriched with 0.2% (or up to 0.8%) sodium pyruvate perform considerably better, both for primary isolation and antigen production of \(\text{Mccp}\) (31, 32). Examples of suitable media are as follows (6, 47, 49):

- **CCPP medium**
  
  A. *Autoclaved portion* (121°C for 15 minutes): Bacto PPLO (pleuropneumonia-like organisms) broth without crystal violet (Difco) (21 g); deionised water (700 ml).

  B. *Membrane-filtered portion*: Horse (alternatively pig or donkey) serum inactivated at 56°C for 30 minutes (200 ml); fresh yeast extract (100 ml); glucose (sterile solution 0.5 g/ml) (2 ml); and sodium pyruvate (sterile solution 0.5 g/ml) (8 ml).

  Part B is added to A aseptically. Ampicillin (0.1 g/litre) and thallium acetate (250 mg/litre) can be added to prevent contamination in primary isolations. The final pH of the medium should be 7.4–7.6.

- **Modified CCPP medium**
  
  A. *Autoclaved portion* (121°C for 15 minutes): Bacto PPLO broth without crystal violet (Difco) (17.5 g); glass distilled water (650 ml).

  B. *Membrane-filtered portion*: Horse (alternatively pig or donkey) serum inactivated at 56°C for 30 minutes (250 ml); fresh yeast extract (100 ml); 50% glucose (4 ml); 25% sodium pyruvate (8 ml); 5% thallium acetate (4 ml); ampicillin (250 mg); and 0.5% phenol red (4 ml). The pH is adjusted to 7.8 with sodium hydroxide or hydrochloric acid. Part B is added to A aseptically.

  Modified Newing’s tryptose broth (19) and agar plates (Gourlay’s medium) (11) are routinely used for isolation and maintenance of \(\text{Mccp}\) in Kenya.

iv) **Medium production, storage and quality control**

Certain medium components, particularly serum, yeast extract and deionised water, should be regularly monitored for growth-promoting capacity before incorporation into mycoplasma media. Low-passage field isolates should be used for this screening purpose.

Broth media may be stored for at least 6 months at –25°C before use, but penicillin or its analogues should not be added until final dispensing. Broth media are dispensed into bijoux (1.8 ml or 2.7 ml) or screw-capped tubes (4.5 ml), and stored for up to 3 weeks at 4°C. Solid media are best made with agarose (0.9% [w/v]), Noble agar (1.5% [w/v]), or purified agar (0.6% [w/v]). Plates, which are poured to a depth of 6–8 mm, should be as fresh as possible when used, and should be stored for no more than 2 weeks at 4°C before use. All culture media should be subjected to quality control and must support the growth of *Mycoplasma* spp. from small inocula. The reference strain should be cultured in parallel with the suspicious samples to ensure that the tests are working correctly.

v) **Cultivation**

Cultures are incubated at 37°C. Plates are best incubated in a humid atmosphere of 5% CO\(_2\), 95% air or N\(_2\), or in a candle jar with a moisture source.

Broth cultures are examined daily for evidence of growth – colour change and the appearance of floccular material. Gross turbidity indicates bacterial contamination; cultures showing this should be

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\(^1\) For example, with the Stomacher 80, A.J. Seward, London, United Kingdom (UK).
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passed through a 0.45-µm membrane filter before subculture. Broth cultures are subcultured by inoculation of fresh broth medium with one-tenth of their volume, or by streaking agar medium with a loop.

Plate cultures are examined every 1–3 days using a stereo microscope (×5–50 magnification) and transmitted and incident light sources. If negative, the plates are discarded after 14 days. Subculture is carried out by the transfer of excised agar blocks bearing isolated colonies to either agar (on which the blocks are pushed, face down) or broth media. Alternatively, an agar plug bearing one colony is drawn into a Pasteur pipette and discharged into fresh broth medium.

Cloning and purification of isolates is performed by repeated transfer of single colonies representing each morphological type seen. Colony morphology varies with the medium used, the mycoplasma species, its passage level and the age of the culture.

In early passage, many mycoplasma species, including *M. capricolum* subsp. *capricolum* (*Mcc*), produce colonies of bizarre morphology, often small, centreless, and of irregular shape. This effect is often associated with the use of marginally suitable medium. With passage, such isolates demonstrate conventional ‘fried egg’ colony morphology, except *M. ovipneumoniae*, which retains centreless colonies. Colonies of *M. mycoides* subsp. *mycoides* Large Colony (*Mmm*LC) and *Mcc* may be up to 3 mm in diameter.

Filtration of broth cultures through 0.45 µm filters before subculture aids purification by excluding cell aggregates.

Cultures suspected of being L-forms of bacteria should be examined for reversion to bacterial form by three to five passages on solid mycoplasma medium from which antibiotics and thallium acetate have been omitted.

Broth media used for primary isolation and which have shown no indication of growth by 7 days, should be subcultured blind.

Cultures of each sample, including one blind subculture, should be examined for a minimum of 3 weeks before being discarded. Titrations in broths, if performed in full (to 10⁻¹⁰), are also read at 3–4 weeks and are expressed as colour-changing units per transfer volume. Growth on plates is expressed as colony-forming units (CFU) per ml.

e) Identification of mycoplasmas

i) **Polymerase chain reaction**

Once the organism has been cultured, verification of *Mccp* can be achieved in 1 day by PCR. There are now various PCR tests that can be applied for an identification of *Mccp* strains. The first test (3) is based on amplification of a segment of the 16S rRNA gene. The amplified fragment is common to the mycoides cluster. However, when the amplicon is digested with endonuclease *Pst*I, a unique cleavage pattern of three fragments for *Mccp* is observed when the enzyme digests are analysed in agarose gel electrophoresis and stained with ethidium bromide (6). The second test is based on a specific amplification and can allow confirmation of CCPP within a few hours (50).

Recently, PCR and sequencing has been used to establish the molecular epidemiology of CCPP. These tests can be performed on dried samples, such as pleural fluid on filter papers. The sequencing allows a precise identification of the species (the cleavage site for the 16S rRNA and a specific detection for the ‘locus H2’) (22, 37).

Identification of *Mccp* strains by PCR (and sequencing) has now superseded all other techniques because of its rapidity and reliability. However PCR reactions must be performed with great care to prevent contaminations.

ii) **Biochemical tests**

Wild strains should be passaged, and preferably cloned, three times before identification is attempted.

Biochemical tests cannot identify an isolate unequivocally, which at present can only be done by serological or genetic means. Intraspecies variation in some biochemical reactions is often considerable (13), but some tests perform a useful function both as a preliminary screening system and in providing supportive data for serological findings.

The tests most commonly used are glucose breakdown, arginine hydrolysis, ‘film and spots’ formation, reduction of tetrazolium chloride (aerobically and anaerobically), phosphatase activity, serum digestion and digitonin sensitivity. The first three of these tests are performed routinely in isolation and cultivation procedures. Glucose breakdown is indicated by acid (yellow) changes, and arginine hydrolysis by alkaline (red) changes in broth media, using phenol red as indicator. Arginine use cannot be assessed on conventional medium for isolation and culture as the media for testing the arginine
deiminase pathway should contain high concentrations of arginine and no glucose. Film and spots describe an apparent wrinkling of the agar surface due to the deposition on it of an iridescent film of lipid, together with the development of black spots within the medium in the vicinity of ageing colonies. This phenomenon, produced by three mycoplasmas of small ruminants, is demonstrated on agar media containing 20% or more serum, preferably of horse or pig origin. Supplementation of the medium with 10% egg yolk emulsion improves the sensitivity of the test.

The remaining biochemical tests require specific media or reagents. The test for tetrazolium reduction provides corroborative evidence of the mycoplasmal nature of \textit{M. agalactiae} isolates, as this organism is neither glycolytic nor arginine-hydrolysing. Serum digestion (9) distinguishes members of ruminant mycoplasmas, and phosphatase production (7) separates \textit{Mcc} from other members of this cluster. Digitonin sensitivity distinguishes members of the order Mycoplasmatales from those of the order Acholplasmatales (10). A diagnostic medium is available that enables the specific detection of \textit{Mccp} growing on agar medium: colonies show a red coloration (35).

iii) **Serological identification**

Mycoplasmal antigens used in hyperimmune serum production are often contaminated with medium constituents. The antibodies stimulated by these contaminants can cause false-positive reactions in serological identification tests. This problem is avoided by absorption of the antiserum with the medium used to produce the antigen (10 mg lyophilised medium per ml of antiserum), or by growing the mycoplasmas to be used as antigens in medium containing homologous animal components, e.g. growth in VFG medium to immunise goats.

Because of the close serological relationships between members of the ‘\textit{M. mycoides} cluster’, isolates from cases of CCPP should, preferably, be identified by at least two of the three tests described below.

• **Growth inhibition test**

The growth inhibition test (GIT) is the simplest and most specific, but the least sensitive, of the tests available. It depends on the direct inhibition of growth on solid medium by specific hyperimmune serum, and detects primarily surface antigens (8).

\textit{Mccp} appears to be highly homogeneous serologically and wide zones of inhibition free of ‘breakthrough’ colonies are observed with antiserum to the type strain, regardless of the source of the test strain (17). \textit{Mccp} cross-reacts with Leach’s bovine group 7 (PG50), \textit{M. equigenitalium} and \textit{M. primatum} in the GIT when polyclonal antisera are used, but an MAb specific for \textit{Mccp} in the GIT has been produced (42). The MAb reagent, WM25, has been reported to be specific for (\textit{Mccp}) isolates by the disc growth inhibition method, which will exclude \textit{M. agalactiae}, \textit{Mcc} and the other members of the ‘\textit{M. mycoides} cluster’ associated with goats, but not bovine group 7 (not usually found in goats): the latter can be excluded, however, by colony indirect fluorescence tests (4). A small proportion of \textit{Mccp} isolates also cross-react in the GIT with antiserum to \textit{Mcc}. Group seven of Leach strains can sometimes be found in goats although it is rare. Results should be interpreted carefully as some bovine strains have been misidentified by the GIT using the ‘specific’ antiserum.

• **Test procedure**

i) Broth culture in mid-to-late logarithmic phase is used at three tenfold dilutions, the selection of which is related to the vigour of growth of the isolate on agar.

ii) Agar plates are dried for 30 minutes at 37°C.

iii) Sterile paper disks of 6–7 mm in diameter are impregnated with a drop (10–20 µl) of undiluted antiserum. Disks may be used wet, in which form they can be stored at –20°C, or they can be lyophilised (8), which allows storage at 4°C.

iv) Using a separate plate for each dilution of culture, 1 ml or 2.5 ml volumes are pipetted on to 5 cm or 10 cm diameter plates, respectively. The inoculum is dispersed evenly over the plate, then the excess is removed.

v) The plates are dried at 20–30°C for 15–20 minutes, preferably under a protective hood, until no visible liquid is present on the surface. Sufficient residual moisture should remain to enable freeze-dried disks to adhere to the agar surface.

vi) Several disks, each impregnated with a different antiserum (selected on the basis of sample source and the biochemical reactions and colony morphology of the isolate), are carefully placed on the agar plates; isolates from CCPP cases should be screened with antisera against \textit{Mccp}, \textit{MmmLC}, \textit{Mcc}, \textit{M. mycoides} subsp. \textit{capri} (\textit{Mcc}) and \textit{M. ovipneumoniae}. A disk containing 1.5% digitonin should also be included on the plates.

vii) The plates are incubated at 37°C for 2–6 days. Initial overnight incubation at 27°C can increase the sensitivity of the test. Inhibition by digitonin is generally readily apparent; however, inhibition by
antiserum may be more difficult to interpret, with suppression rather than total inhibition of growth, depending on the species of mycoplasma, colony density and potency of the antiserum. ‘Breakthrough’ colonies are commonly observed within zones of inhibition. Circular precipitin bands are occasionally seen around disks. Positive inhibition is regarded as a zone of 2 mm or more.

- **Growth precipitation test**

  The growth precipitation test detects soluble cytoplasmic and extramembranous antigens released by growing cultures and allowed to diffuse through solid mycoplasma growth medium towards mycoplasma antiserum during growth (20). As with the gel precipitin test, there are strong cross-reactions within the mycoides cluster. If growth inhibition is performed using MAb WM25², which is specific for Mccp, both specific inhibition and a growth precipitin line are achieved simultaneously.

- **Indirect fluorescence antibody test**

  The direct and indirect fluorescent antibody tests are the most effective of the various serological methods for identifying most mycoplasmas (38). They are simple, rapid, and sensitive, yet economical in the use of antiserum. Several forms have been described, the most commonly used and perhaps best being the indirect fluorescent antibody (IFA) test applied to unfixed colonies on agar. Antiserum against a single strain is sufficient to identify field isolates of that species, and antisera are diluted before use. Cultures do not have to be cloned, but the test is usually applied only after several passages have indicated whether the culture contains more than one species and the growth characteristics of the organism(s) present.

  - **Test procedure**

    i) Two agar plates are predried at 37°C for 30 minutes. Each one is flooded with a different dilution of test broth culture, the dilutions being selected according to the vigour of growth of the strain on agar medium. Alternatively, a drop of undiluted culture is spread over a single 5 cm plate using an L-shaped glass rod.

    ii) The plates are incubated at 37°C until the first evidence of growth is observed. If the IFA test cannot be performed immediately, the plates can be stored at 4°C for up to 4 weeks.

    iii) Several blocks of approximately 0.5–1 cm² are excised from areas where colonies are numerous, but not confluent. The blocks of each agar culture are cut to the same geometric shape to enable recognition of origin, a different shape being used for each isolate. Several blocks of each isolate are distributed (colony surface facing upwards) on to several different slides, each slide being used for a different mycoplasma antiserum. The colony surface of each block is identified for future reference by undercutting one corner.

    iv) Rabbit anti-mycoplasma (ra-m) serum or normal rabbit serum (NRS; as a control on a duplicate block) at a suitable dilution in normal saline or phosphate buffered saline (PBS), pH 7.2, is gently pipetted on to each agar block until the surface area is totally covered. The optimal dilution of ra-m is determined by chequerboard titration against the fluorescein isothiocyanate (FITC)-conjugated anti-rabbit immunoglobulin serum (a-r lg-FITC) used.

    v) The flooded blocks are incubated on their slides at room temperature for 30 minutes in a humid chamber.

    vi) All blocks on one slide are tipped into a 10 ml tube containing approximately 7 ml of PBS.

    vii) The plugged tubes are rotated at 18–30 rpm for 10 minutes. The PBS is then decanted and replaced with fresh PBS, and the tubes are rotated again for 10 minutes.

    viii) The PBS is decanted and the blocks are placed colony surface facing upwards on their respective slides. Excess moisture is blotted off.

    ix) All blocks are flooded with a-r Ig-FITC at optimal dilution.

    x) The blocks are incubated again for 30 minutes at room temperature in a humid chamber, then tipped into tubes containing fresh PBS, and washed twice by rotating, as before.

    xi) The blocks, replaced colony surface facing upwards on their respective slides, are examined by an epi-immunofluorescent microscope using the settings recommended by the manufacturer for FITC.

  - **Notes on the indirect fluorescent antibody test**

    xii) Working dilutions of ra-m and a-r Ig-FITC should be kept at 4°C, which limits their shelf life to approximately 1 week.

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² Available from Kenya Agricultural Research Institute (KARI), P.O. Box 57811, Nairobi, Kenya.
xiii) Isolates from CCPP should be examined using antisera against Mccp, MmmLC, Mmc (M. mycoides subsp. capri) and Mcc, positive control cultures should comprise their type strains, namely Mccp, Y goat, PG3 and California kid, respectively.

xiv) A negative (NRS-treated) control should always be incorporated for each culture.

xv) Interpretation of the IFA test can be difficult. Autofluorescence is produced by some species, particularly acholeplasmas. Even in pure cultures, a proportion of colonies may not stain positively with the relevant antiserum; this is particularly true of Mcc. Otherwise, poor results are usually ascribable to the use of an agar culture that has been allowed to grow for too long, or to the use of antiserum that has deteriorated with dilution and age.

f) Other identification tests

Metabolism inhibition (46) and tetrazolium reduction inhibition (44) are other tests sometimes used in the identification of caprine mycoplasmas. A gene probe, F38-12, capable of distinguishing Mccp has been developed (45).

A polysaccharide-specific antigen detection latex agglutination test has been developed to detect CCPP antigen (29). In this test, latex beads are coated with polyclonal immunoglobulin IgG (rabbit) directed against Mcc polysaccharide and used to detect the antigen in the serum of goats with CCPP. This test is proposed to be inexpensive, easy to carry out in the field and useful for detecting CCPP in its earliest stages.

2. Serological tests

Serology has not been widely applied to identifying the cause of outbreaks of pleuropneumonia in goats and sheep. Endemic infections with MmmLC and Mmc can produce a background of positive titres to these organisms in a proportion of apparently healthy animals (17), and under experimental conditions seroconversion to M. mycoides can occur in goats with no clinical signs of disease. Acute cases caused by Mccp rarely show positive titres to the organism before death (27, 33, 47), perhaps because antibodies are ‘eclipsed’ by circulating mycoplasma antigens (33). Seroconversion to Mccp in experimentally infected animals is observed, by the complement fixation (CF) test and indirect haemagglutination (IHA) test, to start 7–9 days after the appearance of clinical signs, to peak between days 22 and 30, and to decline rapidly thereafter (33). These various observations indicate that serology should be applied on a herd, not an individual basis, and that whenever possible, paired serum samples collected 3–8 weeks apart, should be examined.

a) Complement fixation test (the prescribed test for international trade) (25)

The CF test in various forms remains the most widely used serological test for diagnosis of contagious bovine pleuropneumonia (12, 36). In CCPP, the CF test was used for detection of Mccp infection (25) and it has been found to be more specific, though less sensitive, than the IHA test (33). Its main disadvantage is the high level of technical expertise required to perform the test (12).

One method of performing the test is as follows. To prepare the antigen, 2 litres of culture of titre higher than 10^9 CFU/ml is centrifuged at 40,000 × g for 1 hour at 5°C. The deposit is resuspended and washed three times in physiological saline prior to storage in 0.5–1.0 ml volumes at –20°C. Sterile broth treated as above constitutes sediment antigen, and a freeze-dried broth reconstituted at 200 mg/ml constitutes a second control antigen. Prior to testing, the antigen is diluted 1/60 and ultrasonicated for 3 minutes at low power in a container of iced water. The sonicate is centrifuged at 1250 × g for 30 minutes to remove any debris, and stored at –20°C. If stored for more than 2–3 weeks the antigen should be recentrifuged.

• Test procedure

Microtitre plate tests are performed using 0.025 ml volumes, two volumes containing three mean haemolytic doses of complement, and a 1.5% (v/v) final concentration of sheep red blood cells (SRBCs) in U-bottomed microtitre plates as follows:

i) The following are mixed and incubated at 37°C for 45 minutes:
   • 25 µl of doubling dilutions of test serum (heat inactivated at 56°C for 30 minutes) starting with 1/2 dilution;
   • 25 µl of antigen (the dilution of the antigen must be determined in a chequerboard titration using a known positive serum);
   • 25 µl of complement (3 haemolytic units).
ii) 25 µl of sensitised SRBCs, at a final concentration of 1.5% (v/v), is mixed and the plates are incubated at 37°C for 45 minutes.

iii) The plates are incubated at 4°C for 1 hour to allow the unlysed cells to settle.

iv) Reading the results: The titre will be the highest serum dilution that will fix 50% of the complement, i.e. 50% haemolysis.

• Controls

In all CF tests a number of controls are required:

i) Indicator systems (RBCs + haemolysin) alone to ensure that RBCs do not lyse spontaneously.

ii) Indicator system with complement only to show that enough complement is present to lyse the cells.

iii) Indicator system with antigen only and no complement to show that antigen alone does not lyse the cells.

iv) Indicator system with serum alone and no complement to show that the serum alone does not lyse the cells.

v) Indicator system with complement and antigen to detect any anticomplementary activity of the antigen.

vi) Indicator system with the complement and serum to detect any anticomplementary activity of the serum.

b) Latex agglutination test

Latex beads sensitised with the polysaccharide produced by Mccp and present in culture supernatant have been used in a slide agglutination test (14, 39). This test is presently used routinely in Kenya. It is a very useful test in an outbreak because it can be performed at the penside using a drop of whole blood.

Both CF test and IHA test findings emphasise the difficulties inherent in the serological diagnosis of CCPP when using whole cell or membrane preparations as antigen. The use of the more defined antigen, the polysaccharide elaborated by Mccp, provides greater specificity, as there is no cross-reactivity with sera against the other three principal caprine mycoplasmas.

c) Competitive enzyme-linked immunosorbent assay

A competition ELISA has been developed (48) and proved both specific and sensitive. However due to erratic and low demand the production of this test has been stopped until a new format is developed to ensure longer stability of reagents.

C. REQUIREMENTS FOR VACCINES AND DIAGNOSTIC BIOLOGICALS

The first experimental vaccine against Mccp was a live high passage Mccp (28). When inoculated intratracheally, it proved innocuous and protected goats against experimental challenge. However, more recent work has concentrated on inactivated forms of vaccine. The current form used in Kenya (where inactivated Mccp, vaccines have been in use for several years) contains inactivated Mccp suspended in saponin, has been described as having a shelf life of at least 14 months and the optimal dose of 0.15 mg provides protection for over 1 year (40).

1. Seed management

a) Characteristics of the seed

The master seed was isolated from the lungs of a sick goat. In Kenya the strain used is called ‘Yatta’ and it has been confirmed to be Mccp by GIT and PCR, after 15 passages in culture. The master seed should also be pure and free from other contaminants.

b) Method of culture

The master seed is established and stored in a freeze-dried form in 1 ml ampoules. The working seed is prepared by amplifying the master seed in modified Newing’s broth to make a bulk of 4 litres. Its growth is arrested at the growth phase before filament formation.

This culture is tested for sterility before it is distributed in aliquots of 20 ml volumes and stored frozen at –20°C.
c) **Validation as a vaccine**

The master seed should be prepared from lung specimens or pleural fluids of a goat that dies of pneumonia, showing all the clinical signs of CCPP. The isolate must be confirmed by GIT or by PCR to be \(Mccp\). It must be checked for sterility, safety, potency and extraneous agents.

2. **Method of manufacture**

For vaccine production, a working seed is first established by amplifying an aliquot of freeze-dried master seed in modified Newing’s broth (19), to make a bulk of 4 litres of culture. This culture is aliquoted in 20 ml volumes and frozen at \(-20^\circ C\). The vaccine is grown in 5 litre pots containing 4 litres of modified Newing’s broth. Each pot is sampled aseptically for sterility testing before 20 ml of working seed is inoculated into each 4 litres of medium. These pots are incubated at 37°C for 4–6 days depending on how fast the mycoplasma grows. After the filaments sediment, the antigen is harvested by centrifugation at 2600 \(g\) from pots that are not contaminated. (The filaments are thin and white and sometimes unite to form a resemblance of an inverted pine tree. This occurs on days 4–6 and it is at this point that they become heavy and sediment.) The pellet is resuspended in sterile normal saline and centrifuged to remove the remnants of the growth medium. The pellet is resuspended in a small volume of sterile normal saline to make a viscous suspension.

Saponin is added to inactivate the mycoplasma at 3 mg saponin to 1 ml of antigen. It is left agitating overnight at 4°C using a magnetic stirrer.

**NOTE:** Saponin also acts as an adjuvant. Three samples are taken aseptically from this suspension and tested for sterility, for protein estimation and for the innocuity test.

3. **In-process control**

During production, the following tests are carried out to ensure that the product remains pure and safe. These tests are carried out by quality assurance staff. Each pot is sampled aseptically before inoculation to test for sterility.

After maturity of culture, only pots that have not shown signs of contamination are pooled for centrifugation; the contaminated pots are decontaminated and discarded after autoclaving.

After inactivation with saponin, a sample is taken aseptically for sterility, another one for innocuity and another one for the protein estimation test.

a) **Sterility test**

This test is aimed at verifying the absence of fungal and bacterial contaminants. Two tubes of thioglycollate are inoculated with 1 ml of the sample each. The tubes contain about 15 ml of broth. These are incubated at 37°C to eliminate aerobics, microaerophilics and anaerobics. The other medium used is soybean casein digest broth. Four tubes are inoculated with 12 ml of sample each.

Two tubes are incubated at 37°C and another two at room temperature (25°C) to eliminate bacterial and fungal contaminants. All media are incubated for 14 days with controls. Absence of any growth shows that the sample is not contaminated.

b) **Innocuity test**

This test aims to demonstrate the absence of living mycoplasma in the vaccine: 0.3 ml of inactivated antigen is added to 2.7 ml of modified Newing’s broth in a tube and tenfold dilutions are made from \(10^{-1}\) to \(10^{-9}\). A positive control is set up using a viable culture of \(M. \text{capripneumoniae}\) and a negative control using three uninoculated tubes of medium. All are incubated at 37°C for 12 days. If there is no growth in the tubes inoculated with test sample, the vaccine passes the test.

c) **Protein content estimation**

The protein content is measured by the bicinchoninic acid method by comparison with a bovine albumin standard.

4. **Batch control**

a) **Sterility**

The sterility test on the final batch is carried out as described in Section C.3.a except that four bottles of each batch are pooled together and samples of the pool are used.
b) Safety
Every batch of CCPP vaccine must be proven to be safe in laboratory animals. Two guinea-pigs are each inoculated by the intramuscular route in the hind leg and another two guinea-pigs are inoculated by the peritoneal route with 0.5 ml containing five doses of vaccine.

If the vaccine is safe, the guinea-pigs should not show any sign of disease for a 14-day observation period and, on post-mortem, there should be no abscesses on the site of inoculation and in the peritoneal cavity, respectively. If any vaccine-related deaths occur during the observation period, the vaccine fails. If on post-mortem examination, abscesses are observed at the site of inoculation and the peritoneum, the vaccine also fails.

c) Potency
Research is in progress at the Kenya Agricultural Research Institute, Muguga, Kenya, to develop a test for potency.

d) Duration of immunity
The vaccine protects goats for 14 months. It is, however, recommended to boost immunity after 1 year.

e) Stability
If stored at 4°C, the vaccine has a shelf life of 1 year. Before use the vaccine should be shaken thoroughly, for even distribution of antigen.

f) Preservatives
At present preservatives are not used in the vaccine.

g) Precautions
Side-effects of the CCPP vaccine include development of a swelling at the site of inoculation and fever for 1–2 days following vaccination, accompanied with inappetance. The swelling may last between 1 and 14 days and is due to the saponin adjuvant. Accidental self-injection causes severe irritation.

5. Tests on the final product

a) Safety
Every batch of vaccine should be tested for safety in laboratory animals as described in C.4.b.

b) Potency
Once the potency test has been developed, every batch of vaccine will be required to be tested for potency.

REFERENCES


Chapter 2.7.6. — Contagious caprine pleuropneumonia


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**NB:** There are OIE Reference Laboratories for Contagious caprine pleuropneumonia (see Table in Part 3 of this *Terrestrial Manual* or consult the OIE Web site for the most up-to-date list: www.oie.int).
CHAPTER 2.7.7.

ENZOOTIC ABORTION OF EWES
(ovine chlamydirosis)

SUMMARY

Ovine chlamydirosis, also known as enzootic abortion of ewes (EAE) or ovine enzootic abortion (OEA), is caused by the bacterium Chlamydia abortus. Chlamydial abortion typically occurs in the last 2–3 weeks of pregnancy with the appearance of stillborn lambs and inflamed placentas. However, infection can also result in the delivery of full-term stillborn lambs or weak lambs that do not survive longer than 48 hours. Infected ewes can also give birth to healthy lambs and it is not uncommon to observe delivery of a dead and a weak or healthy lamb. There are rarely any predictive signs that abortion is going to occur, although behavioural changes and a vulval discharge can be observed in the last 48 hours of pregnancy.

Diagnosis of enzootic abortion depends on the isolation and identification of the causative agent or detection of the agent or its nucleic acid in the products of abortion or vaginal excretions of freshly aborted females. A humoral antibody response may be detected following abortion. Goats as well as sheep and, less commonly, cattle, pigs, horses and deer, can be affected. Chlamydirosis of small ruminants is a zoonosis and the organism must be handled with biosafety precautions. Pregnant women are particularly at risk.

Identification of the agent: The basis for a positive diagnosis of infection with C. abortus depends on a history of abortion in sheep or goats (often in late pregnancy), evidence of necrotic placentitis, and the demonstration of large numbers of the organism in stained smears of affected placentae. The still moist fleece of fetuses or vaginal swabs of females that have freshly aborted are also useful. Care is needed to distinguish cotyledonary damage caused by Toxoplasma gondii and, in stained smears, to be aware of the morphological similarities between C. abortus and Coxiella burnetii, the agent of Q fever.

Chlamydial antigen can be detected by enzyme-linked immunosorbent assay, immunohistochemistry or the fluorescent antibody test, whereas chlamydial DNA can be detected by the polymerase chain reaction or by microarray. Some of these methods are available in commercial kit form.

Chlamydia abortus can be isolated only in living cells; thus facilities for growth in chicken embryos or cell cultures, with appropriate biohazard containment, are required.

Serological tests: A rise in antibody titre to C. abortus, detected by the complement fixation (CF) test, is common after abortion or stillbirth, but this does not occur in every case. Chlamydia abortus shares common antigens with C. pecorum and some Gram-negative bacteria, so that the CF test is not wholly specific, nor does it distinguish between responses to vaccination and to infection. Low CF titres need to be interpreted with caution, particularly if these are encountered in individual animals or in flocks with no history of abortion.

Alternative serological tests have been developed and some commercialised, but none has been sufficiently appraised so far for field use. A delayed hypersensitivity reaction to chlamydial antigen can be elicited in infected sheep, but the procedure is not amenable to routine use.

Requirements for vaccines and diagnostic biologicals: Inactivated and live vaccines are available that have been reported to prevent abortion and to reduce excretion. They assist in control of the disease but will not eradicate it. Serological screening during the period after parturition helps to identify infected flocks, to which control measures can then be applied.
A. INTRODUCTION

Ovine chlamydiosis (enzootic abortion of ewes [EAE] or ovine enzootic abortion [OEA]) is caused by the bacterium *Chlamydiophila abortus*. Chlamydial abortion in late pregnancy causes serious reproductive wastage in many sheep-rearing areas of the world, particularly where flocks are closely congregated during the parturient period (1, 19). Abortion typically occurs in the last 2–3 weeks of pregnancy with the appearance of stillborn lambs and grossly inflamed placentas. Infection can also result in the delivery of full-term stillborn lambs and weak lambs that generally fail to survive beyond 48 hours. It is also not uncommon in multiple births for an infected ewe to produce one dead lamb and one or more weak or healthy lambs. Infection is generally established in a ‘clean’ flock through the introduction of infected replacements and results in a small number of abortions in the first year, which is followed by an ‘abortion storm’ in the second year that can affect up to around 30% of ewes.

Infected animals show no clinical illness prior to abortion, although behavioural changes and a vulval discharge may be observed in ewes within the last 48 hours of pregnancy. Pathogenesis commences around day 90 of gestation coincident with a phase of rapid fetal growth when chlamydial invasion of placentalitates produces a progressively diffuse inflammatory response, thrombotic vasculitis and tissue necrosis. Milder changes occur in the fetal liver and lung and, in cases in which placental damage is severe, there may be evidence of hypoxic brain damage (6). Abortion probably results from a combination of impairment of materno-fetal nutrient and gaseous exchange, disruption of hormonal regulation of pregnancy and induced cytokine aggression (10).

Chlamydial abortion also occurs in goats and, less frequently, cattle, pigs, horses and deer may be affected. In sheep, abortion in late pregnancy with expulsion of necrotic fetal membranes are key diagnostic indicators, with care being needed to distinguish the diffuse pattern of necrosis from that caused by *Toxoplasma gondii* (cotyledons only). Distinction from other infectious causes of abortion such as brucellosis (see Chapter 2.7.2), coxiellosis (see Chapter 2.1.12) or other bacterial pathogens (*Campylobacter* [see Chapter 2.9.3], *Listeria* [see Chapter 2.9.7], *Salmonella* [see Chapter 2.9.9]) can be achieved by microscopy and/or culture.

Taxonomically, the family Chlamydiaceae is divided into two genera and nine species based on sequence analysis of the 16s and 23s rRNA genes (12). The genus *Chlamydia* includes *C. trachomatis* (humans), *C. suis* (swine) and *C. muridarum* (mouse and hamster). The genus *Chlamydiophila* includes *C. psittaci* (avian), *C. felis* (cat), *C. abortus* (sheep, goat and cattle), *C. caviae* (guinea-pig), the former species *C. pecorum* (sheep and cattle) and *C. pneumoniae* (humans). The two genera and nine species have merit both on the basis of molecular structure and for the purposes of host range and clinical disease. The species show a marked degree of correlation with host range, disease syndrome and virulence, thus assisting in understanding the epidemiology of the various species and serovars affecting mammals and birds. The terms ‘chlamydiosis’ and ‘chlamydia(e)’ are used to refer to members of either of the two genera. However, a binomial of the generic and specific names is used when referring to a particular chlamydial species.

Infected females shed vast numbers of infective *C. abortus* at the time of abortion or parturition, particularly in the placenta and uterine discharges (23). Environmental contamination, resulting from this shedding, is considered the primary source of infection for other females. There is also limited evidence to suggest that sheep that have experienced an abortive episode following experimental infection, can also excrete reproductive tract during subsequent oestrus cycles (23). Human infection may be acquired from infected placenta and uterine discharges (23). Environmental contamination, resulting from this shedding, is considered

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B. DIAGNOSTIC TECHNIQUES

1. Identification of the agent

a) Smears

Where the clinical history of the flock and the character of lesions in aborted placentae suggest enzootic abortion, a diagnosis can be attempted by microscopic examination of smears made from affected chorionic villi or adjacent chorion. Several staining procedures are satisfactory, for example, modified Machiavello, Giemsa, *Brucella* differential, or modified Ziehl–Neelsen stains (28). In positive cases stained by the latter method and examined under a high-power microscope, large numbers of small (300 nm) cocoid elementary bodies are seen singly or in clumps stained red against the blue background of cellular debris. Under dark-ground illumination, the elementary bodies are pale green. If placental material is not available, smears may be made from vaginal swabs of females that have aborted within the previous 24 hours, or
from the moist fleece of a freshly aborted or stillborn lamb that has not been cleaned by its mother. In
general, such preparations contain fewer organisms than placental smears.

In terms of morphology and staining characteristics, *C. abortus* resembles the rickettsia *Coxiella burnetii*,
which, in some circumstances, may provoke abortion and which, in humans, causes Q fever. Care must be
taken to differentiate between these two organisms in cases lacking a good history or evidence of
chlamydial-induced placental pathology. Antigenic differences between *C. abortus* and *Coxiella burnetii* can
be detected serologically. Fluorescent antibody tests (FATs) using a specific antiserum or monoclonal
antibody may be used for identification of *C. abortus* in smears.

b) Antigen detection

Several chlamydial genus-level antigen-detection tests are available commercially. A comparative
assessment of several such assays, on non-ovine material, indicated that those using enzyme-linked
immunosorbsorbent assay (ELISA) methodology were more sensitive than kits employing a FAT (34). Under the
test conditions used, a kit that detects chlamydial lipopolysaccharide (LPS) was judged to be the most
sensitive of the rapid ELISA-based systems investigated. Though occasionally yielding false-positive
results, particularly with avian faecal samples, the kit also gave satisfactory results with ovine placental
samples (33). In histopathological sections, antigen detection can be performed using commercially
available anti-*Chlamydia* antibodies directed against LPS or MOMP (major outer membrane protein) (5).

c) DNA

Amplification of chlamydial DNA by polymerase chain reaction (PCR) and real time PCR provide alternative
approaches for verifying the presence of chlamydiae in biological samples without resorting to culture. PCR
is highly sensitive for this purpose, but has the attendant risk of cross-contamination between samples, so
appropriate measures must be taken to avoid this happening (see Chapter 1.1.7 Biotechnology in the
diagnosis of infectious diseases and vaccine development). Another potential problem is in the production
of false negatives resulting from PCR-inhibitory substances in the samples. Methods for discriminating
between amplified DNA sequences originating from *C. abortus* and *C. pecorum* have been described (8, 11,
15, 18, 31). Such tests are beginning to be introduced into diagnostic laboratories throughout Europe.
Recently, DNA microarray hybridisation assays using the ArrayTube™ platform have been developed and
hold much promise for the direct detection and identification of organisms from clinical samples (9, 25).

d) Tissue sections

Intracellular chlamydial inclusions can be demonstrated by Giemsa staining of thin (≤ 64 µm) sections taken
from target tissues that have been suitably fixed in fluids such as Bouin or Carnoy. More striking results can
be obtained by immunological staining procedures. The direct immunoperoxidase method (13) is rapid and
simple, while the method with streptavidin–biotin is more complex (29). Electron microscopy can also be
performed using negative contrast, to differentiate chlamydiae from *Coxiella burnetii*.

e) Isolation of the agent

*Chlamydophila abortus* can be isolated in embryonated chicken eggs or in cell culture, the latter being the
method of choice for isolation of new strains. The causative agent of chlamydiosis is zoonotic (19) and thus
isolation and identification procedures must be carried out under the appropriate containment level as
described in Chapter 1.1.2.

Tissue samples, such as diseased cotyledons, placental membranes, fetal lung or liver, or vaginal swabs,
that may be subject to any delay before isolation procedures begin, should be maintained in a suitable
transport medium in the interim period. The most satisfactory medium is sucrose/phosphate/glutamate or
SPG medium (sucrose [74.6 g/litre], KH₂PO₄ [0.512 g/litre], K₂HPO₄ [1.237 g/litre], L-glutamic acid
[0.721 g/litre]) supplemented with 10% fetal bovine serum, antibiotic (streptomycin and gentamycin are
suitable, but not penicillin), and a fungal inhibitor (27). A tissue:medium ratio of 1:10 is commonly employed.
Alternatively, approximately 1 g of tissue is ground with sterile sand in 8 ml of transport medium.

Chicken embryos: Test samples are prepared as 10% suspensions in nutrient broth containing streptomycin
(not penicillin) (200 µg/ml); 0.2 ml of suspension is inoculated into the yolk sac of 6–8-day old embryos,
which are then further incubated at 37°C. Infected embryos die between 4 and 13 days after inoculation.
Smears prepared from their vascularised yolk sac membranes reveal large numbers of elementary bodies.

Cell cultures: *Chlamydophila abortus* of ovine origin can be isolated in a variety of cell types, but McCoy,
Buffalo Green Monkey (BGM) or baby hamster kidney (BHK) cells are most commonly used. For
confirmatory diagnosis, cultured monolayers are suspended in growth medium at a concentration of 2 ×
10⁶ cells/ml. Aliquots of 2 ml of the suspension are dispensed into flat-bottomed glass Universal bottles,
each containing a single 16 mm cover-slip. Confluent cover-slip monolayers are achieved after incubation
for 24 hours at 37°C. The growth medium is removed and replaced by 2 ml of test inoculum, which is then
centrifuged at 2500 \textit{g} for 30 minutes on to the cover-slip monolayer to promote infection. After further incubation for 2–3 days, the cover-slip monolayers are fixed in methanol and stained with Giemsa or according to the method of Gimenez (3, 14). After methanol fixation, infected cultures contain basophilic (Giemsa) or eosinophilic (Gimenez) intracytoplasmic inclusions. Similar procedures are used in culturing \textit{C. abortus} for antigen preparation. FAT techniques can also be used and are equally effective.

Chlamydial activity can be further enhanced by chemical treatment of cultured cells, before or during infection, to favour chlamydial growth. Various substances that have been described for incorporation into the infective inoculum to which cover-slip monolayers are exposed include: cycloheximide (0.5 \mu g/ml) in the maintenance medium, emetine (1 \mu g/ml) for 5 minutes before infection, and 5-ido-2-deoxyuridine (80 \mu g/ml) for 3 days prior to infection. Unless preconditioned cells are available, the latter isolation procedure requires increased time for successful agent isolation.

2. Serological tests

a) Complement fixation test

Complement fixation (CF) is the most widely used procedure for detecting infection (sheep and goats are generally tested within 3 months of abortion or parturition). The test will also detect evidence of vaccination. Infection is evident principally during active placental infection in the last month of gestation and following the bacteraemia that often accompanies abortion. Consequently, paired sera collected at the time of abortion and again at least 3 weeks later may reveal a rising CF antibody titre that will provide a basis for a retrospective diagnosis. Antigenic cross-reactivity between \textit{C. abortus} and \textit{C. pecorum}, as well as with some Gram-negative bacteria (e.g. \textit{Acinetobacter}), can give rise to low false-positive CF test results. Thus, titres less than 1/32 in individual animals should be considered to be nonspecific for \textit{C. abortus}, although they could also be due to a low grade infection with \textit{C. abortus}. Ambiguous results can be investigated further by western blot analysis using purified elementary bodies (17).

Antigen is prepared from heavily infected yolk sac membranes obtained from chicken embryos that have been inoculated in the same manner as those used to isolate the organism from field material. The preparation of the antigen should be carried out in a biosafety cabinet with the appropriate biosecurity precautions to prevent human infection (see Chapter 1.1.2). Chopped and ground membranes are suspended in phosphate buffer, pH 7.6, at the rate of 2 ml per g membrane. After removal of crude debris, the supernatant fluid is centrifuged at 10,000 \textit{g} for 1 hour at 4°C, the deposit is resuspended in a small volume of saline, and a smear of this is examined to ensure a high yield of chlamydiae. The suspension is held in a boiling water bath for 20 minutes, or is autoclaved, and sodium azide (0.3%) is added as a preservative. Antigen may also be prepared from cell cultures infected with \textit{C. abortus}. Infected monolayers are suspended in phosphate buffer, pH 7.6, and the cells are disrupted by homogenisation or ultrasonication. Gross debris is removed and subsequent procedures are as for the preparation of antigen from infected yolk sacs. In either case, CF tests with standardised complement and antisera will establish the optimal working dilution for each batch of antigen.

b) Other tests

The serological responses to \textit{C. abortus} and \textit{C. pecorum} can be resolved by indirect micro-immunofluorescence, but the procedure is too time-consuming for routine diagnostic purposes. ELISAs developed independently by several research groups have not been adapted for general diagnostic work, partly because of difficulties associated with the use of particulate antigens. However, a novel ELISA that incorporates a stable, solubilised antigen has been used to test experimental and field samples, and has given results that, though lacking species specificity, have a higher sensitivity than the CF test (2, 17). Other tests using monoclonal antibody technology in a competitive ELISA (26) and recombinant antigen technology in indirect ELISAs (20) have been developed and shown to be more sensitive and specific than the CF test in differentiating animals infected with \textit{C. abortus} from those infected with \textit{C. pecorum}. However, these tests are currently mainly used as research tools, and have not been developed commercially. A number of commercially available serological tests have been evaluated and compared with these 'in-house' tests with variable results (17, 32). None of the serological tests that is available can differentiate vaccination titres from those acquired as a result of natural infection (4).

C. REQUIREMENTS FOR VACCINES AND DIAGNOSTIC BIOLOGICALS

Currently, two types of vaccine (inactivated and attenuated live vaccines) are available commercially, to be administered intramuscularly or subcutaneously at least 4 weeks before breeding to aid in the prevention of abortion. A multi-component recombinant vaccine against \textit{C. abortus} remains a future goal of chlamydial vaccine research (21).
Inactivated vaccines can be prepared from infected yolk sacs or cell cultures (16) and incorporate whole organisms or fractions of them (30), using the appropriate biosecurity precautions to prevent human infection (see Chapter 1.1.2). Operator care should be observed in handling commercial inactivated vaccines that incorporate mineral oil-based adjuvants, as self-injection can result in severe local inflammation and tissue necrosis. The commercial live, attenuated vaccine is a chemically induced temperature-sensitive mutant strain of the organism that grows at 35°C but not at 39.5°C, the body temperature of sheep (24). This vaccine is supplied lyophilised and must be reconstituted in diluent immediately before administration. Operator care should be observed in handling and administering this live vaccine, particularly by immunocompromised individuals and pregnant women. Importantly, the live vaccine must not be given to animals being treated with antibiotics, particularly teracyclines.

Both types of vaccine have a role to play in controlling disease, but neither confers absolute protection against challenge or completely reduces the shedding of infective organisms. However, vaccinates exposed to infection do experience significantly lower abortion rates and reduced excretion of chlamydiae for at least two to three lambings after vaccination. It has been claimed that the live vaccine could be an aid to eradication of disease (22).

1. Seed management
   a) Characteristics of the seed
      One or more ovine abortion isolates that consistently grow productively in the chosen substrate are suitable, and an early passage of the seed stock can be established. Alternatively, an isolate that has been adapted to the chicken embryo by multiple passage (>100) can be used. This permits more of the embryo to be used for vaccine production. Although adaptation to the embryo may diminish the isolate’s virulence for sheep, there is no evidence that such change reduces its protective efficacy as an inactivated vaccine.
   b) Method of culture
      For low passage isolates, the procedures described for the preparation of CF antigen are suitably adapted and amplified for bulk production. Once the final harvest suspension is obtained, an aliquot is removed for titration of its infectivity. The bulk is treated with formalin to a final concentration of 0.4%, and stored until sterility tests confirm complete inactivation.
   c) Validation as a vaccine
      Before inoculation of large numbers of embryos or cell cultures, the viability and freedom from contamination of seed stock should be verified. It may be convenient to collect the total harvest in separate manageable lots. In this case, the infectivity of an aliquot of each lot should be separately titrated to ensure that each matches the requirements (see Section C.2 below). Store under refrigerated conditions.

2. Method of manufacture
   The inactivated harvest is centrifuged and resuspended in phosphate buffered saline containing 0.2% formalin to a volume representing a preinactivation infectivity titre of approximately $10^8$ infectious units/ml. Usually, the aqueous suspension is blended with an oil adjuvant, either directly or after precipitation by potassium alum ($\text{AlK}_2\text{(SO}_4\text{)}_{12} \cdot 12 \text{H}_2\text{O}$). A preservative, such as 0.01% thiomersal, may also be added.

3. In-process control
   The main requirements are to ensure adequate growth of *C. abortus*, avoidance of extraneous infection of the culture substrate, completeness of inactivation and biohazard awareness by process workers.

4. Batch control
   Each separate batch of manufactured vaccine should be tested for sterility, safety and potency.
   a) Sterility
      Tests for sterility and freedom from contamination of biological materials may be found in Chapter 1.1.9.
   b) Safety
      Subcutaneous inoculation into two or more seronegative sheep of twice the standard dose (usually 1.0 ml) of manufactured vaccine should elicit no systemic reaction, but oil-adjuvant vaccines can cause a nonharmful swelling at the inoculation site.
c) **Potency**

At present, potency is judged by the occurrence of a serological response in previously unvaccinated sheep given 1 ml of vaccine subcutaneously. Blood samples taken before and 28 days after vaccination are compared. Ultimately, potency has to be judged against experimental challenge or field performance, but no *in vitro* correlation of protective efficacy has yet been established.

d) **Duration of immunity**

No firm data are available, but revaccination is recommended after 1–3 years, according to the exposure risk.

e) **Stability**

Vaccine stored under refrigeration (5±3°C) should remain stable for at least 1 year. Before use it should be held at room temperature for 24 hours, and the container should be shaken vigorously immediately before vaccine is withdrawn.

5. **Tests on the final product**

a) **Safety**

See Section C.4.b.

b) **Potency**

See Section C.4.c.

**REFERENCES**


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**NB:** There are OIE Reference Laboratories for Enzootic abortion of ewes (see Table in Part 3 of this *Terrestrial Manual* or consult the OIE Web site for the most up-to-date list: www.oie.int).
CHAPTER 2.7.8.
NAIROBI SHEEP DISEASE

See Chapter 2.9.1. Bunyaviral diseases of animals (excluding Rift Valley fever)

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OIE Terrestrial Manual 2008
SUMMARY

Brucella ovis produces a clinical or subclinical disease in sheep that is characterised by genital lesions in rams, and placentitis in ewes. Accordingly, the main consequences of the disease are reduced fertility in rams, infrequent abortions in ewes, and an increased perinatal mortality. The disease has been reported in Latin American, North American and European countries as well as Australia, New Zealand and South Africa, but probably occurs in most sheep-raising countries.

Identification of the agent: The existence of clinical lesions (unilateral or, occasionally, bilateral epididymitis) in rams may be indicative of the existence of infection, but laboratory examinations are necessary to confirm the disease. Laboratory confirmation may be based on direct or indirect methods. Direct diagnosis is made by means of bacteriological isolation of B. ovis from semen samples or tissues of rams, or vaginal discharges and milk of ewes, on adequate selective media. Molecular biological methods, such as polymerase chain reaction and pulse-field gel electrophoresis, are being developed. However, indirect diagnosis based on serological tests is preferred for routine diagnosis.

Serological tests: The complement fixation test (CFT), agar gel immunodiffusion (AGID) test and indirect enzyme-linked immunosorbent assay (ELISA) using soluble surface antigens obtained from B. ovis, can be used. Some ELISAs using recombinant proteins and monoclonal antibodies are being tested in field trials. The sensitivities of the AGID test and ELISA are similar and sometimes the ELISA has higher sensitivity than the CFT. A combination of the AGID test and ELISA seems to give the best results in terms of sensitivity. However, with regard to simplicity and cost, the AGID test is the most practicable test for diagnosis of B. ovis. However, because of the lack of standardised methods recognised at the international level for ELISA and AGID, the prescribed test for international trade remains the CFT.

Requirements for vaccines and diagnostic biologicals: Seed cultures for antigen or vaccine production should be obtained from internationally recognised laboratories. A single standard dose (10^9 colony-forming units) of the live B. melitensis Rev.1 vaccine, administered subcutaneously or conjunctivally, can be used safely and effectively in rams, for the prevention of B. ovis infection. This vaccine strain should meet minimal quality standards: adequate concentration, absence of dissociation, adequate residual virulence and immunogenicity and free of extraneous agents (see Chapter 2.7.2 Caprine and ovine brucellosis [excluding B. ovis]).

A. INTRODUCTION

Brucella ovis causes a genital infection of ovine livestock manifested by epididymitis, infrequent abortions, and increased lamb mortality. Passive venereal transmission via the ewe appears to be a frequent route of infection, but ram-to-ram transmission is also common. Infected ewes may excrete B. ovis in vaginal discharges and
milk and, accordingly, ewe-to-ram and lactating ewe-to-lamb transmission could also be determinant mechanisms of infection.

The demonstration of the existence of genital lesions (unilateral or, occasionally, bilateral epididymitis) by palpating the testicles of rams may be indicative of the presence of this infection in a given flock. However, this clinical diagnosis is not sensitive enough because only about 50% of rams infected with \textit{B. ovis} present epididymitis (2). Moreover, the clinical diagnosis is extremely unspecific due to the existence of many other bacteria causing clinical epididymitis. The most frequently reported isolates causing epididymitis in rams include \textit{Actinobacillus seminis}, \textit{A. actinomycetemcomitans}, \textit{Histophilus ovis}, \textit{Haemophilus} spp., \textit{Corynebacterium pseudotuberculosis} \textit{ovis}, \textit{B. melitensis} and \textit{Chlamydophila abortus} (formerly \textit{Chlamydia psittaci}) (5, 6, 9, 11, 13, 25, 31, 34). It must be emphasised that many palpable epididymal lesions in rams are sterile, trauma-induced spermatic granulomas.

Although cattle, goats and deer have been proved susceptible to \textit{B. ovis} in artificial transmission experiments, natural cases have been reported only in deer (21). To date, no human cases have been reported, and \textit{B. ovis} is considered to be non-zoonotic. However, in areas where \textit{B. melitensis} infection co-exists with \textit{B. ovis}, special care is required when handling samples, which should be transported to the laboratory in leak-proof containers (for further details see Chapter 2.4.3 Bovine brucellosis).

**B. DIAGNOSTIC TECHNIQUES**

1. **Identification of the agent**
   a) **Collection of samples**
      
      The most valuable samples for the isolation of \textit{B. ovis} from live animals are semen, vaginal swabs and milk. For the collection of vaginal swabs and milk, see the instructions given in Chapter 2.7.2 Caprine and ovine brucellosis (excluding \textit{B. ovis}). Semen (genital fluids) can be collected easily in swabs taken from the preputial cavity after electro-ejaculation. If an electro-ejaculator is not available, swabs can be taken from the vagina of brucellosis-free ewes immediately after natural mating.
      
      For the isolation of \textit{B. ovis} after necropsy, the preferred organs in terms of probability of isolation are the epididymides, seminal vesicles, ampullae, and inguinal lymph nodes in rams, and the uterus, iliac and supra-mammary lymph nodes in ewes. However, to obtain maximum sensitivity, a complete search that includes other organs and lymph nodes (spleen, cranial, scapular, prefemoral and testicular lymph nodes) should be performed. Dead lambs and placentas should also be examined. The preferred culture sites in aborted or stillborn lambs are abomasal content and lung.
      
      Samples for culture should be refrigerated and transported to the laboratory to be cultured as soon as possible after collection. The organism remains viable for at least 72 hours at room temperature and survival is enhanced at 4°C or, preferably, by freezing the tissue samples.
   
   b) **Staining methods**
      
      Semen or vaginal smears can be examined following staining by Stamp’s method (1, 8) (see Chapter 2.7.2), and characteristic coccobacilli should be demonstrated in many infected animals (32). Examination of Stamp-stained smears of suspect tissues (ram genital tract, inguinal lymph nodes, placentas, and abomasal content and lung of fetuses) may also allow a rapid presumptive diagnosis.
      
      However, other bacteria with similar morphology or staining characteristics (\textit{B. melitensis}, \textit{Coxiella burnetii}, and \textit{Chlamydophila abortus}) can also be present in such samples, making the diagnosis difficult for inexperienced personnel. Microscopy results should always be confirmed by culture of the microorganism.
   
   c) **Culture**
      
      The best direct method of diagnosis is bacteriological isolation on adequate culture media. Semen, vaginal swabs, or milk samples can be smeared directly on to plates with adequate culture media and incubated at 37°C in an atmosphere of 5–10% CO\textsubscript{2}. Tissues should be macerated and ground in a small amount of sterile saline or phosphate buffered saline (PBS) with a stomacher or blender, before plating.

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rams, lick the prepuce of these dominant rams as an act of submission. If these dominant rams are infected, the probability of having \textit{B. ovis} in the prepuce (excretion in the semen) is very high.
Growth normally appears after 3–4 days, but cultures should not be discarded as negative until 7 days have elapsed. Colonies of *B. ovis* become visible (0.5–2.5 mm) after 3–4 days of incubation, and are rough, phase, round, shiny and convex.

*B. ovis* can be isolated in nonselective media, such as blood agar base enriched with 10% sterile ovine or bovine sera, or in blood agar medium with 5–10% sterile ovine blood. However, the inoculum frequently contains other bacteria, which often overgrow *B. ovis*. Accordingly, the use of selective media may be preferred. Various *B. ovis* selective media have been described. The modified Thayer–Martin’s medium (4, 15) is recommended. Briefly, it can be prepared with GC medium base (38 g/litre; Biolife Laboratories, Milan, Italy) supplemented with haemoglobin (10 g/litre; Difco) and colistin methane-sulphonate (7.5 mg/litre), vancomycin (3 mg/litre), nitrofurantoin (10 mg/litre), nystatin (100,000 International Units [IU]/litre = 17.7 mg) and amphotericin B (2.5 mg/litre) (all products from Sigma Chemical, St Louis, United States of America [USA]). Working solutions are prepared as follows:

**Solution A:** Add 500 ml of distilled water to the GC medium base, heat the paste carefully while stirring continuously and autoclave at 120°C for 20 minutes.

**Solution B:** Suspend the haemoglobin in 500 ml of distilled water, adding the water slowly to avoid lumps. Once dissolved, add a magnetic stirrer and autoclave at 120°C for 20 minutes.

**Antibiotic solution (prepared daily):** colistin, nystatin and vancomycin are suspended in a mixture of methanol/water (1/1); nitrofurantoin is suspended in 1 ml of a 0.1 M NaOH sterile solution. For amphotericin B, it is recommended to prepare a stock solution of 10 mg/ml amphotericin B with 10 mg dissolved first in 1 ml sterile dimethyl sulphoxide (C₂H₆OS, for analysis; ACS) and then added to 9 ml of PBS (10 mM, pH 7.2). Any stock solution remaining can be stored some days at 4°C. All antibiotic solutions must be filtered through 0.22 µm filters before addition to the culture medium.

Once autoclaved, stabilise the temperature (45–50°C) of both solutions A and B with continuous stirring. Mix both solutions (adding A to B), avoiding bubble formation. Add the antibiotic solutions while stirring continuously and carefully. Dispense into sterile plates.

Once prepared, the plates should not be stored for long periods, and freshly prepared medium is always recommended. This medium is also suitable for the isolation of *B. melitensis* (see Chapter 2.7.2).

All culture media should be subjected to quality control with the reference strain, to show that it supports growth.

Another suitable, but less effective, antibiotic combination is: vancomycin (3 mg/litre); colistin (7.5 mg/litre); nystatin (12,500 IU/litre); and nitrofurantoin (10 mg/litre).

The Farrell’s medium described for the culture of smooth brucellae is not appropriate for the culture of *B. ovis* as it does not usually grow on this medium.

d) **Identification and typing**

*Brucella ovis* colonies are not haemolytic. They are circular, convex, have unbroken edges, are always of the rough type when examined by oblique illumination, and test positive in the acriflavine test (1, 8). For growth, *B. ovis* needs an atmosphere of 5–10% CO₂. It lacks urease activity, fails to reduce nitrate to nitrite, is catalase positive and oxidase negative. It does not produce H₂S and, although it does not grow in the presence of methyl violet, it usually grows in the presence of standard concentrations of basic fuchsin and thionin. The cultures are not lysed by *Brucella*-phages of the Tbilissi (Tb), Weybridge (Wb) and Izatnagar (Iz) groups at the routine test dilution (RTD) or 10⁴ RTD, while they are lysed by phage R/C (1, 8). Most laboratories are not equipped for a complete identification, and a practical schedule for presumptive identification is needed. Most *B. ovis* isolates can be correctly identified on the basis of growth characteristics, direct observation using obliquely reflected light, Gram or Stamp’s staining, catalase, oxidase, urea and acriflavine tests. However, definitive identification should be carried out by reference laboratories with experience in identification and typing of *Brucella*.

The recently developed polymerase chain reaction (PCR) provides an additional means of detection and identification of *Brucella* sp. (3, 14).

A method of pulse-field gel electrophoresis can differentiate *B. ovis* from other *Brucella* species (19). Moreover, *B. ovis* can be differentiated from the other *Brucella* species through its specific PCR restriction fragment length polymorphism (PCR RFLP) patterns for genes omp2a, omp2b, omp25 and omp31, coding for the major outer membrane proteins of all *Brucella* species (30). Pulsed field gel electrophoresis might also distinguish several subtypes of *Brucella ovis* (22).
2. Serological tests

The most efficient and widely used tests are the complement fixation test (CFT), the double agar gel immunodiffusion (AGID) test and the indirect enzyme-linked immunosorbent assay (ELISA). Several countries have adopted various standard diagnostic techniques for \textit{B. ovis}, but the only test prescribed by the OIE and the European Union (EU) for international trade is the CFT. However, it has been demonstrated that the AGID test shows similar sensitivity to the CFT, and it is a simpler test to perform. Although standardisation is lacking, numerous independent studies have shown that the ELISA is more sensitive and specific than either the CFT or AGID test, and with further validation and standardisation studies, the ELISA could become a suitable candidate for future designation as a prescribed test for \textit{B. ovis} diagnosis.

The International Standard anti-\textit{Brucella ovis} Serum (International Standard 1985\textsuperscript{2}) is the one against which all other standards are compared and calibrated. This reference standard is available to national reference laboratories and should be used to establish secondary or national standards against which working standards can be prepared and used in the diagnostic laboratory for daily routine use.

- Antigens

When rough \textit{Brucella} cells are heat-extracted with saline (hot-saline method, HS), they yield water-soluble antigenic extracts, the major component of which precipitates with sera to rough \textit{Brucella} (10, 20). For this reason, the HS extracts have been referred to as the 'rough-specific antigen' or, when obtained from \textit{B. ovis}, as the 'B.-ovis-specific antigen'. However, the chemical characterisation of the HS extracts from \textit{B. ovis} has shown that they are enriched in rough lipopolysaccharide (R-LPS), group 3 outer membrane proteins and other outer membrane components (23). Thus, HS extracts contain LPS determinants specific for \textit{B. ovis}, but also additional antigenic components, some of them shared with rough and smooth \textit{B. melitensis} and other \textit{Brucella} (26). Such components account for the cross-reactivity that is sometimes observed with the HS method and sera of sheep infected with \textit{B. melitensis} or vaccinated with Rev.1 (23).

The HS extract, due to its water solubility and high content of relevant cell-surface epitopes, is the best diagnostic antigen and has been widely used for the serological diagnosis of \textit{B. ovis} infection.

\textit{Brucella ovis} REO 198, a CO\textsubscript{2}- and serum-independent strain, is recommended as a source of the HS antigens to be used in serological tests. This strain can be obtained from INRA\textsuperscript{3}. Solid media described in Section B.1.c. are satisfactory for the growth of \textit{B ovis} REO 198. HS antigen is prepared as follows:

i) Exponentially grow a suitable strain of \textit{B ovis}, preferably aerobic and nonserum dependent, e.g. REO 198, in one of the following ways: for 48 hours in trypti case–soy broth flasks in an orbital incubator at 37°C and 150 rpm; or in Roux bottles of trypticase–soy agar, or other suitable medium, with 5% serum added (not necessary when using the REO 198 strain); or in a batch-type fermenter as described for \textit{B. abortus}, but with the addition of 5% serum to the medium (not necessary when using the REO 198 strain).

ii) Cells are resuspended in 0.85% saline or PBS, then washed twice in 0.85% saline (12 g of dried cells or 30 g of wet packed cells in 150 ml).

iii) The cell suspension is then autoclaved at 120°C for 15–30 minutes.

iv) After cooling, the suspension is centrifuged (15,000 \textit{g}, 4°C, 15 minutes) and the supernatant fluid is filtered and dialysed against distilled water using 100 times the volume of the suspension, at 4°C; the water should be changed three times over a minimum of 2 days.

v) The dialysed fluid can be ultracentrifuged (100,000 \textit{g}, 4°C, 6–8 hours), and the sediment is resuspended in a small amount of distilled water and freeze-dried. The use of control process serum replacement II (CPSRII) prior to freeze drying may assist in stability and anti-complementary activity.

HS is then resuspended either in distilled water (for use in the AGID test), veronal buffered saline (for use in the CFT), or carbonate/bicarbonate buffer or PBS (for use in the ELISA) and titrated against a set of adequate positive and negative sera.

The resuspended HS is kept at 4°C with 0.5% phenol as preservative (only for use in the AGID test) or freeze-dried. Freezing and thawing should be avoided (10). The CFT antigen should be standardised against the International anti-B. \textit{ovis} Standard Serum to give 50% fixation at a 1/100 serum dilution.

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\textsuperscript{2} Obtainable from the OIE Reference Laboratory for Brucellosis, VLA Weybridge, Addlestone, Surrey KT15 3NB, United Kingdom.

\textsuperscript{3} Institut national de la recherche agronomique (INRA) Laboratoire de Pathologie Infectieuse et Immunologie, 37380 Nouzilly, France.
Chapter 2.7.9. – Ovine epididymitis (Brucella ovis)

a) Complement fixation test (the prescribed test for international trade)

There is no standardised method for the CFT, but the test is most conveniently carried out using the microtitrination method. Some evidence shows that cold fixation is more sensitive than warm fixation (7, 24, 27), but that it is less specific. Anticomplementary reactions, common with sheep serum, are, however, more frequent with cold fixation.

Several methods have been proposed for the CFT using different concentrations of fresh sheep red blood cells (SRBCs) (2–3% suspension is usually recommended) sensitised with an equal volume of rabbit anti-SRBC serum diluted to contain several times (usually from two to five times) the minimum concentration required to produce 100% lysis of SRBCs in the presence of a titrated solution of guinea-pig complement. The latter is independently titrated (in the presence or absence of antigen according to the method) to determine the amount of complement required to produce either 50% or 100% lysis of sensitised SRBCs in a unit volume of a standardised suspension; these are defined as the 50% or 100% haemolytic unit of complement (C'H50 or C'H100), respectively. It is generally recommended to titrate the complement before each set of tests, a macromethod being preferred for an optimal determination of C'H50. Usually, 1.25–2 C'H100 or 5–6 C'H50 are used in the test.

Barbital (veronal) buffered saline (VBS) is the standard diluent for the CFT. This is prepared from tablets available commercially, otherwise it may be prepared according to the formula described elsewhere (see Chapter 2.4.3 Bovine brucellosis). The test sera should be inactivated for 30 minutes in a water bath at 60–63°C, and then diluted (doubling dilutions) in VBS. The stock solution of HS antigen (2.5–20 mg/ml in VBS) is diluted in VBS as previously determined by titration (checkerboard titration). Usually, only one serum dilution is tested (generally 1/10).

Using standard 96-well microtitre plates with round (U) bottom, the technique is usually performed as follows:

i) Volumes of 25 µl of diluted inactivated test serum are placed in the well of the first and second rows. Volumes of 25 µl of CFT buffer are added to all wells except those of the first row. Serial doubling dilutions are then made by transferring 25 µl volumes of serum from the second row onwards.

ii) Volumes of 25 µl of antigen, diluted to working strength, are added to each well except wells in the first row.

iii) Volumes of 25 µl of complement, diluted to the number of units required, are added to each well.

iv) Control wells containing diluent only, complement + diluent, antigen + complement + diluent, are set up to contain 75 µl total volume in each case. A control serum that gives a minimum positive reaction should be tested in each set of tests to verify the sensitivity of test conditions.

v) The plates are incubated at 37°C for 30 minutes or at 4°C overnight, and a volume (25 or 50 µl according to the techniques) of sensitised SRBCs is added to each well. The plates are reincubated at 37°C for 30 minutes.

vi) The results are read after the plates have been centrifuged at 1000 g for 10 minutes at 4°C or left to stand at 4°C for 2–3 hours to allow unlysed cells to settle. The degree of haemolysis is compared with standards corresponding to 0, 25, 50, 75 and 100% lysis. The titre of the serum under test is the highest dilution in which there is 50% or less haemolysis.

b) Agar gel immunodiffusion test

The AGID test (2) uses the following reagents: Good grade Noble agar or agarose, sodium chloride (NaCl), and borate buffer (prepared with boric acid [12.4 g]; potassium chloride [14.5 g]; distilled water [1600 ml]; adjusted to pH 8.3 with 0.2 M NaOH solution and made up to 2000 ml with distilled water).
To prepare the gels, dissolve 1 g of agarose (or Noble agar). 10 g of NaCl and 100 ml of borate buffer by boiling while stirring continuously. On a flat surface, cover clean glass slides with the necessary amount of molten gel to form a bed of 2.5 mm depth (3.5 ml approximately for standard microslides). After the gel has solidified (15–20 minutes), wells are cut in it using a gel puncher. The wells should be 3 mm in diameter and 3 mm apart, and should be arranged in a hexagonal pattern around a central well that is also 3 mm in diameter. The test can be adapted to Petri dishes and other patterns.

Sera to be examined are placed in alternate wells separated by a control positive serum (infection proved by bacteriology), with the antigen at its optimum concentration in the central well. The results are read after incubation for 24 and 48 hours at room temperature in a humid chamber. A positive reaction is a clearly defined precipitin line between the central well and the wells of the test sera that gives total or partial identity with that of the positive controls. Precipitin lines not giving total identity may also appear and correspond to minor antigenic components of HS extracts (antibodies to these components can also be common in infections due to B. melitensis). These reactions should also be considered to be positive. Before a definitive reading, it is important to wash the slides for 1 hour in a 5% sodium citrate water solution to clean unspecific precipitin lines.

The HS (2.5–20 mg/ml) diluted in distilled water and containing 0.5% phenol as a preservative is the antigen used in the AGID test (this preserved antigen can be stored at 4°C for at least 1 month). Dilutions of antigen are tested with a panel of 20–30 sera from rams naturally infected with B. ovis and with a panel of Brucella-free sheep. The optimum concentration of antigen is that giving the clearest precipitation line with all the sera from B. ovis-infected rams being negative with the sera from Brucella-free sheep.

c) Enzyme-linked immunosorbent assay

Several variations of this assay have been proposed. The assay described here is an indirect ELISA using ABTS (2,2’-azino-bis-[3-ethylbenzothiazoline-6-sulphonic acid]) as chromogen, but other procedures are also suitable. Tests are performed on 96-well flat-bottomed ELISA plates. Reagent and serum dilutions are made in PBS, pH 7.2, with the addition of 0.05% Tween 20 (PBST). Antigen dilutions are made in a carbonate/bicarbonate buffer, pH 9.6, or, alternatively, in PBS, pH 7.2. Plates are washed after antigen coating and between incubations, where appropriate, usually with PBST. The antigen (HS) and conjugate are checkerboard titrated, and dilutions are selected to give the best discriminating ratio between negative and positive standard sera. Secondary antibodies (anti-ovine IgG [H + L chains]) antibodies are usually conjugated to horseradish peroxidase (HRPO), although other enzymes or conjugates (such as recombinant protein G/HRPO) can be used. A monoclonal antibody to bovine IgG1–HRPO conjugate has also been found to be suitable for use in the ELISA (29). If a peroxidase conjugate is used, the chromogen, usually ABTS, is diluted in a substrate buffer (composed of citric acid trisodium and citric acid; pH 4). The substrate, hydrogen peroxide (H₂O₂), is added to this, and the plates are incubated for 15–25 minutes at room temperature. The reaction may be stopped with 1 mM sodium azide, and the colour change is read at 405–414 nm (for further details see Chapter 2.4.3).

The antigen used in the ELISA is the HS in stock solution at 1 mg/ml in coating buffer, titrated in a checkerboard titration, with different dilutions of antigen, conjugate and substrate, against a standard serum or against serial dilutions of a panel of sera from B. ovis culture positive and Brucella-free sheep to determine the most sensitive and specific dilution (usually 5–10 µg/ml).

- **Test procedure (example)**
  
  i) **Microtitre plates of good quality polystyrene** are coated by the addition of 100 µl of a predetermined antigen dilution in carbonate buffer, pH 9.6, to each well. Plates are incubated for 2 hours at 37°C. Alternatively, the coating can be made overnight at 4°C with 100 µl/well of the predetermined antigen dilution in PBS, pH 7.2. Plates are then washed four times to remove unbound antigen and dried by tapping firmly upside down on an absorbent paper. The coated plates can be used immediately or dried and stored at 4°C (the stability in these conditions is adequate for at least 1 month).

  ii) **Sera:** Dilute test and positive and negative control serum samples 1/200 by the addition of a minimum of 10 µl of serum to 2 ml PBST. This serum dilution is usually the optimal when using both polyclonal and monoclonal conjugates. However, dilutions of 1/50 are the optimal when using the protein G-peroxidase conjugate (16) Add 100 µl/well volumes of samples in duplicate to the microtitre plates. The plates are covered, incubated at 37°C for 1 hour, and washed three times with the PBST washing buffer.

  iii) **Conjugate:** The titrated conjugate is diluted in PBST, added (100 µl) to the wells, and the plate is covered and incubated for 1 hour at 37°C. After incubation, the plates are washed again three times with PBST.

  iv) **Substrate:** The solution of ABTS in substrate buffer is added (100 µl/well) and the plates are incubated for 15–60 minutes at room temperature with continuous shaking.
v) Reading and interpreting the results: Absorbance is read automatically in a spectrophotometer at 405–414 nm. Absorbance values may be expressed as percentages of the mean absorbance of the positive control or, preferably, transformed into ELISA units calculated either manually or by using a computer and a curve-fitting program from a standard curve constructed with the series of positive control dilution results.

The cut-off threshold should be properly established using the appropriate validation techniques (see Chapter 1.1.4 Principles of validation of diagnostic assays for infectious diseases) using an adequate collection of sera from either culture positive or Brucella free animals. The International Standard for anti-Brucella ovis Serum or the corresponding secondary or national standards should be used to check or calibrate the particular test method in question.

Comparative studies have shown that the ELISA has better sensitivity than either the AGID test or the CFT (18, 24, 28, 35, 36). Due to the existence of some ELISA-negative and AGID-positive sera, the combination of the AGID test and ELISA gives optimal sensitivity (18). However, the combination of CF test and ELISA or CF and AGID tests does not improve the sensitivity of ELISA alone (18). Moreover, the CFT has other important disadvantages such as complexity, obligatory serum inactivation, anticomplementary activity of some sera, the difficulty of performing it with haemolysed sera, and prozone phenomena. Because of its sensitivity, simplicity and easy interpretation, the AGID test is very practicable for routine diagnosis in nonspecialised laboratories.

Little is known about the existence of false positive results in B. ovis serological tests as a consequence of infections due to bacteria showing cross-reacting epitopes with B. ovis. The foot rot agent (Dichelobacter nodosus) has been described as showing cross-reactions with B. ovis (33), but the extent and practical consequences of this cross-reactivity in B. ovis diagnostic tests is unknown.

C. REQUIREMENTS FOR VACCINES AND DIAGNOSTIC BIOLOGICAIS

Vaccination is probably the most economical and practical means for medium-term control of B. ovis in areas with a high incidence of infection. For long-term control, consideration should be given to the effect of vaccination on serological testing, and B.-ovis-free accreditation programmes have to be implemented. The live B. melitensis strain Rev.1 (see Chapter 2.7.2) is probably the best available vaccine for the prophylaxis of B. ovis infection (2). A single standard dose (10⁹ colony-forming units) of Rev.1 administered subcutaneously (in a 1 ml volume) or conjunctivally (in a 25–30 µl volume), to 3–5 month-old rams confers adequate immunity against B. ovis. Conjunctival vaccination has the advantage of minimising the intense and long-lasting serological response evoked by subcutaneous vaccination, thereby improving the specificity of serological tests (2). When used in young animals, the safety of the Rev.1 vaccine is adequate and side-effects appear to be rare. However, there is limited information concerning the safety of Rev.1 vaccine when used in adult rams. Two separate studies found that the subcutaneous or conjunctival vaccination of 12 or 13 month-old rams did not produce adverse side-effects and protected rams against B. ovis (ref. 17 and J.M. Blasco pers. comm.). Therefore, in countries with extensive management and high levels of incidence, it would be advisable to vaccinate both young and healthy adult rams. In countries affected by B. ovis but free of B. melitensis, before using the B. melitensis Rev.1 vaccine account should be taken of the possible serological sequel and the conjunctival route should be preferred. The B. abortus RB51 live vaccine has not proven successful against B. ovis in sheep (12) and no alternative vaccines are currently available.

REFERENCES


4 Arcanobacterium pyogenes and Corynebacterium ovis, whose soluble extracts cross-react with B. ovis positive control sera, have been recently isolated from several lymph nodes of rams giving strong positive responses in B. ovis AGID test and I-ELISA (J.M. Blasco, unpublished results).


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**NB:** There are OIE Reference Laboratories for Ovine epididymitis (*Brucella ovis*) (see Table in Part 3 of this Terrestrial Manual or consult the OIE Web site for the most up-to-date list: www.oie.int).
CHAPTER 2.7.10.

OVINE PULMONARY ADENOCARCINOMA
(adenomatosis)

SUMMARY

Ovine pulmonary adenocarcinoma (OPA), also known as ovine pulmonary adenomatosis and jaagsiekte, is a contagious tumour of sheep and, rarely, of goats. It is a progressive respiratory disease, principally affecting adult animals. The disease occurs in many regions of the world. A beta-retrovirus (jaagsiekte sheep retrovirus: JSRV), distinct from the non-oncogenic ovine lentiviruses, has been shown to cause the disease.

**Identification of the agent:** JSRV cannot yet be propagated in vitro, therefore routine diagnostic methods, such as virus isolation, are not available for diagnosis. Diagnosis relies, at present, on clinical history and examination, as well as on the findings at necropsy and by histopathology and immunohistochemistry. Viral DNA or RNA can be detected in tumour, draining lymph nodes, and peripheral blood mononuclear cells by polymerase chain reaction. Lambs become persistently infected by JSRV at an early age, and, in an OPA-affected flock, most sheep are infected.

**Serological tests:** Antibodies to the retrovirus have not been detected in infected sheep and, therefore, serological tests are not available for diagnosis.

**Requirements for vaccines and diagnostic biologicals:** There are no vaccines or diagnostic biologicals available.

A. INTRODUCTION

Ovine pulmonary adenocarcinoma (OPA), also known as ovine pulmonary adenomatosis, jaagsiekte (Afrikaans = driving sickness) and ovine pulmonary carcinoma (OPC), is a contagious lung tumour of sheep and, more rarely, of goats. It is the most common pulmonary tumour of sheep and occurs in many countries around the world. It is absent from Australia and New Zealand and has been eradicated from Iceland.

A number of different viruses have been linked aetiologically to OPA, including a herpesvirus and lentiviruses that have been propagated from tumour tissue. However, the former does not have an aetiological role in OPA and the latter exhibit characteristics of non-oncogenic lentiviruses. It has been demonstrated clearly that OPA is caused by a beta-retrovirus that cannot yet be cultured in vitro, but the virus has been cloned and sequenced. The term jaagsiekte sheep retrovirus (JSRV) is used in referring to this virus.

B. DIAGNOSTIC TECHNIQUES

At present, diagnosis of OPA relies on clinical and pathological investigations, although polymerase chain reaction (PCR) offers hope for ante-mortem diagnosis of OPA as a flock test. In flocks in which the disease is suspected, its presence must be, at least once, confirmed by histopathological examination of affected lung tissue. For such an examination, it is imperative to take specimens from several affected sites and, if possible, from more than one animal. This is because secondary bacterial pneumonia, which might be the immediate cause of death, often masks the lesions (both macroscopic and microscopic) of the primary disease. In the absence of specific serological tests that can be used for the diagnosis of OPA in live animals, disease control relies on regular flock inspections and prompt culling of suspected cases and, in the case of ewes, their offspring.
1. **Identification of the agent**

Although ovine herpesvirus 1 (OvHV-1) had been isolated exclusively from OPA tumours, epidemiological studies and experimental infections provide no evidence for a role in the aetiology of OPA. Ovine herpesvirus 2 (OvHV-2) is the sheep-associated malignant catarrhal fever herpesvirus and has never been linked to OPA.

The association of retroviruses with OPA has been recognised for several years. Ovine lentiviruses have been isolated on a number of occasions, but these viruses have no aetiological role in OPA.

For many years, the inability to culture JSRV and the lack of antibodies to the virus in affected sheep impeded the confirmation of this virus as the aetiologic agent. However, molecular biological techniques provided a key advance, namely, the cloning and sequencing of the 7.5 kb JSRV genome following purification of virions from lung washes of naturally affected sheep (23). JSRV has been designated as a beta-retrovirus because of its genetic organisation and its structural proteins. Although cloned JSRV genes, used as hybridisation probes, have revealed a range of homologous endogenous sequences in the genome of both healthy and OPA-affected sheep (1, 9, 23), JSRV is clearly exogenous and associated exclusively with OPA (13). JSRV is detected constantly in the lung fluid, tumour, peripheral blood mononuclear cells, and lymphoid tissues of sheep affected by OPA or unaffected in-contact flockmates, and never in sheep from unaffected flocks with no history of the tumour. Full-length proviral clones of JSRV have been obtained from OPA tumour DNA and cells. JSRV virus particles, prepared from these clones by transient transfection of a cell line, were used for intratracheal inoculation of neonatal lambs. OPA tumour was induced in the lambs, thus demonstrating that JSRV is the causal agent of OPA (6, 15).

The sheep genome contains many copies of endogenous viral sequences that are highly related to JSRV. Although they are not involved in the aetiology of OPA, their expression in the fetus may, by induction of tolerance, account for the apparent lack of immune response of mature animals to exogenous JSRV (14).

**a) Nucleic acid recognition methods**

Sequencing of JSRV and endogenous sequences in the sheep genome has led to the development of PCRs that specifically detect JSRV (1, 13). Using this sensitive procedure, JSRV has been detected in peripheral blood mononuclear cells of unaffected in-contact sheep from flocks with OPA, as well as experimentally infected lambs (5, 7, 10) and the bronchoalveolar lavage samples from unaffected in-contact sheep (22). Longitudinal studies in OPA-affected flocks have shown that lambs become infected at a very early age. A high proportion of animals in these flocks are infected, yet only a minority develop OPA (2, 16). JSRV has been found in colostrum and milk obtained from sheep in OPA-affected flocks and JSRV can be detected within a few months in the blood of lambs fed artificially with colostrum and milk (De las Heras et al., unpublished observations).

**Control and treatment**

**b) Animal inoculation**

OPA cannot be transmitted to any laboratory animal and can be transmitted to sheep only with material that contains JSRV, such as tumour homogenates, concentrated cell-free lung fluid from natural cases of OPA and virus produced from molecular clones. Following the experimental inoculation of adult sheep, clinical disease develops only after several months or years. In contrast, JSRV infection can be induced in 100% of lambs aged 1–6 months at the time of inoculation and a high proportion of these animals develop clinical signs (62–90%) and lesions (87–100%) of OPA (17).

At this time there is no practical animal inoculation method for the diagnosis of OPA.

**c) Virus isolation**

There are no permissive cell culture systems for propagation of JSRV. Some cell cultures prepared from the tumours occurring in young lambs can support virus replication for a short period (11, 18).

**d) Clinical signs and pathology**

There is no reliable laboratory method for the ante-mortem diagnosis of OPA in individual animals at this time, therefore flock history, clinical signs and post-mortem lesions are the primary method for the diagnosis of the disease. As OPA has a long incubation period, clinical disease is encountered most commonly in sheep over 2 years of age, with a peak occurrence at the age of 3–4 years. In exceptional cases, the disease occurs in animals as young as 2–3 months of age. The cardinal signs are those of a progressive respiratory embarrassment, particularly after exercise; the severity of the signs reflects the extent of tumour development in the lungs. Accumulation of fluid within the respiratory tract is a prominent feature of OPA, giving rise to moist râles that are readily detected by auscultation. Raising the hindquarters and lowering the
head of affected sheep may cause frothy mucoid fluid to run from the nostrils. Coughing and inappetance are not common but, once clinical signs are evident, weight loss is progressive and the disease is terminal within weeks or months. Death is often precipitated by a superimposed bacterial pneumonia, particularly due to Mannheimia (formerly Pasteurella) haemolytica. In clinically affected animals, a peripheral lymphopenia characterised by a reduction in CD4+ T lymphocytes and a corresponding neutrophilia may assist clinical diagnosis, but the changes are not pathognomonic and are not detected during early experimental infection (20).

In some countries, another form of OPA (atypical OPA) occurs, which generally presents as an incidental finding at necropsy or the abattoir (4).

e) Necropsy

OPA lesions are in most cases confined to the lungs, although intra- and extrathoracic metastasis to lymph nodes and other tissues can occur. In typical cases, affected lungs are considerably enlarged and heavier than normal due to extensive nodular and coalescing firm grey lesions affecting much of the pulmonary tissue. Usually lesions are present in both lungs, although the extent on either side does vary. Tumours are solid, grey or light purple with a shiny translucent sheen and often separated from the adjacent normal lung by a narrow emphysematous zone. The presence of frothy white fluid in the respiratory passages is a prominent feature and is obvious even in lesions as small as a few millimetres. In advanced cases, this fluid flows out of the trachea when it is cut or pendant. Samples should be taken at necropsy for histopathology, immunohistochemistry or PCR for JSRV.

Pleurisy may be evident over the surface of the tumour and often abscesses are present in the adenomatous tissue.

In atypical OPA, tumours comprise solitary or aggregated hard white nodules that have a dry cut surface and show clear demarcation from surrounding tissues. The presence of excess fluid is not a prominent feature.

Adult sheep, which on post-mortem examination appear to have died from acute pasteurellosis, should have their lungs examined carefully, as lesions of OPA may be masked by coexisting bronchopneumonia, verminous pneumonia, chronic progressive pneumonia (maedi-visna) or combinations of these. Samples should be taken at necropsy for histopathology.

f) Histopathology

Histologically, the lesions are characterised by proliferation of mainly type II pneumocytes, a secretory epithelial cell in the pulmonary alveoli. Nonciliated (Clara) and epithelial cells of the terminal bronchioli may be involved. The cuboidal or columnar tumour cells replace the normal thin alveolar cells and sometimes form papilliform growths that project into the alveoli. Intrabronchiolar proliferation may be present. In advanced cases, extensive fibrosis may develop and, occasionally, nodules of loose connective tissue in a mucopolysaccharide substance may be present.

A prominent feature is the accumulation of large numbers of alveolar macrophages in the alveoli adjacent to the neoplastic lesions (21).

Where maedi-visna is concurrent, perivascular, peribronchial and interstitial lymphoid infiltrates may be prominent.

The histological appearance of atypical OPA is essentially the same as classical OPA, but with an exaggerated inflammatory response (mostly lymphocytes and plasma cells) and fibrosis (4).

For more detailed accounts of the clinical, post-mortem and histopathological aspects of OPA, the reader is referred elsewhere (4, 19).

There appears to be a synergistic interaction between OPA and maedi-visna. Lateral transmission of maedi-visna virus appears to be enhanced in sheep affected by OPA (3, 8).

2. Serological tests

At present, there are no laboratory tests to support a clinical diagnosis of OPA in the live animal. JSRV has been associated exclusively with both typical and atypical forms of OPA, but antibodies to this virus have not been detected in the sera of affected sheep, even with highly sensitive assays such as immunoblotting or enzyme-linked immunosorbent assay (12, 20).
C. REQUIREMENTS FOR VACCINES AND DIAGNOSTIC BIOLOGICALS

There are no vaccines or diagnostic biologicals available at the present time.

REFERENCES


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PESTE DES PETITS RUMINANTS

CHAPTER 2.7.11.

SUMMARY

Peste des petits ruminants (PPR), is an acute contagious disease caused by a Morbillivirus in the family Paramyxoviridae. It affects mainly sheep and goats and occasionally small ruminants living in the wild. PPR occurs in Africa in countries lying between the Equator and the Sahara, in the Arabian Peninsula, throughout most of the Near East and Middle East, and in south-west Asia.

The clinical disease resembles rinderpest in cattle. It is usually acute and characterised by serous ocular and nasal discharges. PPR is characterised by severe pyrexia, erosive lesions on different mucous membranes and particularly in the mouth, diarrhoea and pneumonia. At necropsy, characteristic zebra markings may occur in the large intestine, but are not a consistent finding. Lesions also occur in the lungs showing congestion or bronchopneumonia when associated with bacterial infection.

The disease must be differentiated from rinderpest, bluetongue, foot and mouth disease and other exanthemous conditions.

Identification of the agent: The collection of specimens at the correct time is important to achieve diagnosis by virus isolation and they should be obtained in the acute phase of the disease when clinical signs are still apparent. The specimens can be swabs of conjunctival discharges, nasal secretions, buccal and rectal mucosae, and unclotted blood.

Rapid diagnosis is done by immunocapture enzyme-linked immunosorbent assay (ELISA), counter immunoelectrophoresis and agar gel immunodiffusion. Polymerase chain reaction may also be used.

Serological tests: The serological tests that are routinely used are the virus neutralisation and the competitive ELISA.

Requirements for vaccines and diagnostic biologicals: In the past, control of PPR was ensured through vaccination with the rinderpest tissue culture vaccine because of the existence of a strong antigenic relationship between PPR and rinderpest viruses. The use of this heterologous vaccine has been abandoned in favour of the live attenuated PPR virus vaccine, which is now widely commercially available.

A. INTRODUCTION

Peste des petits ruminants (PPR) is an acute viral disease of small ruminants characterised by fever, ocular-nasal discharges, stomatitis, diarrhoea and pneumonia with foul offensive breath. Infected animals present clinical signs similar to those of rinderpest in cattle, from which it must be differentiated. Because of the respiratory signs, PPR can be confused with contagious caprine pleuropneumonia (CCPP) or pasteurellosis. In many cases, pasteurellosis is a secondary infection of PPR, a consequence of the immunodepression that is induced by the causal agent of PPR, the PPR virus (PPRV). PPRV is transmitted mainly by aerosols between animals living in close contact (20).

On the basis of its similarities to the viruses of rinderpest, canine distemper and measles, the PPRV has been classified within the genus Morbillivirus in the family Paramyxoviridae (16). Virus members of this group have six structural proteins: the nucleocapsid protein (Np), which encapsulates the virus genomic RNA, the phosphoprotein (P), which associates with the polymerase (L for large protein) protein, the matrix (M) protein, the fusion (F) protein and the haemagglutinin (H) protein. The matrix protein, intimately associated with the internal face of the viral envelope, makes a link between the nucleocapsid and the virus external glycoproteins: H and F, which are responsible, respectively, for the attachment and the penetration of the virus into the cell to be
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infected. PPR was first described in Côte d’Ivoire (14), but it occurs in most African countries south of the Sahara and north of the equator (20), and in nearly all Middle Eastern countries up to Turkey (13, 21, 26, 27, 37). PPR is also wide-spread in India and south-west Asia (34).

The natural disease affects mainly goats and sheep, but it is usually more severe in goats where it causes heavy losses and is only occasionally severe in sheep. It is generally admitted that cattle can only be infected subclinically. However, in poor conditions it might be possible that cattle develop lesions following PPRV infection, clinical signs of which would be ascribed to rinderpest. Indeed, in the 1950s, disease and death were recorded in calves experimentally infected with PPRV-infected tissue (25). Moreover, PPRV was isolated from an outbreak of rinderpest-like disease in buffaloes in India in 1995 (17). It was also suspected to be involved in the epizootic disease that affected one-humped camels in Ethiopia in 1995–1996 (28, 29). Indeed PPRV antigen and PPRV nucleic acid were detected in some pathological samples collected during that outbreak, but no live virus was isolated. Cases of clinical disease have been reported in wildlife resulting in deaths of gazelles in captivity (11, 13). The American white-tailed deer (Odocoileus virginianus) can be infected experimentally (18).

The incubation period is 4–6 days, but may range between 3 and 10 days. The clinical disease is acute, with a pyrexia up to 41°C that can last for 3–5 days; the animals become depressed, anorexic and develop a dry muzzle. The serous oculonasal discharges become progressively mucopurulent and, if death does not ensue, persist for around 14 days. Within 4 days of the onset of fever, the gums become hyperaemic, and erosive lesions develop in the oral cavity with excessive salivation. These lesions may become necrotic. A watery blood-stained diarrhoea is common in the later stage. Pneumonia, coughing, pleural rales and abdominal breathing also occur. The morbidity rate can be up to 100% with a mortality rate of up to 100% in severe cases. However, this may not exceed 50% during milder outbreaks. A tentative diagnosis of PPR is made on these clinical signs, but laboratory confirmation is required for differential diagnosis with other diseases with similar signs.

At necropsy, the lesions are very similar to those observed in cattle affected with rinderpest, except prominent crusty scabs along the outer lips and severe interstitial pneumonia frequently occur with PPR. Erosive lesions may extend from the mouth to the reticulo–rumen junction. Characteristic linear haemorrhages or zebra stripes occur in the large intestine, commonly at the caeco–colic junction, but they are not a consistent finding; necrotic or haemorrhagic enteritis is usually present. Lymph nodes are enlarged, the spleen may show necrotic lesions, and there is an apical pneumonia.

There are no known health risks to humans working with PPRV as no report of human infection with the virus exists.

B. DIAGNOSTIC TECHNIQUES

1. Identification of the agent
   a) Collection of samples
      In live animals, swabs are made of the conjunctival discharges and from the nasal and buccal mucosae. During the very early stage of the disease, whole blood is also collected in anticoagulant for virus isolation, polymerase chain reaction (PCR) and haematology. At necropsy (two to three animals), lymph nodes, especially the mesenteric and bronchial nodes, lungs, spleen and intestinal mucosae should also be collected aseptically, chilled on ice and transported under refrigeration. Fragments of organs collected for histopathology are placed in 10% formalin. At the end of the outbreak, blood can be collected for serological diagnosis.

   b) Agar gel immunodiffusion
      Agar gel immunodiffusion (AGID) is a very simple and inexpensive test that can be performed in any laboratory and even in the field. Standard PPR viral antigen is prepared from mesenteric or bronchial lymph nodes, spleen or lung material and ground up as 1/3 suspensions in buffered saline. These are centrifuged at 500 g for 10–20 minutes, and the supernatant fluids are stored in aliquots at –20°C. The cotton material from the cotton bud used to collect eye or nasal swabs is removed using a scalpel and inserted into a 1 ml syringe. With 0.2 ml of phosphate buffered saline (PBS), the sample is extracted by repeatedly expelling and filling the 0.2 ml of PBS into an Eppendorf tube using the syringe plunger. The resulting eye/nasal swab extracted sample, like the tissue ground material prepared above, may be stored at –20°C until used. They may be retained for 1–3 years. Negative control antigen is prepared similarly from normal tissues. Standard antisera is made by hyperimmunising sheep with 1 ml of PPRV with a titre of 10^6 TCID50 (50% tissue culture infective dose) per ml given at weekly intervals for 4 weeks. The animals are bled 5–7 days after the last injection (9). Standard rinderpest rabbit hyperimmune antisera is also effective in detecting PPR antigen.
i) Dispense 1% agar in normal saline, containing thiomersal (0.4 g/litre) or sodium azide (1.25 g/litre) as a bacteriostatic agent, into Petri dishes (6 ml/5 cm dish).

ii) Wells are punched in the agar following a hexagonal pattern with a central well. The wells are 5 mm in diameter and 5 mm apart.

iii) The central well is filled with positive antiserum, three peripheral wells with positive antigen, and one well with negative antigen. The two remaining peripheral wells are filled with test antigen, such that the test and negative control antigens alternate with the positive control antigens.

iv) Usually, 1–3 precipitin lines will develop between the serum and antigens within 18–24 hours (10). These are intensified by washing the agar with 5% glacial acetic acid for 5 minutes (this procedure should be carried out with all apparently negative tests before recording a negative result). Positive reactions show lines of identity with the positive control antigen.

Results are obtained in one day, but the test is not sensitive enough to detect mild forms of PPR due to the low quantity of viral antigen that is excreted.

c) Counter immunoelectrophoresis

Counter immunoelectrophoresis (CIEP) is the most rapid test for viral antigen detection (24). It is carried out on a horizontal surface using a suitable electrophoresis bath, which consists of two compartments connected through a bridge. The apparatus is connected to a high-voltage source. Agar or agarose (1–2%, [w/v]) dissolved in 0.025 M barbitone acetate buffer is dispensed on to microscope slides in 3-ml volumes. From six to nine pairs of wells are punched in the solidified agar. The reagents are the same as those used for the AGID test. The electrophoresis bath is filled with 0.1 M barbitone acetate buffer. The pairs of wells in the agar are filled with the reactants: sera in the anodal wells and antigen in the cathodal wells. The slide is placed on the connecting bridge and the ends are connected to the buffer in the troughs by wetted porous paper. The apparatus is covered, and a current of 10–12 milliamperes per slide is applied for 30–60 minutes. The current is switched off and the slides are viewed by intense light: the presence of 1–3 precipitation lines between pairs of wells is a positive reaction. There should be no reactions between wells containing the negative controls.

d) Immunocapture enzyme-linked immunosorbent assay

The immunocapture enzyme-linked immunosorbent assay (ELISA) (22) using three monoclonal antibodies (MAb) anti-N protein, allows a rapid differential identification of PPR or rinderpest viruses, and this is of great importance as the two diseases had until recently a similar geographical distribution and may affect the same animal species.

i) Microtitre ELISA plates (e.g. high adsorption capacity Nunc Maxisorb) are coated with 100 µl of a capture MAb solution (diluted according to the instructions of the Reference Laboratory providing the kit). This MAb reacts with both rinderpest virus and PPRV.

ii) After washing, 50 µl of the sample suspension is added to four wells, and control wells are filled with buffer.

iii) Immediately, 25 µl of a detection biotinylated MAb for PPR and 25 µl of streptavidin/peroxidase are added to two wells, and 25 µl of a detection MAb for rinderpest and 25 µl of streptavidin/peroxidase are added to the two other wells.

iv) The plates are incubated at 37°C for 1 hour with constant agitation.

v) After three vigorous washes, 100 µl of ortho-phenylenediamine (OPD) in hydrogen peroxide is added, and the plates are incubated for 10 more minutes at room temperature.

vi) The reaction is stopped by the addition of 100 µl of 1 N sulphuric acid, and the absorbance is measured at 492 nm on a spectrophotometer/ELISA reader.

The cut-off above which samples are considered to be positive is calculated from each blank (PPR blank and rinderpest blank) as three times the mean absorbance values.

A sandwich ELISA can also be performed: the sample is first allowed to react with the detection MAb and the immunocomplex is then captured by the second MAb adsorbed on to the ELISA plate.

The test is very specific and sensitive (it can detect $10^{0.6} \text{TCID}_{50}$/well for the PPRV and $10^{2.2} \text{TCID}_{50}$ for the rinderpest virus). The results are obtained in 2 hours.

Another immunocapture test, based on the use of a single MAb anti-H, has been described (31).
e) **Nucleic acid recognition methods**

cDNA 32P labelled clones have been used to differentiate PPR and rinderpest (6), but their use in routine diagnosis is not recommended due to the short half-life of the 32P and the need for special equipment to protect the users.

A reverse transcription PCR (RT-PCR) technique based on the amplification of the Np and F protein genes has been developed for the specific diagnosis of PPR (5, 12, 33). This technique is very sensitive compared with other tests and results are obtained in 5 hours, including the RNA extraction. The OIE and FAO1 Reference Laboratory for PPR in France (see Table given in Part 3 of this Terrestrial Manual) can advise on the use of this technique. A multiplex RT-PCR, based on the amplification of fragments of N and M protein genes, has been reported (15). Another format of the N gene-based RT-PCR has also been described (19). Instead of analysing the amplified product – the amplicon – by agarose gel electrophoresis, it is detected on a plate by ELISA through the use of a labelled probe. This new format, RT-PCR-ELISA, is ten times for sensitive that the classical RT-PCR.

f) **Culture and isolation methods**

Even when diagnosis has been carried out by rapid techniques, the virus should always be isolated from field samples in tissue cultures for further studies (10, 20).

PPRV may be isolated in primary lamb kidney or in African green monkey kidney (Vero) cell tissue cultures. Monolayer cultures are inoculated with suspect material (swab material, buffy coat or 10% tissue suspensions) and examined daily for evidence of cytopathic effect (CPE). The CPE produced by PPRV can develop within 5 days and consists of cell rounding and aggregation culminating in syncytia formation in lamb kidney cells. In Vero cells, it is sometimes difficult to see the syncytia. If they exist, they are very small. However, in stained, infected Vero cells, small syncytia are always seen. Syncytia are recognised by a circular arrangement of nuclei giving a ‘clock face’ appearance. Cover-slip cultures may give a CPE earlier than day 5. There are also intracytoplasmic and intranuclear inclusions. Some cells are vacuolated. Similar cellular changes may be seen in stained histopathological sections of infected tissues. After 5–6 days, blind passages should always be carried out as CPE may take time to appear.

g) **Other virus detection techniques**

Other virus detection techniques have potential benefits, but they are not yet widely used. While virus isolation needs pathological samples to be kept in cold conditions until the start of their processing, it is possible to keep them at ambient temperature in a formalin-fixed solution and later analyse them directly by immunofluorescence (IF) or immunochromical test (3, 4, 35). IF has been used successfully on conjunctival smears and tissues collected at necropsy; the smears are fixed in cold acetone. It has now been demonstrated that unlike the rinderpest virus but like the measles virus, PPRV has haemagglutination capability. This characteristic has been used for specific, rapid and inexpensive diagnosis of PPR infection (17, 39).

2. **Serological tests**

Goats and sheep infected with PPRV develop antibodies that may be demonstrated to support a diagnosis by the antigen-detection tests. Tests that are routinely used are the virus neutralisation (VN) test and the competitive ELISA.

a) **Virus neutralisation (the prescribed test for international trade)**

This test is sensitive and specific, but it is time-consuming. The standard neutralisation test is carried out in roller-tube cultures of primary lamb kidney cells, or Vero cells when primary cells are not available.

i) Dilute 1 ml of inactivated serum in a twofold dilution series and mix with a stock virus suspension containing approximately 10^3 TCID_{50}/ml.

ii) Incubate the virus/serum mixtures either for 1 hour at 37°C or overnight at 4°C.

iii) Inoculate 0.2 ml of the mixture into each of five roller tubes, followed immediately by 1 ml of Vero cell suspension in growth medium at a rate of 2 × 10^5 cells/ml.

iv) Incubate the sloped tubes for 3 days at 37°C.

v) Discard the tubes showing virus-specific CPE; replace the medium in the remaining tubes with maintenance medium, and roll the tubes for a further 7 days. The virus-challenge dose is acceptable if

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1 Food and Agriculture Organization of the United Nations.
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it falls between $10^{1.8}$ and $10^{2.8}$ TCID_{50}/tube. Any detectable antibody at a dilution of 1/8 is considered
to be positive.

Usually, a cross-neutralisation test is carried out with rinderpest virus and a serum is considered to be
positive for PPR when the neutralisation titre is at least twofold higher for PPR than for rinderpest.

Instead of using the roller tubes, the VN test can also be performed in 96-well microtitre plates (30).

b) Competitive enzyme-linked immunosorbent assay

Competitive ELISA based on the use of MAb anti-nucleoprotein and a recombinant nucleoprotein produced
in the baculovirus has been described (23).

i) Coat microtitre plates (e.g. high adsorption capacity Nunc Maxisorb) with 50 µl of a predetermined
dilution of N-PPR protein (produced by a recombinant baculovirus) for 1 hour at 37°C with constant
agitation.

ii) Wash the plates three times and blot dry.

iii) Distribute 45 µl of blocking buffer (PBS + 0.5% Tween 20 + 0.5 fetal calf serum) to all wells, and then
add 5 µl of test sera to test wells (at a final dilution of 1/20) and 5 µl of the different control sera (strong
positive, weak positive and negative serum) to control wells.

iv) Add 50 µl of MAb diluted 1/100 in blocking buffer, and incubate at 37°C for 1 hour.

v) Wash the plates three times and blot dry.

vi) Add 50 µl of anti-mouse conjugate diluted 1/1000, and incubate at 37°C for 1 hour.

vii) Wash the plates three times.

viii) Prepare OPD in hydrogen peroxide solution. Add 50 µl of substrate/conjugate mixture to each well.
Stop the reaction after 10 minutes with 50 µl of 1 M sulphuric acid.

ix) Read on an ELISA reader at 492 nm.

The absorbance is converted to percentage inhibition (PI) using the formula:

$$ PI = 100 – \left( \frac{\text{absorbance of the test wells}}{\text{absorbance of the MAb control wells}} \right) \times 100 $$

Sera showing PI greater than 50% are positive.

Two other competitive ELISA techniques, based on the use of monoclonal anti-haemagglutinin (H), have
also been described (1, 32).

C. REQUIREMENTS FOR VACCINES AND DIAGNOSTIC BIOLOGICALS

Sheep and goats that recover from PPR develop an active immunity against the disease. As antibodies have
been demonstrated 4 years after infection, this immunity is probably life-long (9). A homologous PPR vaccine is
available. In 1998, the OIE International Committee endorsed the use of this vaccine in countries that have
decided to follow the ‘OIE pathway’ for epidemiological surveillance for rinderpest in order to avoid confusion
when serological surveys are performed. There have been two published reports on the preliminary results of the
development of recombinant capripox-based PPR vaccine able to protect against both capripox and PPR (2, 7).
The production of the commercially available attenuated PPRV vaccine is described here.

1. Seed management

a) Characteristics of the seed

The PPRV vaccine Nigeria 75/1 strain is a live vaccine cultured in Vero cells. The original strain of the virus
was isolated in Nigeria in 1975 (36). It has been attenuated by serial passages in Vero cell cultures (8). The
strain provided for vaccine production is the 70th passage in Vero cells (PPRV 75/1 I.K6 BK2 Vero 70). It is
stored in freeze-dried form at –20°C and may be obtained from Reference Laboratories (see Table given in
Part 3 of this Terrestrial Manual). Tests of vaccine activity show that it retained the ability to protect (at a
dose of $10^5$ TCID_{50}) up to the 120th passage in Vero cells, the latest passage tested so far.

b) Method of culture

- Cells

PPR vaccine is produced in Vero cells, which must be free from all bacterial, fungal and viral contamination.
• Culture medium

The culture medium consists of minimal essential medium (MEM) supplemented with antibiotics (for example penicillin + streptomycin at final concentrations of 100 IU [International Units]/ml and 100 µg/ml, respectively), and an antifungal agent (nystatin [Mycostatin] at a final concentration of 50 µg/ml). The medium is enriched with 10% fetal calf serum (complete medium) for cell growth. This proportion of serum is reduced to 2% for maintenance medium when the cell monolayer is complete.

• Primary seed batch of vaccine virus

This consists of virus in its 70th passage in Vero cells (PPRV 75/1 LK6 BK2 Vero 70). The freeze-dried contents of a flask from the seed bank are reconstituted with 2 ml of sterile water (or cell culture medium without serum). This liquid is mixed with Vero cells suspended in complete culture medium to provide at least 0.001 TCID₅₀ per cell. Cell culture dishes are filled with this virus/cell mixture (around 2 × 10⁷ Vero cells in a 175 cm² dish), and are incubated at 37°C. The cultured cells are examined regularly to detect a CPE. The medium is renewed every 2 days, reducing the proportion of serum to 2% once the cell monolayer is complete. Virus is first harvested when there is 40–50% CPE. This viral suspension is stored at −70°C. Successive harvesting is made every 2 days until the CPE reaches 70–80%, which is the time for final freezing of the culture dishes (in general, at least two further harvestings can be made before final freezing of the culture dishes). All suspensions of virus collected are submitted to two freeze–thaw cycles, then added to form a single batch, which serves as the primary seed batch. This is divided into small volumes in bottles and stored at −70°C. All suspensions of virus collected are submitted to two freeze–thaw cycles, then added to form a single batch, which serves as the primary seed batch. This is divided into small volumes in bottles and stored at −70°C. All suspensions of virus collected are submitted to two freeze–thaw cycles, then added to form a single batch, which serves as the primary seed batch. This is divided into small volumes in bottles and stored at −70°C.

• Preparation of the working seed batch

This is done under the same conditions as for the primary seed batch. A large stock of virus is formed, from which the final vaccine will be produced. This batch is distributed into receptacles and stored at −70°C. It must satisfy tests for sterility. Five samples are titrated (minimum titre required: 10⁶ TCID₅₀/ml).

c) Validation as a vaccine

It is necessary to confirm or rule out the presence of PPRV in the product under test. For this purpose, anti-PPR serum is used to neutralise the virus in cell culture.

• Test procedure

i) Mix the contents of two vaccine bottles with sterile double-distilled water to provide a volume equal to the volume before freeze-drying.

ii) Make tenfold dilutions of the reconstituted vaccine in serum-free culture medium (0.5 ml of viral suspension + 4.5 ml of medium).

iii) Make two series of mixtures for virus dilutions from each bottle on a 96-well plate as follows:

<table>
<thead>
<tr>
<th>Series 1:</th>
<th>Dilutions of viral suspension:</th>
<th>–1</th>
<th>–2</th>
<th>–3</th>
<th>–4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Viral suspension (in µl)</td>
<td>50</td>
<td>50</td>
<td>50</td>
<td>50</td>
<td></td>
</tr>
<tr>
<td>Culture medium (in µl)</td>
<td>50</td>
<td>50</td>
<td>50</td>
<td>50</td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>Series 2:</th>
<th>Dilutions of viral suspension:</th>
<th>–1</th>
<th>–2</th>
<th>–3</th>
<th>–4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Viral suspension (in µl)</td>
<td>50</td>
<td>50</td>
<td>50</td>
<td>50</td>
<td></td>
</tr>
<tr>
<td>PPR antiserum (in µl)</td>
<td>50</td>
<td>50</td>
<td>50</td>
<td>50</td>
<td></td>
</tr>
</tbody>
</table>

(Note: PPR antiserum used for this purpose is prepared in goats and freeze-dried. It is reconstituted with 1 ml of sterile double-distilled water in a dilution of 1/10.)

iv) Incubate the mixtures at 37°C for 1 hour

v) Add to each well 100 µl of cells suspended in complete culture medium (30,000 cells/well).

vi) Incubate the microplate at 37°C in the presence of CO₂.

vii) Read the plate after 10–15 hours of incubation.

Normally a CPE is present only in the wells containing cells infected with the mixture of virus and culture medium. If it is detected in the wells of Series 2, it will be necessary to identify PPRV by immunofluorescence, using a PPR MAb, or by immunocapture (specific PPR MAb, and the immunocapture
test kit are available from the OIE Reference Laboratory for PPR in France [see Table given in Part 3 of this Terrestrial Manual]. If this identification confirms the presence of PPRV, the PPR antiserum used must have been too weak, or the batch must be changed. If immunofluorescence or immunocapture is negative, a viral contaminant must be present, and the material under test must be destroyed.

2. Method of manufacture

a) Vaccine production

This operation is performed on a larger scale. Cells can be infected with virus at a multiplicity of infection as before or with high doses, e.g. up to 0.01. Products of the various harvests, after two freeze–thaw cycles, are brought together (to form the final product) and stored at −70°C pending the results of titration and tests for sterility. If these results are satisfactory, the vaccine is freeze-dried.

b) Freeze-drying

The freeze-drying medium (Weybridge medium) is composed of 2.5% (w/v) lactalbumin, 5% (w/v) sucrose and 1% (w/v) sodium glutamate, pH 7.2.

This medium is added to an equal volume of viral suspension for freeze-drying (which may have been diluted beforehand to provide the desired number of vaccine doses per bottle). The resulting mixture is kept cool, homogenised, then distributed into bottles and freeze-dried. At the end of a freeze-drying cycle, the probe is adjusted and kept at 35°C for 4 hours. Once this operation has been completed, the bottles are capped under vacuum. Randomly selected samples (e.g. 5%) of this final batch are submitted to tests for innocuousness, efficacy and sterility, and residual moisture is estimated by the Karl Fisher method (optimum ≤3.5%). If the tests give unsatisfactory results, the entire batch is destroyed.

3. In-process control

Cells used in cultures must be checked for normal appearance and shown to be free from contaminating viruses, especially bovine viral diarrhoea virus. A virus titration must be undertaken on the seed lot: using MEM (serum-free) medium, a series of tenfold dilutions is made (0.5 ml virus + 4.5 ml diluent) down to 10⁻⁶ of the product to be titrated. Vero cells from one flask are trypsinised and suspended in complete culture medium at 300,000/ml. They are distributed on a 96-well plate (30,000 cells per well, equivalent to 100 µl of cell suspension). Then, 100 µl of virus diluted tenfold is added to the cells (dilutions ranging from 10⁻² to 10⁻⁶). One row of wells serves as a control for uninfected cells to which virus-free culture medium (100 µl) is added. The plate is incubated at 37°C in the presence of CO₂. The plates are read (by examining for CPE) 10–15 days after infection.

Virus titre is determined by the Spearman–Kärber method. The minimum titre per dose is 10²⁻⁵.

4. Batch control

a) Identity

The contents of one container from each filling lot must be checked for identity by culture after neutralisation with specific antiserum.

b) Sterility

This consists of testing for viral, bacterial or fungal contaminants. It is done on cells and sera before their use in vaccine production, and on the seed stock and the vaccine before and after freeze-drying. Any product that fails this test for sterility is destroyed.

Tests for sterility and freedom from contamination of biological materials may be found in Chapter 1.1.9.

c) Safety

This test is done in rodents in order to detect any nonspecific toxicity associated with the product. The test requires reconstituted vaccine in solvent (mixed contents of five bottles), six guinea-pigs, each weighing 200–250 g; ten unweaned mice (17–22 g, Swiss line or similar).

Vaccine, 0.5 ml, is injected intramuscularly into a hind limb of two guinea-pigs, 0.5 ml into the peritoneal cavity of two guinea-pigs, and 0.1 ml into the peritoneal cavity of six mice. Two guinea-pigs and four mice are kept as un inoculated controls. The animals are observed for 3 weeks. If one guinea-pig or two mice die, the test must be repeated. Dead animals undergo post-mortem examination to ascertain the cause of death. At the end of 3 weeks of observation, all animals are killed for post-mortem examination. All the
results are recorded. The vaccine is considered to be satisfactory if, during the first or second test, at least
80% of animals remain in good health during the period of observation, and no significant post-mortem
lesion is found.

d) Potency and efficacy in small ruminants

This test requires the following: vaccine reconstituted with normal saline (the mixed contents of five bottles)
to provide 100 doses and 0.1 dose/ml; six goats and six sheep, all approximately 1-year old and free from
antibodies to rinderpest or PPR; sterile syringes and needles; and pathogenic PPRV previously titrated in
goats, diluted with sterile normal saline to provide 10^3 of the 50% lethal dose for goats (LD_{50}).

Vaccinate two goats and two sheep subcutaneously with 100 doses per animal; vaccinate two goats and
two sheep subcutaneously with 0.1 dose per animal; keep the remaining animals as in-contact controls. The
animals are observed and temperature measurements are recorded daily for 3 weeks. At the end of this
period, blood is taken from all animals for the preparation of sera. All animals are challenged by
subcutaneous injection of a 1 ml suspension of pathogenic PPRV (10^3 LD_{50} per animal). The animals are
observed and their body temperature measurements are recorded daily for 2 weeks.

The vaccine is considered to be satisfactory if all vaccinated animals resist the challenge infection, while at
least half of the in-contact controls develop signs of PPR. The serum neutralisation test must be positive for
PPR antibody (in serum diluted at least 1/10) in vaccinated animals only in samples taken 3 weeks after
vaccination. If any of the controls are also positive, the experiment must be repeated using another batch of
pathogenic PPRV. The batch of vaccine is destroyed if vaccinated animals react to the virulent challenge.

• Titration of neutralising PPR antibody

This test requires the following: cell suspensions at 600,000/ml; 96-well cell culture plates; sera to be
titrated (inactivated by heating to 56°C for 30 minutes); complete cell culture medium; PPRV diluted to give
1000, 100, 10 and 1 TCID_{50}/ml.

Dilute the sera at 1/5, then make a twofold dilution in cell culture medium. Mix 100 µl of virus at
1000 TCID_{50}/ml (to give 100 TCID_{50} in each well) and 100 µl of a given dilution of serum (using six wells per
dilution) in the wells of the cell culture plate. Arrange a series of control wells for virus and uninfected cells
as follows: six wells with 100 TCID_{50} (100 µl) per well; six wells with 10 TCID_{50} (100 µl) per well; six wells
with 1 TCID_{50} (100 µl) per well; six wells with 0.1 TCID_{50} (100 µl) per well; and six wells with 200 µl of virus-
free culture (control cells) per well.

Make the wells containing the virus controls up to 100 µl with complete culture medium, and incubate the
plates for 1 hour at 37°C. Add 50 µl of cell suspension to each well. Incubate the plates at 37°C in the
presence of CO_2. Read the plates after 1 and 2 weeks of incubation. The results should be as follows: 100% CPE in
virus control wells of 100 and 10 TCID_{50}; 50% CPE for the 1 TCID_{50} dilution, no CPE for the
0.1 TCID_{50} dilution, no CPE in wells where the virus had been neutralised by serum during the test, and
CPE in wells where the virus had not been neutralised by serum during the test.

e) Duration of immunity

Duration of immunity is at least 3 years.

f) Stability

Freeze-dried vaccine can be kept for at least 2 years at 2–8°C (although storage at –20°C is better),
provided it is stored under vacuum and protected from light. Recently, it has been demonstrated that this
vaccine, suspended in medium containing trehalose and submitted to the ultra rapid method of dehydration,
can resist at 45°C for a period of 14 days with minimal loss of potency (38).

5. Tests on the final product

a) Safety

See Section C.4.c.

b) Potency

See Section C.4.d.
REFERENCES


* * *

**NB:** There are OIE Reference Laboratories for Peste des petits ruminants (see Table in Part 3 of this *Terrestrial Manual* or consult the OIE Web site for the most up-to-date list: www.oie.int).
CHAPTER 2.7.12.

SALMONELLOSIS  
(S. abortusovis)

See Chapter 2.9.9, Salmonellosis
CHAPTER 2.7.13.

SCRAPIE

SUMMARY

Scrapie is a naturally occurring infectious neurodegenerative disease of sheep and goats characterised by vacuolar or spongy changes in the central nervous system (CNS). It has been recognised as a clinical disorder for over two and a half centuries. It was defined much later as a transmissible spongiform encephalopathy (TSE) and a prion disease. TSEs, or prion diseases are defined by the accumulation of an abnormal form of a host membrane glycoprotein (prion protein or PrP), in certain tissues, notably the CNS and lymphoreticular organs. This abnormal isomorph is referred to as PrPSc, which, according to the prion hypothesis, is a component, perhaps the sole molecule, of the causative agent.

Polymorphisms within the protein-coding region of the sheep PrP gene are associated with the occurrence of classical scrapie in several breeds of sheep. PrP genotyping has been used as a tool in the control of classical scrapie, but no genotype appears to be completely resistant to infection. A recently detected variant called atypical scrapie has been reported in sheep of PrP genotypes that are apparently resistant to classical scrapie. Some possess a polymorphism at codon 141 of the PrP gene. Classical scrapie is endemic in many parts of the world, where it has often been introduced by importation. Australia and New Zealand have maintained freedom by use of strict restrictions on imports and other measures. The infection in sheep may be passed from ewe to lamb in the period from parturition to weaning. Infection can also pass horizontally to unrelated sheep or goats, especially when parturition occurs in confined areas. Fetal membranes are known to be a source of infection. The incubation time between primary infection and clinical disease is nearly always longer than 1 year and may sometimes exceed the commercial lifespan of the sheep. The majority of cases occur in sheep between 2 and 5 years of age. Clinical disease only develops if the infection enters the CNS.

Identification of the agent: The disease is recognised by the clinical signs, which start insidiously with behavioural abnormalities. These may initially go unrecognised, but usually progress to more obvious neurological signs, including pruritus and incoordination. Loss of bodily condition is also common. The clinical disorder is variable in duration and in the range of signs presented, but it is inevitably fatal. Clinical diagnosis is supported by the immuno-detection of disease-specific accumulations of prion protein in the brain or lymphoreticular tissues or by histopathological diagnosis of spongiform encephalopathy or by detection of scrapie-associated fibrils by electron microscopy. Brain pathology is characterised by bilateral and usually symmetrical neuronal vacuolation and spongiform change of grey matter, which, in classical scrapie, is mainly in the brain stem and in atypical scrapie, may predominate in the cerebellum. Detection of PrPSc in tissue sections or extracts of diseased brains is a disease-specific diagnostic criterion. Immunohistochemistry may be used to detect abnormal accumulations of PrP in routine histologically prepared brain and lymphoreticular tissue sections. PrPres, a partially protease-resistant truncated form of the prion protein, can be detected in unfixed brain material by detergent extraction, enzymatic digestion, electrophoresis and immunoblotting. Automated immunochromatography detection of the protein in brain samples forms the basis of rapid tests used in active surveillance programmes.

Detection of PrPSc in lymphoreticular tissues during the incubation period of scrapie in some animals offers a means of preclinical diagnosis of scrapie infection by proxy and may be particularly useful for surveillance purposes when performed on biopsied tissue.

Atypical forms of scrapie, identified predominantly through active surveillance, are partially characterised for diagnostic purposes, but remain the object of further research.
Most, but not all, currently recognised forms of scrapie can be transmitted to laboratory rodents by injecting them with infected brain tissue, but the variable efficiency of transmission coupled with long incubation times preclude this as a practical diagnostic procedure.

**Serological tests:** Scrapie infection is not known to elicit any specific immune response and there is no basis for establishing a diagnosis by detecting specific antibodies.

**Requirements for vaccines and diagnostic biologicals:** There are no biological products available.

### A. INTRODUCTION

Scrapie is a naturally occurring progressive, fatal, infectious, neurodegenerative disease of sheep and goats, that has been recognised as a clinical entity affecting sheep populations in countries of Western Europe for at least the past two and a half centuries. The histological definition of the lesions in the central nervous system (CNS) was achieved in the late 19th century and experimental transmission in the 1930s, but only later in the 20th century did the term transmissible spongiform encephalopathy (TSE) come into generic use to describe scrapie and other related diseases in humans and animals. Following discovery of the prion protein in the 1980s, these diseases are now known as prion diseases (see Chapter 2.4.6 Bovine spongiform encephalopathy [BSE]). The occurrence of scrapie preceded recognition of other prion diseases of mammals and so, in retrospect, it is the archetype of prion disorders (18). Prion diseases are defined by the presence of an abnormal isoform of the host-encoded membrane protein (PrP\(_{\text{C}}\)), designated PrP\(_{\text{Sc}}\), which accumulates in the CNS and in the lymphoreticular system (LRS) and sometimes also in other tissues. The prion hypothesis proposes that the altered protein is a major component or the sole molecule of the aetiological agent. It is proposed that different strains of the agent result from different conformational forms of the protein. Alternative hypotheses for the agent structure, such as a slow virus, an unconventional virus, or the virino, all of which demand the existence of a nucleic acid genome (which has not been identified) have been suggested in the past (18).

It has long been known, as in other infectious diseases, that in the TSEs, the interaction of agent variables (particularly strain) and host variables (particularly the sequence of the PrP gene and the presence or absence of certain alleles of the PrP gene within breeds) determines the disease phenotype. In sheep, different alleles of the gene (PrP genotypes) are associated with susceptibility to TSEs (17). The sheep PrP gene is highly polymorphic. Common polymorphisms have been identified within the protein-coding region of the sheep PrP gene at codons 136, 154, and 171. The incidence of scrapie in various breeds of sheep has been associated with some of these polymorphisms. The codon 171 polymorphisms are of particular significance in determining overall risk.

Detection and characterisation of different strains of scrapie isolates have, historically, relied upon transmission to rodents, principally inbred (wild-type) mice. Some twenty or more murine strains of scrapie have been derived from natural scrapie case isolates; characterised phenotypically in mice on incubation period data and brain lesion profiles in selected grey and white matter areas (biological strain typing) (6). While the reproducibility and stability of the disease phenotype produced on serial transmission of an isolate in mice has provided the basis of such typing, the murine scrapie strains obtained do not necessarily represent the original ovine scrapie strains (see Chapter 2.4.6 BSE) and it is unclear as to how many different strains might occur naturally in sheep. As some natural scrapie isolates do not transmit to wild-type mice, strain characterisation by this method has limitations. The use of transgenic mice, expressing the natural host PrP gene instead of the mouse PrP gene provides further scope for biological typing but as yet does not have the breadth of data accumulated from wild-type mouse studies. Molecular methods of typing TSE sources are based upon biochemical properties of PrP\(_{\text{Sc}}\) for example, by using immunohistochemistry or Western blotting. Immunohistochemical patterns of PrP\(_{\text{Sc}}\) accumulation in the brain have been used to differentiate disease caused by scrapie agents from that caused experimentally by the BSE agent (19). Considerably more data are required on correlations of results from different typing methods to provide an understanding of scrapie strain characterisation. The ability to distinguish scrapie agent strains from the (classical) BSE agent strain is of particular importance in small ruminants because of the zoonotic nature of the latter and the potential for past exposure of small ruminants to the foodborne agent, the clinical congruence between experimental BSE and scrapie in small ruminants and the single confirmed report of the occurrence of BSE in a goat in France (9). Isolation of the BSE agent has not however been reported from a TSE of sheep and epidemiological data would indicate that if present, it is at a prevalence that is undetectable by current surveillance programmes.

The introduction of active surveillance for prion disorders of small ruminants in the European Union (EU) in 2002, with the use of rapid immunochemical methods previously approved for surveillance for BSE in cattle, has provided evidence for PrP\(_{\text{Sc}}\) distribution patterns in healthy sheep and goats slaughtered for human consumption, and in fallen stock, that were not previously detected by traditional sampling approaches, or criteria for the diagnosis of scrapie. Such cases, colloquially referred to as ‘atypical scrapie’, have features in common with a scrapie phenotype termed Nor98 and were present before 2002 (12). Atypical scrapie has been identified
Scrapie is endemic in many European countries and has also been reported in Asia, Africa and North America. Because of the known inadequacies of baseline (passive) surveillance and the absence of active surveillance components, the true scrapie status of many countries is unknown. Some countries have never recorded the disease against a background of general and/or targeted surveillance, while others have maintained freedom for various periods through rigorous preventative policies and monitoring. Notably, scrapie has been considered absent from Australia and New Zealand for more than 50 years (31). Within the EU, since January 1993, and now also in many other countries, scrapie is a compulsory notifiable disease. The disease usually occurs in sheep 2–5 years of age (17). Rarely are cases present in sheep less than 1 year of age. In atypical scrapie, significant numbers of cases have been reported in sheep over 5 years of age. In some instances, the commercial lifespan of the sheep may be too short or exposure has occurred too late in life to allow the clinical disease to develop. Scrapie has also been described in goats, and captive moufflon (Ovis musimon), a primitive type of sheep. Most breeds of sheep may be affected, although in some there is a clear genetic basis for resistance or low prevalence of clinical disease. Flock records indicate that in sheep the disease tends to be linked with certain family lines. The infection in sheep may be passed from ewe to lamb in the period from parturition to weaning. Infection can also pass horizontally to unrelated sheep or goats, especially when parturition occurs in confined areas. Fetal membranes are known to be a source of infection, but pasture previously grazed by or buildings previously inhabited by infected sheep may also represent a risk. Animals incubating the disease, and even animals that never develop clinical signs, may still be a source of infection to others.

The biohazard for humans from scrapie diagnostic testing appears to be limited, but appropriate precautions should be taken. The long historical existence of natural scrapie in domestic sheep and the failure of many investigations to show any epidemiological link between scrapie and the human TSEs, provide a strong indication of negligible risks to those working with the agent (5). Most specifically, Creutzfeldt-jakob disease (CJD) has been found to occur at no greater frequency in those with occupations providing closest contact with the agent than in other population groups. However, the extreme chemical and physical resistance of the scrapie agent and the fact that it is experimentally transmissible by injection to a wide spectrum of mammalian species suggest the prudence of preventing human exposure. Because of the now established link between BSE and the variant form of the human CJD, BSE and related agents are now categorised, with respect to the level of biohazard designated for working with the human TSE agents (see Chapter 2.4.6 BSE). While natural scrapie is excluded from this categorisation, adoption of containment measures similar to those used for the other TSE agents is recommended when dealing with tissues from natural scrapie cases in the laboratory. This is particularly relevant in those countries that have experienced cases of BSE in their endemic cattle population. As sheep may have been exposed to the same contaminated feedstuffs considered to be the source of infection in cattle, those working directly with infected tissues should therefore wear appropriate personal protective equipment and clothing and observe standard decontamination procedures for the spongiform encephalopathy agents (see Chapter 2.4.6 BSE). Any contaminated tissues or single use equipment must be disposed of in a safe and approved manner and reusable equipment cleaned and decontaminated using effective approved methods. Such an approach also ensures that attempts to characterise the scrapie agent are not compromised by cross-contamination. The soundness of such precautionary measures is reinforced by the occurrence of atypical scrapie, which has been transmitted experimentally to sheep and transgenic mice over-expressing the ovine PrP gene (23), but where the risk of natural transmission or the potential pathogenicity for humans is unknown.

**B. DIAGNOSTIC TECHNIQUES**

1. **Presumptive diagnosis based on clinical signs**

The clinical signs of scrapie (26) usually start insidiously, often with behavioural changes that are evident only from repeated inspections. These subtle presenting features, which may include apparent confusion, disengagement from the flock and a staring gaze, progress to a more definite neurological illness frequently characterised by signs of pruritus and ataxia or incoordination of gait. Either the pruritus or the ataxia usually emerges to dominate the clinical course. Death may occur after a protracted period of only vague neurological signs or may even occur without premonitory signs.

The variety of names that have been used in different languages to describe scrapie, for example, la tremblante (trembling), Traberkrankheit (trotting disease), or Gnubberkrankheit (nibbling disease) reflects the diversity of the presenting and predominant clinical signs in different sheep populations.

Pruritus is recognised principally by compulsive rubbing or scraping against fixed objects, nibbling at the skin and scratching with a hind foot. This results in extensive loss of wool, particularly over the lateral thorax, flanks and hindquarters. The persistence of pruritus often results in localised self-inflicted skin lesions. These may occur in areas of wool loss and on the poll, face, ears and limbs. A characteristic ‘nibbling reflex’ can often be elicited by palpation of the lumbar region, and may also be evoked by the sheep’s own scraping movements.
Ataxia or incoordination of gait may first become apparent as difficulty in positioning the hind limbs on turning, swaying of the hindquarters and a high stepping or trotting gait of the forelimbs. Stumbling and falling occur, but the sheep is generally able to quickly regain a standing posture. These signs progress to weakness and recumbency. Information on the phenotypic variants of scrapie, termed atypical scrapie, or Nor98 is as yet insufficient to suggest clinical features distinct from classical scrapie, but dominance of ataxia has been reported (20, 26). Other neurological signs may include teeth grinding (bruxism), an abnormally low carriage of the head and ears, a fine tremor, seizures, and blindness. There may also be apparent hyperaesthesia to sound, movement, or touch. In most cases, there is also a loss of bodily condition, but significant weight loss may be apparent only in the late clinical stages.

Progress of the clinical disease is very variable, lasting for a week or up to several months, with an inevitably fatal outcome. There is also variation in the clinical signs among individual animals and in different breeds of sheep. These variations may be due to the influence of host genotype and strain of agent. Environmental factors may also have an influence on the disease course. The clinical diagnosis of individual cases of scrapie can therefore be difficult. The clinical signs may, especially in the early phase of the disease, resemble those of some other conditions of adult sheep, including ectoparasitism, pseudorabies (Aujeszky’s disease), rabies, cerebral listeriosis, ovine progressive pneumonia (maedi-visna), pregnancy toxaemia (ketosis), hypomagnesaemia and chemical and plant intoxications.

Video-clips illustrating the clinical signs of scrapie can be viewed on the web-site of the European Commission (EC) TSE Community Reference Laboratory/Veterinary Laboratories Agency (VLA) (10). Other historical videotape footage of classical scrapie signs can also be sourced (27). Video-clips of atypical scrapie signs are also available (20).

2. Identification of the agent

According to the prion hypothesis, demonstration of PrP\textsuperscript{Sc} would constitute identification of the agent, but, by definition transmission from infected tissues, usually to laboratory rodents by injection, is the only available means of detection of infectivity. Long incubation periods and the failure of some natural scrapie sources to transmit to specific mice strains mean that it is impractical to use the criterion of transmissibility for diagnosis. However, biological characterisation on transmission is an important component of the definition of any emerging new phenotypic variants of scrapie and for discriminatory approaches to distinguish cases of scrapie from BSE in sheep or goats (10).

The laboratory diagnosis of scrapie (14) has in the past, been reliant on the demonstration of pathological changes as there are no \textit{in-vitro} methods for isolation of the causative agent. In the absence of specific gross pathological changes, the only practical diagnostic method was demonstration of the histopathological changes in the CNS. Prior to the routine use of immunochemical detection of PrP\textsuperscript{Sc}, morphological demonstration of the pathological PrP in the form of scrapie-associated fibrils (SAF) was employed as an adjunct to the histopathological diagnosis. SAF are visualised in unfixed brain extracts using negative-stain electron microscopy. SAF detection was particularly useful when available brain tissue was autolysed.

The histopathological diagnosis was usually based on examination of a single section of medulla oblongata taken at the level of the obex, considered the predilection site for morphological changes (29). Immunohistochemical (IHC) examination of the medulla section and Western blotting techniques, performed on adjacent fresh tissue, were introduced with recognition of the diagnostic specificity of PrP\textsuperscript{Sc} and increasing efficiency of detection methods. While clinically suspect cases of scrapie should, where suitable samples are available, continue to be investigated initially by histopathological examination for morphologic changes, diagnostic criteria must now include the demonstration of PrP\textsuperscript{Sc} in the CNS. Detectable PrP\textsuperscript{Sc} precedes vacuolation and clinical signs, making the immuno-based tests a more sensitive option (16). This approach also assists in the characterisation of the prion protein that is present, and contributes to discrimination of disease phenotypes, particularly between classical and atypical scrapie, and indeed BSE (2, 30).

Commercially available rapidly performed methods for the detection of PrP\textsuperscript{Sc}, introduced originally for the diagnosis of BSE, are approved for scrapie diagnosis (11). They take the form of Western blot methods or enzyme-linked immunosorbent assay (ELISA)-based methods (14). These rapid tests provide a preliminary screening from which positive or inconclusive results are subject to examination by confirmatory immunohistochemical or Western blot methods.

Failure to observe characteristic histological changes or to detect disease-specific PrP/SAF does not confirm the absence of the disease; agreement between the results of multiple diagnostic approaches provides the best assurance of accuracy. Clearly, in surveillance situations where monitoring is for the purpose of obtaining evidence of freedom from scrapie in small ruminant species, it may be necessary to apply multiple diagnostic criteria and to use at least two laboratory methods on accurately sampled CNS tissue (histopathological and immunohistochemical, or immunoblotting) to maintain a high degree of confidence in negative results.
Passive surveillance of scrapie, comprising the examination of CNS material from clinically suspect cases, has, in recent years, been complemented in many countries by active surveillance, targeting healthy adult culled and fallen stock (diseased or dead animals, also called risk animals). The principal approach for active surveillance diagnosis has been the application of the rapid methods to post-mortem CNS material. However, unlike in BSE, the opportunity exists in scrapie for screening examinations that do not rely solely on examination of the CNS tissue from dead animals to detect exposed animals as the widespread presence of PrPSc in lymphoreticular tissue enables demonstration by biopsy of some peripherally accessible lymphoreticular tissues.

PrPSc detection in lymphoreticular tissue by immunohistochemistry has been applied for the preclinical diagnosis of scrapie in sheep using palatine tonsil, nictitating membrane, superficial lymph nodes and, most recently, rectal mucosa (15) lymphoreticular tissue biopsies. Applications of PrP detection to the preclinical diagnosis of scrapie require a better understanding of the variability of pathogenesis before their sensitivity relative to incubation period can be assessed. In addition, occasional scrapie cases of sheep appear to lack a peripheral lymphoreticular phase of replication (1, 15). Whereas large-scale testing to determine freedom from scrapie should include targeted examination of peripheral tissues of younger animals, surveillance for prevalence of disease could potentially limit tissue examination to the CNS of adult sheep. However, testing to estimate disease prevalence needs to take into account a number of factors, including the stratification of the sheep-farming industry, dose or level of infection within particular flocks, frequency of disease and relative involvement of the LRS in different genotypes and the effect of genotype/agent strain combination on incubation period.

The need to distinguish between cases of scrapie and possible BSE in sheep and goats has required development of diagnostic methods with the potential to discriminate between the agents causing these infections. Studies suggest that the conformation of disease-specific PrP produced in BSE-infected sheep is different from that of disease-specific PrP found in natural sheep scrapie (12, 19, 25). These conformational differences may be detected by immunoblotting or immunohistochemical techniques using peptide-specific antibodies. Within the European Union, the strategy for this distinction comprises examination of source CNS material after initial detection through active or passive surveillance (initial screening), in a primary, secondary and tertiary phase procedure (10), involving a Western blot method capable of such discrimination, followed by peer review and further investigation by biochemical and immunohistochemical methods, and finally, if necessary, mouse transmission to a standard panel of wild-type mice (see Chapter 2.4.6 BSE). Interpretation of the in-vitro methods (Western blot or ELISA) is reliant on differences between BSE and scrapie in the N-terminal cleavage site for Proteinase-K digestion of PrPSc. The in-situ immunohistochemical approach relies on distribution and peptide-specific labelling patterns of PrPSc in brain and lymphoreticular tissues. Increasingly, for biological characterisation of agent strain, appropriate transgenic mouse constructs are being used, but they lack the wealth of background data available from studies of the long established wild-type mouse panel.

Quality control (QC) and quality assurance (QA) are essential parts of testing procedures and advice can be supplied by the OIE Reference Laboratories (30).

a) Sample preparation

Concerns regarding BSE in small ruminant populations and, more recently, the recognition of atypical scrapie, have influenced the strategies for sampling and diagnosis. Although the most comprehensive sampling and multiple testing methods would provide contingencies for these and possible future uncertainties in the diagnosis of prion diseases of small ruminants, operational factors also determine what is practically and economically possible. The relative implementation of passive and active surveillance programmes and the diagnostic methods applied further influence sampling strategy. Selection and recommendation of methods is necessarily therefore under constant review.

For routine diagnosis, the sampled CNS material is either stored fresh or frozen for subsequent biochemical tests or it is fixed for histological preparations. Where programmes are in place to identify possible infections with BSE in small ruminant populations, all sampling must be conducted aseptically, using new sterile disposable instruments, or instruments sterilised under conditions specified for the decontamination of prions, (see Chapter 2.4.6 BSE). Cross contamination at necropsy must be avoided. Thus, in the following procedures where fresh tissue is sampled for biochemical methods, an aliquot should, if required, be reserved for transmission studies. Although in many instances disease can be confirmed on autolysed or suboptimally preserved material, such samples can only provide limited evidence of the absence of scrapie.

**Sheep in which clinical disease is suspected (passive surveillance)** should be killed by intravenous injection of barbiturate and the whole brain removed by standard necropsy procedures as soon after death as possible. Whole brain removal is advisable to allow pathological examinations for differentiation between possible different TSE phenotypes and differential diagnosis of non-TSE brain disorders. Methods of subdividing the brain tissue for application of PrP-detection techniques requiring fresh tissue and for histological techniques are dependent on the optimum sensitivities of each of the tests for different brain areas and the compromise that precisely the same area cannot be used for both fresh/frozen and fixed tissue approaches. The following protocol is suggested but may be subject to modification to satisfy the particular portfolio of tests. Further information can be obtained from OIE Reference Laboratories (10, 30).
Initially, a coronal block of medulla oblongata inclusive of the obex (see Chapter 2.4.6 BSE, Figure 1) is taken for fixation in 10% formal saline for 3–5 days prior to trimming and histological processing to paraffin wax for histopathological and immunohistochemical examinations. Care should be taken to ensure that this sample is not frozen. For the detection of PrP (or scrapie-associated fibrils), fresh tissue samples are taken and stored frozen (–20°C or below) prior to extraction. Samples should, if possible, provide 5 g of tissue. This should be taken initially from the caudal medulla and, if necessary supplemented with brainstem immediately rostral to the medulla – obex sample. Subdivision of this tissue to accommodate multiple biochemical methods can be achieved by hemisecting in the median plane or by transverse sectioning. Possible variation in sampling for rapid test requirements at the level of the obex is dealt with below in relation to active surveillance approaches. Where the whole brain is available additional fresh samples are advocated to minimise false-negative diagnoses, taking into account the possibility that there may be strain-specific targeting of other parts of the brain. For example, in atypical scrapie, cerebellum, thalamus and basal ganglia regions provide useful additional sites for testing.

The remaining brain tissue is fixed in approximately 10 times its volume of 10% formal saline for at least 1 week and then cut transversely as required to obtain blocks for histological processing to paraffin wax. The initial sampling of the single block of the medulla may well be sufficient for the immunohistochemical and the morphological diagnosis (see Chapter 2.4.6 BSE, Fig. 1). Requirements for pathological phenotypic characterisation or differential diagnosis can be fulfilled by taking additional areas of the brain stem and, as necessary, representative blocks of all major brain regions. Sections 5 µm in thickness, are stained with haematoxylin and eosin and examined initially for the morphological changes and, as required, for immunohistochemical detection of PrPSc, as outlined below.

For active surveillance programmes, methods for removal of the brainstem via the foramen magnum using proprietary spoon shaped instruments, similar to those employed in cattle for sampling for BSE diagnosis (see Chapter 2.4.6 BSE, Figure 2) have been devised for sheep. Although not advisable, the approach can also be used for clinical suspect cases. The minimum sampling is the brainstem at the level of the obex and, if it is required to also diagnose atypical scrapie, the cerebellum. The brainstem portion is either hemisectioned in the median plane to provide half (fresh/frozen) for a rapid test and half (fixed) for histopathology. Alternatively a complete coronal slice inclusive of the obex is fixed and a similar adjacent caudal medulla slice taken for the rapid test. The complete coronal slice has been recommended in the past, to establish the symmetry of morphological changes, but with the use of rapid molecular techniques there is competition between tests for the optimal diagnostic sites at the obex. Some rapid test kits use a core sampling approach to obtain an appropriate mass of material from the obex region. While hemisectioning of the obex region of the brainstem will result in loss of the ability to assess vacuolar lesion symmetry, the greater specificity provided by immunohistochemistry to detect PrPSc largely offsets this disadvantage. However if this, or a core sampling approach, is adopted, it becomes critical to ensure that the contralateral target site is not compromised. The dorsal nucleus of the vagus nerve (the optimal target area for most cases of scrapie) is a narrow column and lies close to the midline (see Chapter 2.4.6 BSE, Figure 3). The options are also dependent on the specific sampling instruments provided by the test kit manufacturer. For all sampling methods it is vital operators are trained and that the training includes instruction in the gross and cross-sectional neuroanatomy of the brainstem and the precise location of the target areas for disease-specific PrPSc accumulation.

For differentiation of classical and atypical cases portions of cerebellum are required fixed and fresh/frozen.

b) Histological examination

Morphological changes in the CNS are those of a spongiform encephalopathy comprising principally vacuolation of neuronal perikarya and neuropil accompanied by a variable and usually less conspicuous gliosis (particularly an astrocytic reaction). Typically, the lesions have a bilaterally symmetrical distribution. There is considerable variation in the distribution pattern of vacuolation and other changes, but lesions, at least in classical scrapie, are usually most apparent in the brain stem and frequently affect the dorsal nucleus of the vagus nerve. Vacuolation of neuronal perikarya is not pathognomonic because it is often present in the brains of apparently healthy sheep, albeit at a low frequency (28). Furthermore, none of the individual patho-morphological features of scrapie can be considered to be strictly disease specific, but in combination and with abundant vacuolar changes they are undoubtedly pathognomonic. Severe clinical signs are not always observed with the presence of severe neuropathological changes. Conversely, even when clinical signs are severe, morphological changes may be undetectable by light microscopy (3, 13). A clinical diagnosis of suspected scrapie cannot be refuted by a failure to find significant vacuolar changes in the brain. The absence of lesions is therefore not evidence of the absence of scrapie infection, as this can exist without either clinical signs or detectable morphological changes.

Despite such reservations, the histological examination of sections of medulla oblongata at the obex may be sufficient to confirm a diagnosis in most cases of clinically suspect scrapie (14, 29). While this has been used successfully for routine diagnosis of classical scrapie, equivocal findings in the medulla would require examination of additional sections of the CNS. This has become particularly apparent with the recognition of atypical scrapie, in which morphologic changes in the brain may be sparse or entirely absent and
c) Detection of disease-specific forms of PrP

Methods for the demonstration of accumulation of disease-specific forms of PrP in specified target areas now provide the principal approach for the diagnosis of natural scrapie (14). In suspect clinical cases the combined use of immunohistochemistry and Western blotting is advocated to confirm the diagnosis. Immunohistochemistry on tissue sections to demonstrate accumulation of PrPSc should be carried out in parallel with routine histology in suspected cases. Also, the truncated, partially protease resistant form of PrP, PrPres, can be detected after partial purification from detergent and protease-treated extracts of unfixed, affected brain by electrophoresis and immunoblotting. Combined use of immunohistochemistry and Western blotting is also recommended where histological lesions are mild in severity and considered equivocal. In active surveillance programmes, the primary diagnosis will usually be accomplished using rapid test methods and, in the case of positive or inconclusive results, confirmatory methods should also be applied. Test methods are detailed on the web sites of OIE Reference Laboratories (see Chapter 2.4.6 BSE).

- Immunohistochemical methods

Disease-specific PrP in scrapie-affected brain is demonstrated immunohistochemically on routinely formalin-fixed material by the application of a variety of epitope demasking techniques and the use of appropriate antibodies to PrP (10, 30). Recognition of morphological disease-specific immunolabelling configurations, their cellular associations and neuroanatomical distribution patterns provide the basis for a confirmatory diagnosis in classical (21) and in atypical (4) scrapie.

- Western blot methods

A diagnosis based on PrPres detection by Western blotting requires that a wide region of immunostained bands corresponding to proteins of molecular mass 27–30 kDa be present in the proteinase-K-treated scrapie sample lanes only and that control sample lanes provide appropriate comparisons. Several sensitive Western immunoblot methods for the detection of PrPres have been published (24). The original technique used for diagnosis of BSE, which has been referred to as ‘the OIE Western immunoblotting technique’ (10) relies on detergent extraction of a large amount of fresh brain material (4 g) followed by ultracentrifugation to concentrate the PrP and then treatment with proteinase K enzyme to digest any normal host protein. This leaves only PrPres to be bound by a specific antibody and detection system in positive brain samples (see also Chapter 2.4.6 BSE).

If histopathological examination and immunohistochemistry results cannot be achieved due to the poor state of the sample, i.e. severely autolysed cases, immunoblotting, the ELISA methods and SAF detection are the remaining test options available. Similarly, these methods can also be applied in circumstances when, sometimes in error at necropsy, CNS material intended for fixation and histological examination has been frozen. IHC methods can still be applied to such samples, but the ability to identify anatomical sites may be compromised, which means that any ‘negative’ result must be qualified. With modification the method for SAF detection may also be applied successfully to formalin-fixed tissue (7). SAF detection should not be used as a sole test if an immunochemical method is possible.

A wide range of antisera and monoclonal antibodies for PrP detection by immunochemical methods are now in use and some are commercially available. Positive and negative controls are essential.

- Rapid tests methods

Rapid immunodiagnostic test formats for the detection of PrPSc in small ruminant brain tissue have been developed and have been evaluated for diagnostic use (11). The rapid tests rely on the optimisation of the reagents used for extraction and digestion and a specific monoclonal antibody for detection, which negates the need for lengthy ultracentrifugation steps. The tests require fresh medulla taken at the obex or just caudal to the obex. Most rapid tests use less than 0.5 g of material and many sampling tools are designed to sub-sample precise amounts. However, to allow for possible additional testing at least 1 g of initial sample, or sample ‘in total’ is advised. Some laboratories use the OIE Western immunoblotting technique (if enough tissue is available) to confirm any weak-positive samples that are initially detected using a rapid test (10). The increased amount or concentration of PrPres extracted by ultracentrifugation from the larger aliquot of brain tissue can give improved sensitivity. Rapid test methods have been used to identify atypical scrapie cases (12) and detection is optimised if the appropriate brain sample (such as cerebellum) is tested.

Prospects for more sensitive diagnostic tests for scrapie and other TSEs are mainly directed at present on the refinement of existing methods and the development of new approaches to the detection of disease-specific forms of PrP. Achievement of the consistent performance of rapid test methods for the primary diagnostic approach is paramount, particularly with regard to the capacity of tests to recognise both
classical and atypical scrapie phenotypes. Overall diagnostic sensitivity is strongly influenced by the accuracy of sampling.

d) Other diagnostic tests

As for BSE of cattle (see Chapter 2.4.6 BSE) tests that can be applied effectively to the live animal to detect scrapie cases in the early stage of incubation remain elusive, despite several avenues of research. The pursuit of non-prion protein biomarkers, including possibly through metabolomic or proteomic approaches may offer prospects but there are constraints, including that the key tissue is accessible and that specificity is demonstrable.

2. Serological tests

A serological immune response to the scrapie agent has not been detected.

3. Genetic screening

Scrapie control and elimination strategies based on genetic selection for resistance to classical scrapie have been deployed in some countries. Selection is made on determination of the common polymorphisms of the sheep PrP gene. As an aid to the control of classical scrapie: breeding stock, particularly rams, of appropriate PrP genotype can be selected to produce progeny with reduced risk of developing disease (8). Such genotyping services are available on a commercial basis in North America and in several countries in Europe. The test is performed using DNA extracted from white blood cells obtained from ethylene diamine tetra-acetic acid (EDTA)-treated blood samples. Selection of breeding stock can be made on the most scrapie-resistant animals, homozygous for arginine at codon 171, thereby reducing the incidence of classical scrapie in individual flocks. These animals are not common in most flocks, and in some breeds the genotype is actually absent.

A strategic approach to eliminating scrapie infection from national flocks or geographical regions by adopting a national genetic breeding programme is possibly premature. The shortage of sheep that are homozygous for arginine at codon 171 is one factor. The lack of data on the effects of a high prevalence of such a genotype on productivity, resistance to diseases other than scrapie and viability in general is another factor. Furthermore, there is, as yet insufficient knowledge concerning the prevalence and epidemiology of atypical scrapie. Decisions on the appropriateness of such programmes must take account of a thorough evaluation of the current national/regional/local scrapie situation, the availability of replacement resistant sheep, the sheep importation policy, availability of testing facilities and the desirability and support of the sheep industry; especially the willingness of sheep breeders to commit themselves to the programme for a long period of time.

C. REQUIREMENTS FOR VACCINES AND DIAGNOSTIC BIOLOGICALS

There are no biological products available.

REFERENCES


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**NB:** There are OIE Reference Laboratories for Scrapie (see Table in Part 3 of this Terrestrial Manual or consult the OIE Web site for the most up-to-date list: www.oie.int).
CHAPTER 2.7.14.
SHEEP POX AND GOAT POX

SUMMARY

Sheep pox and goat pox are viral diseases of sheep and goats characterised by fever, generalised papules or nodules, vesicles (rarely), internal lesions (particularly in the lungs), and death. Both diseases are caused by strains of capripoxvirus, all of which can infect sheep and goats. Although most of the strains examined cause more severe clinical disease in either sheep or goats, some strains have been isolated that are equally pathogenic in both species. It has been proposed that the malignant pox diseases of sheep and goats caused by capripoxvirus as well as Kenyan sheep and goat pox, Indian goat dermatitis and north African stone pox of sheep and goats be referred to as capripox.

Capripox is endemic in Africa north of the Equator, the Middle East, Turkey, Iran, Afghanistan, Pakistan, India, Nepal, parts of the People’s Republic of China and, since 1984, Bangladesh. Recently, it has made frequent incursions into southern Europe.

Identification of the agent: Laboratory confirmation of capripox is most rapid by the identification of typical capripox virions using the transmission electron microscope in combination with a clinical history consistent with generalised capripox infection. The capripox virion is distinct from that of the other poxvirus commonly infecting sheep and goats – a parapoxvirus that causes orf or contagious pustular dermatitis. A precipitating antigen can be identified by an agar gel immunodiffusion test (AGID) using lymph gland biopsy material taken from an early case of capripox and specific immune sera; however, there is a cross-reaction with parapoxvirus. Capripoxvirus will grow on tissue culture of ovine, caprine or bovine origin, although field isolates may require up to 14 days to grow or require one or more additional tissue culture passage(s). The virus causes intracytoplasmic inclusions, clearly seen using haematoxylin and eosin staining. The antigen can also be detected in tissue culture using specific sera and immunoperoxidase or immunofluorescence techniques. Capripoxvirus antigen and inclusion bodies may be seen in stained cryostat or paraffin sections of biopsy or post-mortem lesion material.

An antigen-detection enzyme-linked immunosorbent assay (ELISA) using a polyclonal detection serum raised against a recombinant immunodominant antigen of capripoxvirus has been developed. Genome detection using capripoxvirus-specific primers for the fusion protein gene and attachment protein gene has also been reported.

Serological tests: The virus neutralisation test is the most specific serological test, but because immunity to capripox infection is predominantly cell mediated, the test is not sufficiently sensitive to identify animals that have had contact with the virus and developed only low levels of neutralising antibody. The AGID and indirect immunofluorescence tests are less specific due to cross-reactions with antibody to other poxviruses. Western blotting using the reaction between the P32 antigen of capripoxvirus with test sera is both sensitive and specific, but is expensive and difficult to carry out. The use of this antigen, expressed by a suitable vector, in an ELISA offers the prospect of an acceptable and standardised serological test.

Requirements for vaccines and diagnostic biologicals: Live and inactivated vaccines have been used for the control of capripox. All strains of capripoxvirus so far examined share a major neutralisation site and will cross protect. Inactivated vaccines give, at best, only short-term immunity.
A. INTRODUCTION

Sheep pox and goat pox (capripox) are endemic in central and North Africa, the Middle East and India. Capripox is caused by strains of capripoxvirus and produces a characteristic clinical disease in fully susceptible breeds of sheep and goats and would usually be difficult to confuse with any other disease. In indigenous animals, generalised disease and mortality are less common, although they are seen where disease has been absent from an area or village for a period of time, when intensive husbandry methods are introduced, or in association with other disease agents, such as peste des petits ruminants virus or foot and mouth disease virus.

Capripox is a major constraint to the introduction of exotic breeds of sheep and goats, and to the development of intensive livestock production. Strains of capripoxvirus that cause lumpy skin disease (such as Neethling), are also found in cattle, but there is no evidence that these strains will naturally cause disease in sheep and goats. The geographical distribution of lumpy skin disease differs from that of sheep pox and goat pox.

Strains of capripoxvirus do pass between sheep and goats, although most cause more severe clinical disease in only one species; recombination also occurs between these strains, producing a spectrum showing intermediate host preferences and a range of virulence. Some strains are equally pathogenic in both sheep and goats. Capripox has the potential to spread and become established in countries outside its normal distribution. In 1983 it spread into Italy, in 1985 and 1989 into Cyprus, and in 1988 and numerous subsequent occasions into Greece, but did not become established in these countries. In 1984, however, it spread into Bangladesh where it has persisted. In 2005, an outbreak in goats in Vietnam indicated that capripox has a larger distribution than previously recognised.

The incubation period is between 8 and 13 days following contact between an infected and susceptible animals. It may be as short as 4 days following experimental infection by intradermal inoculation or mechanical transmission by insects. Some breeds of European sheep, such as Soay, may die of acute infection before the development of skin lesions. In other breeds there is an initial rise in rectal temperature to above 40°C, followed in 2–5 days by the development of, at first, macules – small circumscribed areas of hyperaemia, which are most obvious on unpigmented skin – and then of papules – hard swellings of between 0.5 and 1 cm in diameter – which may cover the body or be restricted to the groin, axilla and perineum. Papules may be covered by fluid-filled vesicles, but this is rare. A flat haemorrhagic form of capripox has been observed in some breeds of European goat, in which all the papules appear to coalesce over the body; this form is always fatal.

Within 24 hours of the appearance of generalised papules, affected animals develop rhinitis, conjunctivitis and enlargement of all the superficial lymph nodes, in particular the prescapular lymph nodes. Papules on the eyelids cause blepharitis of varying severity. As the papules on the mucous membranes of the eyes and nose ulcerate, so the discharge becomes mucopurulent, and the mucosae of the mouth, anus, and prepuce or vagina become necrotic. Breathing may become laboured and noisy due to pressure on the upper respiratory tract from the swollen retropharyngeal lymph nodes, due to the developing lung lesions.

If the affected animal does not die in this acute phase of the disease, the papules start to become necrotic from ischaemic necrosis following thrombi formation in the blood vessels at the base of the papule. In the following 5–10 days the papules form scabs, which persist for up to 6 weeks, leaving small scars. The skin lesions are susceptible to fly strike, and secondary pneumonia is common. Anorexia is not usual unless the mouth lesions physically interfere with feeding. Abortion is rare.

On post-mortem examination of the acutely infected animal, the skin lesions are often less obvious than on the live animal. The mucous membranes appear necrotic and all the body lymph nodes are enlarged and oedematous. Papules, which may be ulcerated, can usually be found on the abomasal mucosa, and sometimes on the wall of the rumen and large intestine, on the tongue, hard and soft palate, trachea and oesophagus. Pale areas of approximately 2 cm in diameter may occasionally be seen on the surface of the kidney and liver, and have been reported to be present in the testicles. Numerous hard lesions of up to 5 cm in diameter are commonly observed throughout the lungs, but particularly in the diaphragmatic lobes.

The clinical signs and post-mortem lesions vary considerably with breed of host and strain of capripoxvirus. Indigenous breeds are less susceptible and frequently show only a few lesions, which could be confused with insect bites or contagious pustular dermatitis. However, lambs that have lost their maternally derived immunity, animals that have been kept isolated and animals brought into endemic areas from isolated villages, particularly if they have been subjected to the stress of moving long distances and mixing with other sheep and goats, and their pathogens, can often be seen with generalised and sometimes fatal capripox. Invariably there is high mortality in unprotected imported breeds of sheep and goats following capripoxvirus infection. Capripox is not infectious to humans.
B. DIAGNOSTIC TECHNIQUES

1. Identification of the agent

- Sample collection, submission and preparation

Material for virus isolation and antigen detection should be collected by biopsy or at post-mortem from skin papules, lung lesions or lymph nodes. Samples for virus isolation and antigen-detection enzyme-linked immunosorbent assay (ELISA) should be collected within the first week of the occurrence of clinical signs, before the development of neutralising antibodies. Samples for genome detection by polymerase chain reaction (PCR) may be collected when neutralising antibody is present. Buffy coat from blood collected into heparin or EDTA (ethylene diamine tetra-acetic acid) during the viraemic stage of capripox (before generalisation of lesions or within 4 days of generalisation), can also be used for virus isolation. Samples for histology should include tissue from the surrounding area and should be placed immediately following collection into ten times the sample volume of 10% formalin.

Tissues in formalin have no special transportation requirements. Blood samples, for virus isolation from theuffy coat, should be collected in tubes containing anticoagulant, placed immediately on ice and processed as soon as possible. In practice, the blood samples may be kept at 4°C for up to 2 days prior to processing, but should not be frozen or kept at ambient temperatures. Tissues for virus isolation, antigen detection and genome detection should be kept at 4°C, on ice or at –20°C. If it is necessary to transport samples over long distances without refrigeration, the medium should contain 10% glycerol; the samples should be of sufficient size that the transport medium does not penetrate the central part of the biopsy, which should be used for virus isolation/detection.

Material for histology should be prepared by standard techniques and stained with haematoxylin and eosin (H&E). Lesion material for virus isolation and antigen detection are homogenised. The following is an example of one technique for homogenisation: The tissue is minced using sterile scissors and forceps, and then ground in a sterile pestle and mortar with sterile sand and an equal volume of sterile phosphate buffered saline (PBS) containing sodium penicillin (1000 international units [IU]/ml), streptomycin sulphate (1 mg/ml), mycostatin (100 IU/ml) or fungizone (2.5 µg/ml) and neomycin (200 IU/ml). The homogenised suspension is freezethawed three times and then partially clarified by centrifugation using a bench centrifuge at 600 g for 10 minutes. Buffy coats may be prepared from 5–8 ml uncleotted blood by centrifugation at 600 g for 15 minutes; the Buffy coat is carefully removed into 5 ml of cold double-distilled water using a sterile Pasteur pipette. After 30 seconds, 5 ml of cold double-strength growth medium is added and mixed. The mixture is centrifuged at 600 g for 15 minutes, the supernatant is discarded and the cell pellet is suspended in 5 ml of growth medium, such as Glasgow’s modified Eagle’s medium (GMEM). After centrifugation at 600 g for a further 15 minutes, the resulting pellet is suspended in 5 ml of fresh GMEM. Alternatively, the Buffy coat may be separated from a heparinised sample using a Ficoll gradient.

a) Culture

Capripoxvirus will grow in tissue culture of bovine, ovine or caprine origin, although primary or secondary cultures of lamb testis (LT) or lamb kidney (LK) cells are considered to be the most susceptible, particularly those derived from a wool sheep breed. The following is an example of an isolation technique; Either 1 ml of buffy coat cell suspension or 1 ml of clarified biopsy preparation supernatant is inoculated on to a 25 cm² tissue culture flask of 90% confluent LT or LK cells, and allowed to absorb for 1 hour at 37°C. The culture is then washed with warm PBS and covered with 10 ml of a suitable medium, such as GMEM, containing antibiotics and 2% fetal calf serum. If available, tissue culture tubes containing LT or LK cells and a flying cover-slip, or tissue culture microscope slides, are also infected.

The flasks should be examined daily for up to 14 days for evidence of cytopathic effect (CPE), and the medium is replaced if it appears to be cloudy. Infected cells develop a characteristic CPE consisting of retraction of the cell membrane from surrounding cells, and eventually rounding of cells and margination of the nuclear chromatin. At first only small areas of CPE can be seen, sometimes as soon as 4 days after infection; over the following 4–6 days these expand to involve the whole cell sheet. If no CPE is apparent by day 7, the culture should be freeze-thawed three times, and clarified supernatant inoculated on to fresh LT or LK culture. At the first sign of CPE in the flasks, or earlier if a number of infected cover-slips are being used, a cover-slip should be removed, fixed in acetone and stained using H&E. Eosinophilic intracytoplasmic inclusion bodies, which are variable in size but up to half the size of the nucleus and surrounded by a clear halo, are indicative of poxvirus infection. Syncytia formation is not a feature of capripoxvirus infection. If the CPE is due to capripoxvirus infection of the cell culture, it can be prevented or delayed by inclusion of specific anti-capripoxvirus serum in the medium; this provides a presumptive identification of the agent. Some strains of capripoxvirus have been adapted to grow on African green monkey kidney (Vero) cells, but these are not recommended for primary isolation.

- Electron microscopy

Material from the original suspension is prepared for transmission electron microscope examination, prior to centrifugation, by floating a 400-mesh hexagon electron microscope grid, with pileiform-carbon substrate
activated by glow discharge in pentylamine vapour, on to a drop of the suspension placed on parafilm or a wax plate. After 1 minute, the grid is transferred to a drop of Tris/EDTA buffer, pH 7.8, for 20 seconds and then to a drop of 1% phosphotungstic acid, pH 7.2, for 10 seconds. The grid is drained using filter paper, air-dried and placed in the electron microscope. The capripox virion is brick shaped, covered in short tubular elements and measures approximately 290 × 270 nm. A host-cell-derived membrane may surround some of the virions, and as many as possible should be examined to confirm their appearance (19).

The virions of capripoxvirus are indistinguishable from those of orthopoxvirus, but, apart from vaccinia virus, no orthopoxvirus causes lesions in sheep and goats. However, capripoxvirus is distinguishable from the virions of parapoxvirus, that cause contagious pustular dermatitis, as they are smaller, oval in shape, and each is covered in a single continuous tubular element, which appears as striations over the virion.

- **Histology**

Following preparation, staining with H&E, and mounting of the formalin-fixed biopsy material, a number of sections should be examined by light microscopy. On histological examination, the most striking aspects of acute-stage skin lesions are a massive cellular infiltrate, vasculitis and oedema. Early lesions are characterised by marked perivascular cuffing. Initially infiltration is by macrophages, neutrophils and occasionally eosinophils, and as the lesion progresses, by more macrophages, lymphocytes and plasma cells. A characteristic feature of all capripoxvirus infections is the presence of variable numbers of ‘sheep pox cells’ in the dermis. These sheep pox cells can also occur in other organs where microscopic lesions of sheep and goat pox are present. These cells are large, stellate cells with eosinophilic, poorly defined intracytoplasmic inclusions and vacuolated nuclei. Vasculitis is accompanied by thrombosis and infarction, causing oedema and necrosis. Epidermal changes consist of acanthosis, parakeratosis and hyperkeratosis. Changes in other organs are similar, with a predominant cellular infiltration and vasculitis. Lesions in the upper respiratory tract are characterised by ulceration.

- **Animal inoculation**

Clarified biopsy preparation supernatant (see Section B.1.a Culture) may also be used for intradermal inoculation into susceptible lambs. These lambs should be examined daily for evidence of a skin reaction.

### b) Immunological methods

- **Fluorescent antibody tests**

Capripoxvirus antigen can also be identified on infected cover-slips or tissue culture slides using fluorescent antibody tests. Cover-slips or slides should be washed and air-dried and fixed in cold acetone for 10 minutes. The indirect test using immune sheep or goat sera is subject to high background colour and nonspecific reactions. However, a direct conjugate can be prepared from sera from convalescent sheep or goats or from rabbits hyperimmunised with purified capripoxvirus. Uninfected tissue culture should be included as a negative control because cross-reactions, due to antibodies to cell culture antigens, can cause problems. The fluorescent antibody tissue section technique has also been used on cryostat-prepared slides.

- **Agar gel immunodiffusion**

An agar gel immunodiffusion (AGID) test has been used for detecting the precipitating antigen of capripoxvirus, but has the disadvantage that this antigen is shared by parapoxvirus. Agarose (1%) is prepared in borate buffer, pH 8.6, dissolved by heating, and 2 ml is poured on to a glass microscope slide (76 × 26 mm). When the agar has solidified, wells are cut to give a six-well rosette around a central well. Each well is 5 mm in diameter, with a distance of 7 mm between the middle of the central well and the middle of each peripheral well. The wells are filled as follows: 18 µl of the lesion suspension is added to the central well. The slides are placed in a humidified chamber at room temperature for 48 hours, and examined for visible precipitation lines using a light box. The test material is positive if a precipitation line develops with the control serum that is confluent with that produced by the positive control antigen. This test will not, however, distinguish between capripox infection and contagious pustular dermatitis (orf).

To prepare antigen for the AGID, one of two 125 cm² flasks of LT or LK cells is infected with capripoxvirus, and harvested when there is 90% CPE (8–12 days). The flask is freeze–thawed twice, and the cells are shaken free of the flask. The contents are centrifuged at 4000 g for 15 minutes, most of the supernatant is decanted and stored, and the pellet is resuspended in the remaining supernatant. The cells should be lysed using an ultrasonic probe for approximately 60 seconds. This homogenate is then centrifuged as before, the resulting supernatant being pooled with that already collected. The pooled supernatant is added to an equal volume of saturated ammonium sulphate at pH 7.4 and left at 4°C for 1 hour. This solution is centrifuged at 4000 g for 15 minutes, and the precipitate is collected and resuspended in a small volume of 0.8% saline for use in the AGID test. The uninfected flask is processed in an identical manner throughout, to produce a tissue culture control antigen (17).
• **Enzyme-linked immunosorbent assay**

Following the cloning of the highly antigenic capripoxvirus structural protein P32, it is possible to use expressed recombinant antigen for the production of diagnostic reagents, including the raising of P32 monospecific polyclonal antiserum and the production of monoclonal antibodies (MAbs). These reagents have facilitated the development of a highly specific ELISA (5). Using hyperimmune rabbit antiserum raised by inoculation of rabbits with purified capripoxvirus, capripox antigen from biopsy suspensions or tissue culture supernatant can be trapped on an ELISA plate. The presence of the trapped antigen can then be detected using guinea-pig serum raised against the group-specific structural protein P32, commercial horseradish-peroxidase-conjugated rabbit anti-guinea-pig immunoglobulin and a chromogen/substrate solution.

c) **Nucleic acid recognition methods**

It is not possible to distinguish between strains of capripoxvirus from cattle, sheep or goats using serological techniques. However, strains can be characterised by comparing the genome fragments generated by HindIII digestion of their purified DNA (2, 16). Using this techniques and by sequencing of the genome, differences between isolates from the different species have been identified (12), but these are not consistent and there is evidence for the movement of strains between species and recombination between strains in the field (10).

The PCR can be used to detect the capripoxvirus genome in biopsy or tissue culture samples. Primers for the viral attachment protein gene and the viral fusion protein gene are specific for capripoxvirus, and the nature of the PCR products can be confirmed using restriction enzyme recognition sites (11, 13).

2. **Serological tests**

a) **Virus neutralisation**

A test serum can either be titrated against a constant titre of capripoxvirus (100 TCID$_{50}$ [50% tissue culture infective dose]) or a standard virus strain can be titrated against a constant dilution of test serum in order to calculate a neutralisation index. Because of the variable sensitivity of tissue culture to capripoxivirus, and the consequent difficulty of ensuring the use of 100 TCID$_{50}$, the neutralisation index is the preferred method. The test is described using 96-well flat-bottomed tissue culture grade microtitre plates, but it can be performed equally well in tissue culture tubes with the appropriate changes to the volumes used, although it is more difficult to read an end-point in tubes. The use of Vero cells in the virus neutralisation test has been reported to give more consistent results (20).

• **Test procedure**

i) Test sera including a negative and a positive control are diluted 1/5 in Eagle’s/HEPES (N-2-hydroxyethylpiperazine, N-2-ethanesulphonic acid) and inactivated at 56°C for 30 minutes.

ii) Next, 50 µl of the first inactivated serum is added to columns 1 and 2, rows A to H of the microtitre plate. The second serum is placed in columns 3 and 4, the third in columns 5 and 6, the positive control serum is placed in columns 7 and 8, the negative control serum is placed in columns 9 and 10, and 50 µl of Eagle’s/HEPES without serum is placed in columns 11 and 12 and to all wells of row H.

iii) A reference strain of capripoxvirus, usually a vaccine strain known to grow well in tissue culture, with a titre of over log$_{10}$ 6 TCID$_{50}$ per ml is diluted in Eagle’s/HEPES in bijoux bottles to give a log dilution series of log$_{10}$ 5.0; 4.0; 3.5; 3.0; 2.5; 2.0; 1.5 TCID$_{50}$ per ml (equivalent to log$_{10}$ 3.7; 2.7; 2.2; 1.7; 1.2; 0.7; 0.2 TCID$_{50}$ per 50 µl).

iv) Starting with row G and the most diluted virus preparation, 50 µl of virus is added to each well in that row. This is repeated with each virus dilution, the highest titre virus dilution being placed in row A.

v) The plates are covered and incubated for 1 hour at 37°C.

vi) LT cells are prepared from pregrown monolayers as a suspension of $10^5$ cells/ml in Eagle’s medium containing antibiotics and 2% fetal calf serum. Following incubation of the microtitre plates, 100 µl of cell suspension is added to all the wells, except wells H11 and H12, which serve as control wells for the medium. The remaining wells of row H are cell and serum toxicity controls.

vii) The microtitre plates are covered and incubated at 37°C for 9 days.

viii) Using an inverted microscope, the monolayers are examined daily starting at day 4 for evidence of CPE. There should be no CPE in the cells of row H. Using the 0240 KSGP vaccine strain of capripoxvirus, the final reading is taken on day 9, and the titre of virus in each duplicate titration is calculated according to Kärber (14). If left longer, there is invariably a ‘breakthrough’ of virus in which that was at first neutralised appears to disassociate from the antibody.
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Interpretation of the results: The neutralisation index is the log titre difference between the titre of the virus in the negative serum and in the test serum. An index of ≥1.5 is positive. The test can be made more sensitive if serum from the same animal is examined before and after infection. Because immunity to capripox is predominantly cell mediated, a negative result, particularly following vaccination in which the response is necessarily mild, does not imply that the animal from which the serum was taken is not protected.

A constant-virus/varying-serum method has been described using serum dilutions in the range 1/5 to 1/500 and fetal calf muscle cells. Because these cells have a lower sensitivity to capripoxvirus than LT cells, the problem of virus ‘breakthrough’ is overcome.

b) Agar gel immunodiffusion

The AGID test cannot be recommended as a serological test for the diagnosis of capripox because of the cross-reaction with antibody to contagious pustular dermatitis virus, which is the main differential diagnosis. A consequence of this cross-reaction is many false-positive results.

c) Indirect fluorescent antibody test

Capripoxvirus-infected tissue culture grown on flying cover-slips or tissue culture microscope slides can be used for the indirect fluorescent antibody test. Uninfected tissue culture control, and positive and negative control sera, should be included in the test. The infected and control cultures are fixed in acetone at –20°C for 10 minutes and stored at 4°C. Dilutions of test sera are made in PBS, starting at 1/5, and positives are identified using an anti-sheep gamma-globulin conjugated with fluorescein isothiocyanate (FITC). Cross-reactions can occur with orf, bovine papular stomatitis virus and perhaps other poxviruses.

d) Western blot analysis

Western blotting of test sera against capripoxvirus-infected cell lysate provides a sensitive and specific system for the detection of antibody to capripoxvirus structural proteins, although the test is expensive and difficult to carry out.

Capripoxvirus-infected LT cells should be harvested when 90% CPE is seen, freeze–thawed three times, and the cellular debris pelleted by centrifugation. The supernatant should be decanted, and the proteins should then be separated by SDS/PAGE (sodium dodecyl sulphate/polyacrylamide gel electrophoresis). A vertical discontinuous gel system, using a stacking gel made up of acrylamide (5%) in Tris (125 mM), pH 6.8, and SDS (0.1%), and a resolving gel made up of acrylamide (10–12.5%) in Tris (560 mM), pH 8.7, and SDS (0.1%), is recommended for use with a glycine running buffer containing Tris (250 mM), glycine (2 M), and SDS (0.1%). Samples of supernatant should be prepared by boiling for 5 minutes with an appropriate lysis buffer prior to loading. As an alternative to tissue culture antigen, it is possible to use purified virus or expressed recombinant P32.

Molecular weight markers should be run concurrently with the protein samples. The separated proteins in the SDS/PAGE gel should then be transferred by electroblotting to a nitrocellulose membrane (NCM). After blotting, the NCM is rinsed thoroughly in PBS and blocked in 3% bovine serum albumin (BSA) in PBS, or 5% skimmed milk powder in PBS, on a rotating shaker at 4°C overnight. The NCM can then be separated into strips by employing a commercial apparatus to allow the concurrent testing of multiple serum samples, or may be cut into strips and each strip can be incubated separately thereafter. The NCM is washed thoroughly with five changes of PBS for 5 minutes on a rotating shaker, and then incubated at room temperature on the shaker for 1.5 hours, with the appropriate serum at a dilution of 1/50 in blocking buffer (3% BSA and 0.05% Tween 20 in PBS; or 5% milk powder and 0.05% Tween 20 in PBS). The membrane is then thoroughly washed and incubated (in blocking buffer) with anti-species immunoglobulin horseradish-peroxidase-conjugated immunoglobulins at a dilution predetermined by titration. After further incubation at room temperature for 1.5 hours, the membrane is washed and a solution of diaminobenzidine tetrahydrochloride (10 mg in 50 ml of 50 mM Tris-HCl, pH 7.5, and 20 µl of 30% [v/v] hydrogen peroxide) is added. This is then incubated for approximately 3–7 minutes at room temperature on a shaker with constant observation, and the reaction is stopped by washing in PBS before excessive background colour is seen. A positive and negative control serum should be used on each occasion.

Positive test samples and the positive control will produce a pattern consistent with reaction to proteins of molecular weights 67, 32, 26, 19, and 17 kDa – the major structural proteins of capripoxvirus – whereas negative serum samples will not react with this pattern. Hyperimmune serum prepared against parapoxvirus (orf virus) will react with some of the capripoxvirus proteins, but not the 32 kDa protein that is specific for capripoxvirus.

e) Enzyme-linked immunosorbsent assay

A capripoxvirus antibody ELISA has been developed using the expressed structural protein P32 of capripoxvirus and MAbs raised against the P32 protein.
C. REQUIREMENTS FOR VACCINES AND DIAGNOSTIC BIOLOGICALS

A variety of attenuated live and inactivated capripoxvirus vaccines has been used to provide protection to sheep and goats against capripox (see refs 4 and 15 for reviews). All strains of capripoxvirus of ovine, caprine or bovine origin examined so far share a major neutralising site, so that animals recovered from infection with one strain are resistant to infection with any other strain (3). Consequently, it is possible to use a single strain of capripoxvirus to protect both sheep and goats against all field strains of virus, regardless of whether their origin was in Asia or Africa (18, 20).

There are two antigenic forms of capripoxvirus, the intact virion covered in short tubular elements, and the intact virion additionally covered in a host-cell-derived membrane. The latter is the form usually produced by the infected animal, whereas the former is that seen when virus is produced by freeze–thawing infected tissue culture. Dead vaccines produced from tissue culture are almost entirely naked virions, and when used as a vaccine do not stimulate immunity to the membrane-bound virion. This in part explains the poor success of inactivated vaccines. An additional factor is that inactivated vaccines are less effective than live, replicating vaccine virus in stimulating the cell-mediated immune response, which is the predominant protective response to poxvirus infection. Dead capripox vaccines provide, at best, only temporary protection. A number of strains of capripoxvirus have had widespread use as live vaccines (9), for example the 0240 Kenya sheep and goat pox strain used in sheep and goats, the Romanian and RM-65 strains used mainly in sheep, and the Mysore and Gorgan strains used in goats. Immunity in sheep and goats against capripox following vaccination with the 0240 strain lasts over a year, and will probably provide lifelong protection against lethal challenge. The 0240 strain should not be used in Bos taurus breeds of cattle.

A new generation of capripox vaccines is being developed that uses the capripoxvirus genome as a vector for the genes of other ruminant pathogens, for instance genes of rinderpest and peste des petits ruminants (PPR) viruses. The recombinant vaccine will provide protection against capripox, rinderpest and PPR in a single vaccination (1).

1. Seed management

a) Characteristics of the seed

A strain of capripoxvirus used for vaccine production must be accompanied by a history describing its origin and tissue culture or animal passage. It must be safe to use in all breeds of sheep and goats for which it is intended, including pregnant animals. It must be nontransmissible, remain attenuated after further tissue culture passage, and provide complete protection against challenge with virulent field strains for a minimum of 1 year. A quantity of master seed vaccine virus should be prepared and stored in order to provide a consistent working seed for regular vaccine production.

b) Method of culture

Vaccine seed should be lyophilised and stored in 2 ml vials at –20°C. It may be stored wet at –20°C, but when wet, is more stable at –70°C or lower. The virus should be cultured in primary or secondary LT or LK cells of wool sheep origin for maximum yield. Vero cells may also be used.

c) Validation as a vaccine

Seed lots must be shown to be:

i) **Pure**: Free from adventitious viruses, in particular pestiviruses, such as border disease and bovine viral diarrhoea virus, and free from contamination with bacteria, fungi and/or mycoplasmas.

ii) **Safe**: Produce no clinical reaction in all breeds of sheep or goats when given by the recommended route.

iii) **Efficacious**: Stimulate complete immunity to capripox in all breeds of sheep and goats for at least 1 year.

The necessary tests are described in Section C.4.

2. Method of manufacture

Vaccine batches are produced on fresh monolayers of secondary LT or primary LK cells. A vial of seed virus is reconstituted with GMEM and inoculated on to an LT or LK monolayer that has been previously washed with warm PBS, and allowed to adsorb for 15 minutes at 37°C before being overlaid with additional GMEM. After 4–6 days, there will be extensive (50–70%) CPE. The culture should be examined for any evidence of nonspecific CPE, medium cloudiness or change in medium pH. The culture is freeze–thawed three times, the suspension
removed and centrifuged at 600 g for 20 minutes. A second passage may be required to produce sufficient virus for a production batch (to produce enough for $10^6$ doses, the yield from five 175 cm$^2$ flasks is required).

The procedure is repeated and the harvests from individually numbered flasks are each mixed separately with an equal volume of sterile and chilled 5% lactalbumin hydrolysate and 10% sucrose, and transferred to individually numbered bottles for storage at −20°C. Prior to storage, 0.2 ml is removed from each bottle for sterility control. An additional 0.2 ml is removed for virus titration; 2 ml pools composed of 0.2 ml samples taken from ten bottles are used. A written record of all the procedures must be kept for all vaccine batches.

Inactivated vaccines are produced, usually from unattenuated field strains of capripoxvirus, grown in tissue culture as described above, inactivated with 0.03% formaldehyde, and mixed with an equal volume of alhydrogel as adjuvant. Formaldehyde is no longer considered to be a suitable inactivant for certain other viral vaccines because its mode of action cannot be guaranteed to be totally effective in inactivating all the live virus. This has not been fully investigated for capripoxvirus.

3. In-process control

Cells: Cells should be obtained from the testis or kidney of a healthy young lamb from a scrapie-free flock of a wool sheep breed. During cultivation, cells must be observed for any evidence of CPE, and for normal morphology (predominantly fibroblastic). They can usually be passaged successfully up to ten times. When used for vaccine production, uninfected control cultures should be grown in parallel and maintained for at least one additional passage for further observation. They should be checked for the presence of noncytopathic strains of bovine virus diarrhoea or border disease viruses by immunofluorescence or immunoperoxidase techniques. If possible, cells should be prepared and screened prior to vaccine production, and a stock stored in sterile DMSO (dimethyl sulphoxide) in liquid nitrogen (1–2 ml aliquots containing $20 \times 10^6$ cells/ml). Serum used in the growth medium must be free from antibody to capripoxvirus or contamination with pestivirus.

Virus: Seed virus and final vaccine must be titrated in tissue culture tubes or microtitre plates. Vaccine samples must be examined for the presence of adventitious viruses including cytopathic and noncytopathic strains of pestivirus, and should be mixed with a high-titre capripoxvirus-immune serum that has tested negative for antibody to pestivirus to prevent the vaccine virus itself interfering with the test. The vaccine bulk can be held at −20°C until all sterility tests and titrations have been completed, at which time it should be freeze-dried in 1 ml aliquots in vials sufficient for 100 doses. The vaccine harvest diluted with lactalbumin hydrolysate and sucrose should have a minimum titre $\log_{10} 4.5$ TCID$_{50}$ per ml after freeze-drying, equivalent to a field dose of $\log_{10} 2.5$ TCID$_{50}$. A further titration is carried out on five randomly chosen vials of the freeze-dried preparation to confirm the titre.

4. Batch control

a) Sterility

Tests for sterility and freedom from contamination of biological materials may be found in Chapter 1.1.9.

b) Safety and efficacy

Four sheep and four goats of known susceptibility to capripox are placed in a high containment level animal unit and serum samples are collected. Five randomly chosen vials of the freeze-dried vaccine are reconstituted in 1 ml of sterile PBS each, and pooled. One sheep and one goat are inoculated intradermally with 0.2 ml of the concentrated vaccine. The remaining vaccine is diluted 20 times with sterile PBS and two sheep and two goats are inoculated subcutaneously with 0.2 ml – the recommended field dose. The remaining sheep and goat are control animals. The animals are clinically examined daily and rectal temperatures are recorded. On day 21 after vaccination, the eight animals are again serum sampled and challenged with a known virulent capripoxvirus strain by intradermal inoculation. The clinical response is recorded during the following 14 days. Control animals should develop the typical clinical signs of capripox, whereas there should be no local or systemic reaction in the vaccinates other than a delayed-type hypersensitivity reaction, which will disappear within 4 days. Serum samples are again collected on day 30 after vaccination. The day 21 serum samples are examined for seroconversion to selected viral diseases that could have contaminated the vaccine, and the day 0 and 30 samples are compared to confirm the absence of antibody to pestivirus.

The fully reconstituted vaccine is also tested in mice and guinea-pigs. Two guinea-pigs are inoculated intramuscularly with 0.5 ml into the hind leg, and two guinea-pigs and six mice are inoculated intraperitoneally with 0.5 ml and 0.1 ml, respectively. Two guinea-pigs and four mice are kept as uninoculated controls. The animals are observed for 3 weeks, humanely killed and a post-mortem examination is carried out. There should be no evidence of pathology due to the vaccine.
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**c) Potency tests**

Less than 1 TCID$_{50}$ of the 0240 strain is sufficient to immunise a sheep or goat. However, potency tests must be undertaken for other strains of capripoxvirus if the minimum immunising dose is not known. This is usually carried out by comparing the titre of a virulent challenge virus on the flanks of vaccinated and control animals. Following vaccination, the flanks of at least three animals and three controls are shaved of wool or hair. Log$_{10}$ dilutions of the challenge virus are prepared in sterile PBS and six dilutions are inoculated intradermally (0.1 ml per inoculum) along the length of the flank; four replicates of each dilution are inoculated down the flank. An oedematous swelling will develop at possibly all 24 inoculation sites on the control animals, although preferably there will be little or no reaction at the four sites of the most dilute inocula. The vaccinated animals should develop an initial hypersensitivity reaction at sites of inoculation within 24 hours, which should quickly subside. Small areas of necrosis may develop at the inoculation site of the most concentrated challenge virus. The macule/papule is measured at between 8 and 10 days post-challenge. The titre of the challenge virus is calculated for the vaccinated and control animals; a difference of log titre $>$log$_{10}$ 2.5 is taken as evidence of protection.

**d) Duration of immunity**

Immunity to virulent field virus following vaccination of sheep or goats with the 0240 strain lasts over 1 year, and protection against generalised infection following intradermal challenge lasts at least 3 years and is effective lifelong. The duration of immunity produced by other vaccine strains should be ascertained in both sheep and goats by undertaking controlled trials in an environment in which there is no possibility of field strains of capripoxvirus confusing the results.

The inactivated vaccines provide immunity for less than 1 year, and for the reasons given at the beginning of this section, may not give immunity to the form of capripoxvirus usually associated with natural transmission.

**e) Stability**

Properly freeze-dried preparations of capripox vaccine, particularly those that include a protectant, such as sucrose and lactalbumin hydrolysate, are stable for over 25 years when stored at –20°C and for 2–4 years when stored at 4°C. There is evidence that they are stable at higher temperatures, but no long-term controlled experiments have been reported.

The inactivated vaccines must be stored at 4°C, and their shelf life is usually given as 1 year.

**f) Preservatives**

No preservatives other than a protectant, such as sucrose and lactalbumin hydrolysate, are required for the freeze-dried preparation.

**g) Precautions (hazards)**

There are no precautions other than those described above for sterility and freedom from adventitious agents. The 0240 vaccine strain should not be used in *Bos taurus* breeds of cattle.

Capripoxvirus is not infectious to humans.

5. Tests on the final product

**a) Safety**

Safety tests should be carried out on the final product of each batch as described in Section C.4.b.

**b) Potency**

Once the potency of the particular strain being used for vaccine production has been determined in terms of minimum dose required to provide immunity, it is not necessary to repeat this on the final product of each batch, provided the titre of virus present has been ascertained.

**REFERENCES**

Chapter 2.7.14. — Sheep pox and goat pox


* * *

**NB:** There are OIE Reference Laboratories for sheep pox and goat pox (see Table in Part 3 of this *Terrestrial Manual* or consult the OIE Web site for the most up-to-date list: www.oie.int).
SECTION 2.8.

SUIDAE

CHAPTER 2.8.1.

AFRICAN SWINE FEVER

SUMMARY

African swine fever (ASF) is an infectious disease of domestic and wild pigs of all breeds and ages, caused by a virus that produces a range of syndromes. Acute disease is characterised by high fever, haemorrhages in the reticuloendothelial system, and a high mortality rate. Soft ticks of the Ornithodoros genus, especially O. moubata and O. erraticus, have been shown to be both reservoirs and transmission vectors of ASF virus (ASFV).

ASFV is the only member of the Asfarviridae family, genus Asfivirus.

Laboratory diagnostic procedures for ASF fall into two groups: the first contains the tests for virus isolation and the detection of virus antigens and genomic DNA, while the second contains the tests for antibody detection. The selection of the tests to be carried out depends on the disease situation and laboratory diagnostic capacity in the area or country.

Identification of the agent: Laboratory diagnosis must be directed towards isolation of the virus by simultaneously carrying out the inoculation of pig leukocyte or bone marrow cultures, the detection of antigen in smears or cryostat sections of tissues by fluorescent antibody test (FAT) and the detection of genomic DNA by the polymerase chain reaction (PCR). The PCR is an excellent, highly sensitive, and rapid technique for ASFV detection and it is very useful under a wide range of circumstances. It is especially useful if the tissues are unsuitable for virus isolation and antigen detection.

In doubtful cases, the material is passaged in leukocytes cell cultures and the procedures described above are repeated.

Serological tests: Pigs that survive natural infection usually develop antibodies against ASFV from 7–10 days post-infection and these antibodies persist for long periods of time. Where the disease is endemic, or where a primary outbreak is caused by a strain of low virulence, the investigation of new outbreaks should include the detection of specific antibodies in serum or extracts of the tissues submitted. A variety of methods such as indirect fluorescent antibody (IFA), the enzyme-linked immunosorbent assay (ELISA) and the immunoblotting test are available for antibody detection.

Requirements for vaccines and diagnostic biologicals: At present, there is no vaccine for ASF.

A. INTRODUCTION

African swine fever virus (ASFV) is a complex icosahedral enveloped DNA virus that exhibits many features common to both Iridovirus and Poxvirus families (4, 28). This virus is currently classified as the only member of a family called Asfarviridae. At least 28 structural proteins have been identified in intracellular virus particles (200 nm) (26). More than a hundred infectious proteins have been identified in infected porcine macrophages, and at least 50 of them react with sera from infected or recovered pigs. The virus genome comprises between 170 and 192 kilobases (kb), with a conserved central region of about 125 kb and variable ends. These variable regions encode five multigene families that are directly involved with the variability of the virus genome (7).
complete analysis of the sequence of several ASFV strains has been accomplished. Different strains of ASFV vary in their ability to cause disease, but at present there is only one recognised serotype of the virus detectable by antibody tests.

The molecular epidemiology of the disease is investigated firstly by partial sequencing of the VP72 gene, which differentiates up to 22 distinct genotypes (9, 16), followed by intra-genotypic resolution of viral relationships by analysis of the variable regions located in the conserved central region of ASFV genome (18, 23).

ASF viruses produce a range of syndromes varying from peracute, acute to chronic disease and apparently healthy virus carriers. Pigs are the only domestic animal species that is naturally infected by ASFV. European wild boars and feral pigs are also susceptible to the disease, exhibiting clinical signs and mortality rates similar to those observed in domestic pigs. In contrast African wild pigs such as warthogs (Phacochoerus aethiopicus), bush pigs (Potamochoerus porcus) and giant forest hogs (Hylochoerus meinertzhageni) are resistant to the disease and show few or no clinical signs. These species of wild pigs act as reservoir hosts of ASFV in Africa (26).

The incubation period is usually 3–15 days. The more virulent strains produce peracute or acute haemorrhagic disease characterised by high fever, loss of appetite, haemorrhages in the skin and internal organs, and death in 3–10 days, sometimes even before the first clinical signs are observed. Mortality rates may be as high as 100%. Less virulent strains produce mild clinical signs – slight fever, reduced appetite and depression – which can be readily confused with many other conditions in pigs and may not lead to suspicion of ASF. Low virulent, nonhaemadsorbing strains occasionally produce mainly subclinical nonhaemorrhagic infection and seroconversion, but some animals may develop discrete lesions in the lungs or on the skin in areas over bony protrusions and other areas subject to trauma. Animals which have recovered from either acute or chronic infections may become persistently infected, acting as virus carriers. The biological base for the persistence of ASFV is still not well understood (10). Recovered ASFV carrier pigs and persistently infected wild pigs constitute the biggest problems in controlling the disease. The serological recognition of carrier pigs has been vital for the success of eradication programmes (5).

ASF cannot be differentiated from classical swine fever (hog cholera; CSF) by either clinical or post-mortem examination, and both diseases should be considered in the differential diagnosis of any acute febrile haemorrhagic syndrome of pigs. Bacterial septicaemias may also be confused with ASF and CSF. Laboratory tests are essential to distinguish between these diseases.

In countries free from ASF but suspecting its presence, the laboratory diagnosis must be directed towards isolation of the virus by simultaneously carrying out the inoculation of pig leukocyte or bone marrow cultures, the detection of antigen in smears or cryostat sections of tissues by the fluorescent antibody test (FAT) and, by the detection of genomic DNA by the polymerase chain reaction (PCR), which is the most sensitive technique for detecting the presence of the agent in persistently infected animals and is particularly useful if samples submitted are unsuitable for virus isolation and antigen detection because they have undergone putrefaction. ASFV can be detected by PCR from a very early stage of infection in tissues, ethylene diamine tetra-acetic acid (EDTA)-blood and serum samples. Pigs recovered from acute or chronic infections usually exhibit a viraemia for several weeks making the PCR test a very useful tool for the detection of ASFV in pigs infected with low or moderately virulent strains.

As no vaccine is available, the presence of ASFV antibodies is indicative of previous infection and, as antibodies are produced from the first week of infection and persist for long periods, they are a good marker for the diagnosis of the disease. The early appearance (from 7 to 10 days post-infection) and subsequent long-term persistence of antibodies make antibody detection techniques, such as ELISA, immunoblotting or IFA test, very useful in diagnosing the subacute and chronic forms of disease.

In regions where Ornithodorus soft ticks are present, the detection of ASFV in these reservoirs of infection contributes to a better understanding of the epidemiology of the disease. This is of major importance in establishing effective control and eradication programmes (6).

**B. DIAGNOSTIC TECHNIQUES**

1. **Identification of the agent**

Where ASF is suspected, the following samples should be sent to the laboratory: blood in anticoagulant (EDTA), spleen, lymph nodes, tonsil and kidney. These should be kept as cold as possible, without freezing, during transit. After the samples arrive at the laboratory, they should be stored at –70°C if processing is going to be delayed. As maintaining a cold chain is not always possible, samples can be submitted in glycerosaline; this may slightly decrease the likelihood of virus identification, but it may facilitate the submission of samples to the laboratory so that an outbreak can be confirmed.
Sample preparation for haemadsorption and pig inoculation

i) Prepare suspensions of tissues by grinding 0.5–1.0 g pieces with a pestle and mortar containing sterile sand, then add 5–10 ml of a buffered salt solution or tissue culture medium containing antibiotics.

ii) Clarify the suspensions by centrifugation at 1000 \( g \) for 5 minutes.

Use the supernatant for haemadsorption (Section B.1.a below) and pig inoculation (Section B.1.d below), although pig inoculation is not recommended.

Haemadsorption test

The haemadsorption (HAD) test (17) is based on the fact that pig erythrocytes will adhere to the surface of pig monocyte or macrophage cells infected with ASFV and that most virus isolates produce this phenomenon of haemadsorption. A positive result in the HAD test is definitive for ASF diagnosis. A very small number of ‘nonhaemadsorbing’ viruses have been isolated, most of which are avirulent, but some do produce typical acute ASF. The test is carried out by inoculating blood or tissue suspensions from suspect pigs into primary leukocyte cultures, (Procedure 1 below) or into alveolar macrophages cell cultures, and also by preparing leukocyte cultures from the blood of pigs inoculated at the laboratory or from the blood of suspect pigs collected in the field (Procedure 2 below). Up to 300 cultures in tubes can be prepared from each 100 ml of defibrinated or heparinised blood collected. It is essential to carry out all procedures in such a way as to prevent contamination of the cultures.

Procedure 1: Haemadsorption test in primary leukocyte cultures

i) Collect the required volume of fresh defibrinated or heparinised (100 International Units [IU/ml blood]) pig blood.

ii) Centrifuge at 700 \( g \) for 30 minutes and collect the buffy coat cells. Add three volumes of 0.83% ammonium chloride to the leukocytes obtained. Mix and incubate at room temperature for 15 minutes. Centrifuge at 650 \( g \) for 15 minutes and carefully remove the supernatant. Wash pellet in medium or phosphate buffered saline (PBS).

iii) Resuspend the cells at a concentration of \( 10^6–10^7 \) cells/ml in tissue culture medium containing 10–30% pig serum and antibiotics. In order to prevent nonspecific haemadsorption, the medium should contain serum or plasma from the same pig from which the leukocytes were obtained. If a large volume of samples is to be tested, the homologous serum can be replaced by serum that has been identified by pre-screening as capable of preventing the nonspecific auto-rosette formation.

iv) Dispense the cell suspension in aliquots of 1.5 ml in 160 × 16 mm tubes and incubate in a sloping position (5–10° from the horizontal) at 37°C. This procedure can also be performed in 96-well plates with 200 µl of \( 10^6–10^7 \) cells/ml per well.

Note: For routine diagnosis, only 2–4-day-old cultures are sufficiently sensitive.

v) Inoculate three tubes or well plates by adding 0.2 ml/tube or 0.02 ml (1/10 final dilution)/well of prepared samples. It is advisable to inoculate ten-fold and hundred-fold dilutions into cultures, and this is especially important when the field material submitted is in poor condition.

vi) Inoculate positive control cultures with haemadsorbing virus. Uninoculated negative controls are essential to monitor the possibility of nonspecific haemadsorption.

vii) After 3 days, add 0.2 ml of a fresh preparation of 1% pig erythrocytes in buffered saline to each tube. In the case of the 96-well plates, after 1 day add 0.02 ml of 1% pig erythrocytes.

viii) Examine the cultures daily for 7–10 days under a microscope for cytopathic effect (CPE) and haemadsorption.

ix) Reading the results: Haemadsorption consists of the attachment of large numbers of pig erythrocytes to the surface of infected cells. A CPE consisting of a reduction in the number of adherent cells in the absence of haemadsorption may be due to the cytotoxicity of the inoculum, Aujeszky’s disease virus or nonhaemadsorbing ASFV, which can be detected by the FAT on the cell sediment or by use of PCR (see below). If no change is observed, or if the results of the immunofluorescence and PCR tests are negative, subinoculate the supernatant up to three times into fresh leukocyte cultures. All isolations should be confirmed by PCR and sequencing.

Procedure 2: Haemadsorption ‘autorosette’ test with peripheral blood leukocytes from infected pigs

This procedure is quicker than the preparation and inoculation of primary pig leukocyte cultures (described in Procedure 1 above) and will give more rapid results in positive cases. It can be performed in laboratories that are not equipped for routine virological examinations; the minimum requirements are slides and coverslips, a microscope and sterile medium, tubes or bottles and pipettes. Blood from suspect pigs in the field,
or those inoculated in the laboratory, is collected in heparin and leukocyte cultures are prepared for direct examination for haemadsorption. However, the results of the test are difficult to evaluate and it is now being replaced by the PCR.

i) Collect 20 ml of whole blood in a syringe containing 2000 IU heparin in 2 ml of saline, mix and transfer to a glass tube or narrow bottle.

ii) Place the tube/bottle vertically in an incubator or water bath at 37°C, and allow the cells to settle. Sedimentation is improved by the addition of 2 ml of a plasma volume expander, such as 'Dextravan 150' which is a solution of Dextran 150 in 0.9% NaCl for injection (Fisons, United Kingdom).

iii) Incubate the cultures for 6–8 hours at 37°C, and then examine the cultures at 2–3-hour intervals by transferring small aliquots of the white-cell-rich supernatant, together with some erythrocytes, on to a glass slide.

iv) Reading the results: The presence of haemadsorbing cells identified under a microscope indicates the presence of ASFV. Haemadsorption consists of the attachment of large numbers of pig erythrocytes to the surface of infected cells. Any evidence of haemadsorption would be enough to consider repeating the assay or confirming the presence of ASFV by another test such as PCR.

b) Antigen detection by fluorescent antibody test

The FAT (8) can be used as an additional method to detect antigen in tissues of suspect pigs in the field or those inoculated at the laboratory. Positive FAT plus clinical signs and appropriate lesions can provide a presumptive diagnosis of ASF. It can also be used to detect ASFV antigen in leukocyte cultures in which no HAD is observed and can thus identify nonhaemadsorbing strains of virus. It also distinguishes between the CPE produced by ASFV and that produced by other viruses, such as Aujeszky’s disease virus or a cytotoxic inoculum. However, it is important to note that in subacute and chronic disease, FAT has a significantly decreased sensitivity. This reduction in sensitivity may be related to the formation of antigen-antibody complexes in the tissues of infected pigs which block the interaction between the ASFV antigen and ASF conjugate (26).

• Test procedure

i) Prepare cryostat sections or impression smears of test tissues, or spreads of cell sediment from inoculated leukocyte cultures on slides, air dry and fix with acetone for 10 minutes at room temperature.

ii) Stain with fluorescein isothiocyanate (FITC)-conjugated anti-ASFV immunoglobulin at the recommended or pretitrated dilution for 1 hour at 37°C in a humid chamber.

iii) Fix and stain positive and negative control preparations similarly.

iv) Wash by immersing four times in fresh clean PBS, mount stained tissues in PBS/glycerol, and examine under an ultraviolet light microscope with suitable barrier and exciter filters.

v) Reading the results: Tissues are positive if specific granular cytoplasmic fluorescence is observed in paracortical tissue of lymphoid organs or in fixed macrophages in other organs.

c) Detection of virus genome by the polymerase chain reaction

PCR techniques have been developed, using primers from a highly conserved region of the genome, to detect and identify a wide range of isolates belonging to all the known virus genotypes, including both nonhaemadsorbing viruses and isolates of low virulence. The PCR techniques are particularly useful for identifying virus DNA in pig tissues that are unsuitable for virus isolation or antigen detection because they have undergone putrefaction, or when there is good reason to believe that virus may have been inactivated before samples are received in the laboratory.

Three validated PCR procedures are described and consist of a sample preparation followed by the test procedure. These procedures serve as a general guideline and a starting point for the PCR protocol. Optimal reaction conditions (incubation times and temperatures, models and suppliers of equipment, concentrations of assay reagents such as the primers and dNTPs) may vary so the described conditions should be evaluated first.

• Sample preparation procedure 1

This sample preparation procedure is simple and inexpensive, but may produce false-negative results due to the presence of PCR inhibitors (14).

i) Prepare suspensions of tissue by grinding up small pieces of tissue with a pestle and mortar containing sterile sand, and make a 1/10 dilution by adding 5–10 ml of PBS containing 1% ox serum and antibiotics.
ii) Centrifuge at 500 g for 5 minutes.

iii) Extraction for control samples: 1/10 tissue homogenates (same tissue as the samples to be analysed):
(a) a negative control: use 500 µl of a homogenate of ASFV-negative tissue; (b) a positive control: use 500 µl of a homogenate of ASFV-positive tissue.

iv) Transfer 500 µl to a screw-capped Eppendorf tube and boil for 10 minutes.

v) Centrifuge at 13,000 g in a microfuge for 5 minutes.

The resultant tissue supernatant can be used directly in the PCR assay.

• Sample preparation procedure 2 (1, 2)

An alternative more expensive but more sensitive extraction procedure using the commercial High Pure PCR Template Preparation Kit (Roche Diagnostics) is described below. A number of other DNA extraction kits are commercially available for the preparation of template suitable for PCR depending on the sample submitted for analysis and may be appropriate for use. Different samples can be used in this procedure such as cell culture supernatants, EDTA-blood, serum and tissue homogenates, even if the latter have been kept in warm conditions and undergone a degree of putrefaction. This procedure has the advantage in that it can be used for both the extraction of ASFV DNA and CSFV RNA which enables the simultaneous detection of both viruses in a multiplex PCR assay (2).

The High Pure PCR Template Preparation Kit (Roche Diagnostics) includes the following reagents: Binding Buffer, Proteinase K, Inhibitor Removal Buffer, Wash Buffer, and High Pure Filter Tubes and collection tubes.

For organ and tissue samples, first prepare a 1/10 homogenate of the material in PBS, then centrifuge to clarify at 12,000 g for 5 minutes. Extract DNA/RNA from the resultant supernatant fluid. Sometimes it is recommended to process a 1/10 dilution of the supernatant in parallel.

Extraction for control samples: 1/10 tissue homogenates (same tissue as samples to be analysed): (a) a negative control: use 200 µl of a homogenate of ASFV-negative tissue; (b) a positive control: use 200 µl of a homogenate of ASFV-positive tissue. Process both controls together with the test samples.

i) Pipette 200 µl of sample into a 1.5 ml microcentrifuge tube.

ii) Add 200 µl of binding buffer and 40 µl of proteinase K. Mix immediately. Incubate for 10 minutes at 72°C.

iii) Briefly centrifuge the 1.5 ml microcentrifuge tube to remove drops from the inside of the lid.

iv) Add 100 µl of isopropanol to the sample tube.

v) Place the High Pure filter tube in a collection tube and pipette the sample in the upper reservoir. Centrifuge for 1 minute at 8000 rpm. (With blood samples, repeat the centrifugation step if sample remains in the filter tube.)

vi) Discard the collection tube and place the filter tube into a clean collection tube.

vii) Add 500 µl of Inhibitor Removal Buffer to the upper reservoir and centrifuge for 1 minute at 8000 rpm.

viii) Discard the collection tube and place the filter tube into a clean collection tube.

ix) Add 450 µl of wash buffer to the upper reservoir and centrifuge for 1 minute at 8000 rpm.

x) Discard the collection tube and repeat the washing step.

xi) Discard the collection tube and place the filter tube into a clean collection tube. Centrifuge for 10 seconds at 13000 rpm to remove residual wash buffer.

xii) Discard the collection tube and place the filter tube in a clean 1.5 ml microcentrifuge tube.

xiii) For the elution of nucleic acids, add 50 µl of prewarmed (70°C) sterile water to the upper reservoir (be careful not to use the Elution Buffer included in the kit for CSFV RNA). Centrifuge for 1 minute at 8000 rpm.

xiv) Use immediately or store at –20°C for future use.

• PCR Procedure 1 (validated for use with sample preparation procedure 1 and 2)

• Stock solutions

i) Taq DNA polymerase and PCR amplification buffer (10×) are commercially available.

ii) Stock 1.25 mM dNTP: Prepare 50 mM stock solutions of each of the following nucleotides: dATP, dCTP, dGTP and dTTP. Add 10 µl of each of these stock solutions to 360 µl sterile distilled water.
iii) Primers at a concentration of 20 pmol/µl: Primer 1 sequence 5’-ATGGA-TACCG-AGGGA-ATAGC-3’ (positive strand); Primer 2 sequence 5’-CTTAC-CGATG-AAAAT-GATAC-3’ (negative strand).

iv) Loading buffer: 0.25% Orange G in an aqueous solution of 30% glycerol.

v) TAE buffer (50×) for agarose gel: Tris base (242 g); glacial acetic acid (57.1 ml); 0.5 M EDTA, pH 8.0 (100 ml); distilled water (to 1 litre).

vi) Marker DNA: 100 base-pair ladder is commercially available.

• PCR amplification assay
   i) Add the following reagents to the required number of 0.75 ml polypropyl Eppendorf tubes:
   Sterile distilled water (24.5 µl); (10× conc.) PCR amplification buffer (5 µl); magnesium chloride 25 mM (4 µl); 1.25 mM stock dNTP solution (8 µl); primer 1, 20 µM (1 µl); primer 2, 20 µM (1 µl); Taq DNA polymerase 5 U/µl (0.25 µl), tissue supernatant (10 µl)
   ii) Control tubes contain no tissue supernatant.
   Negative control (no DNA): Add 10 µl of distilled water.
   Positive control: Add 2 µl of ASFV DNA and 8 µl of distilled water.
   iii) Overlay the mixture with 60 µl of mineral oil if necessary.
   iv) Place all the tubes in an automated DNA thermal cycler and run the following programme:
   One cycle at 94°C for 5 minutes.
   Thirty-five cycles at 94°C for 1 minute, 50°C for 1 minute and 72°C for 1 minute.
   One cycle at 72°C for 10 minutes.
   Hold at 4°C.
   v) At the end of the programme, carefully remove 20 µl of each reaction mixture from below the mineral oil, transfer to a clean tube and add 2 µl of loading buffer.
   vi) Load all the samples in a 2% agarose gel in TAE buffer containing ethidium bromide at a final concentration of 0.5 µg/ml.
   Add marker DNA to one lane on each side of the gel.
   vii) Run the gel at a constant voltage of 100 volts for 20–30 minutes.
   viii) Reading the results: Examine the gel over a UV light source. In a positive sample, a discrete band will be present that should co-migrate with the PCR product of the positive control. Calculate the size of the PCR products in the test samples and the positive control by reference to the standard markers.
   The PCR product of the positive control has a size of 278 base pairs. No bands should be seen in the negative control.

• PCR Procedure 2 (validated for use with sample preparation procedure 2) (1)
   In order to gain maximum levels of sensitivity this PCR method described in reference 1 is recommended for use with sample preparation procedure 2. The ASFV primer set described in this procedure can be combined with a specific primer set for CSFV in a multiplex RT-PCR method that allows the simultaneous and differential detection of both virus genomes in a single reaction (2).

• Stock solutions
   Nuclease-free sterile water.
   Taq Gold DNA polymerase, 10× PCR Buffer II, and magnesium chloride are commercially available from Applied Biosystems.
   PCR nucleotide mix containing 10 mM of each dNTP is commercially available from Roche Diagnostics.
   Primers at a concentration of 20 pmol/µl: Primer 1 sequence 5’-AGT-TAT-GGG-AAA-CCC-GAC-CC-3’ (forward primer); primer 2 sequence 5’-CCC-TGA-ATC-GGA-GCA-TCC-T-3’ (reverse primer).
   10× Loading buffer: 0.2% xylene cyanol, 0.2% bromophenol blue, 30% glycerol.
   TAE buffer (50×) for agarose gel: Tris base (242 g); glacial acetic acid (57.1 ml); 0.5 M EDTA, pH 8.0 (100 ml); distilled water (to 1 litre).
   Marker DNA: 100 base-pair ladder is commercially available.

• PCR amplification assay
   i) In a sterile 1.5 ml microcentrifuge tube prepare the PCR reaction mixture described below for each sample. Prepare the reaction mixture in bulk for the number of samples to be assayed allowing for one extra sample.
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ii) Nuclease-free or sterile distilled water (17.375 µl), 10 × PCR Buffer II (2.5 µl), magnesium chloride 25 mM (2 µl), dNTP mix 10mM (0.5 µl), primer 1, 20 pmol/µl (0.25 µl), primer 2, 20 pmol/µl (0.25 µl), Taq Gold DNA polymerase 5 U/µl (0.125 µl).
iii) Add 23 µl of the PCR reaction mix to the required number of 0.2 ml PCR tubes.
iv) Add 2 µl of extracted sample template to each PCR tube. Include a positive reaction control (2 µl of ASFV DNA) and a negative reaction control (2 µl of distilled water) for each PCR run.
v) Place all the tubes in an automated thermal cycler and run the following programme:
   One cycle at 95°C for 10 minutes.
   40 cycles at 95°C for 15 seconds, 62°C for 30 seconds and 72°C for 30 seconds.
   One cycle at 72°C for 7 minutes.
   Hold at 4°C.
vi) At the end of the programme, remove PCR tubes and add 2.5 µl of 10 × loading buffer to each tube.
vii) Load all the samples in a 2% agarose gel in TAE buffer containing ethidium bromide at a final concentration of 0.5 µg/ml.
viii) Add marker DNA to one lane on each side of the gel.
ix) Run the gel at a constant voltage of 150-200 volts for about 30 minutes.

Reading the results: Examine the gel over a UV light source. In a positive sample, a discrete band will be present that should co-migrate with the PCR product of the positive control. Calculate the size of the PCR products in the test samples and the positive control by reference to the standard markers. The PCR product of the positive control has a size of 257 base pairs. No bands should be seen in the negative control.
x) Optional: An additional confirmatory assay could be performed by BsmA I restriction endonuclease digestion of the amplified products. For this assay, incubate for 2.5 hours at 55°C a total of 5 µl of amplified DNA product in a final volume of 20 µl digestion mix: 2 µl of 10 × buffer, 1 µl of BsmA I (5 U/µl) and 12 µl of sterile distilled water. Then, run the samples in a 3% agarose gel as described above. The restriction pattern should include two fragments of 173–177 and 84–80 base pairs in the positive samples.

• PCR Procedure 3: TaqMan® PCR protocol (15, 30)
  • Sample preparation
This PCR method is described in reference 15. A number of other DNA extraction kits are commercially available for the preparation of template suitable for PCR depending on the sample submitted for analysis and may be appropriate for use in Reference Laboratories.

The QIAamp® Viral RNA Mini Kit (QIAGEN) procedure (spin protocol) is described below. This kit can be used for blood from suspected swine fever animals. Blood from infected swine should be taken in EDTA. In these cases, detection of ASFV can be performed in parallel to CSF virus (see Chapter 2.8.3 for CSFV molecular detection methods).

i) Pipette 560 µl of the supplied buffer AVL into a 1.5 ml microcentrifuge tube.
ii) Add 140 µl of test or control sample and mix by pulse-vortexing for about 15 seconds. Negative ASF control samples consisting of spleen homogenates from uninfected pigs and uninfected porcine bone marrow (PBM) and peripheral blood mononuclear (PBL) cells should be processed alongside the test samples. Additional extraction negative controls can also be prepared for each test sample and uninfected negative control by running parallel extractions of nuclease-free water (all controls should subsequently be assayed by the PCR procedure along with the test samples).
iii) Incubate at room temperature for at least 10 minutes.
iv) Briefly centrifuge the 1.5 ml microcentrifuge tube to remove drops from the inside of the lid.
v) Add 560 µl ethanol to the sample, pulse-vortex for approximately 15 seconds and briefly centrifuge to remove drops from the inside of the lid.
v) Add 630 µl of the solution from v) to a QIAamp spin column (in a 2-ml collection tube) without wetting the rim. Close the cap and centrifuge for 1 minute at 6000 g. Place the spin column into a clean 2 ml collection tube and discard the tube containing the filtrate.
vii) Carefully open the QIAamp spin column and repeat step vi.
viii) Carefully open the QIAamp spin column and add 500 µl of Buffer AW1. Close the cap and centrifuge for 1 minute at 6000 g. Place the spin column into a clean 2-ml collection tube and discard the tube containing the filtrate.

ix) Carefully open the QIAamp spin column and add 500 µl of Buffer AW2. Close the cap and centrifuge for 3 minutes at 20,000 g.

x) Place the QIAamp spin column in a new 1.5 ml microcentrifuge tube. Discard the old collection tube containing the filtrate. Carefully open the QIAamp spin column and add 60 µl of Buffer AVE. Close the cap and incubate at room temperature for 1 minute. Centrifuge for 1 minute at 6000 g.

xi) Discard the QIAamp spin column. Store the extracted DNA (60 µl) at –20°C until required for PCR amplification procedure.

• Stock solutions
  
i) Nuclease-free or another appropriate sterile water and TaqMan® PCR reaction master mix (2×).
  
ii) Primers at a concentration of 50 pmol/µl: Primer 1 sequence 5’-CTGCT-CATGG-TATCA-ATCTT-ATCGA-3’ (positive strand); Primer 2 sequence 5’-GATAC-CACAA-GATC(AG)-GCCGT-3’ (negative strand).
  
iii) TaqMan® probe at a concentration of 5 pmol/µl: (5’-[6-carboxy-fluorescein (FAM)]-CCACG-GAGG-AATAC-CAACC-CAGTG-3’-[6-carboxy-tetramethyl-rhodamine (TAMRA)].

• PCR amplification by TaqMan® assay (15)
  
i) In a sterile 1.5 ml microcentrifuge tube prepare the PCR reaction mixture described below for each sample. Prepare the reaction mixture in bulk for the number of samples to be assayed but allowing for one extra sample.

  Nuclease-free or sterile water (7.5 µl); (2× conc.) TaqMan® PCR reaction master mix (12.5 µl); primer 1, 50 pmol (1.0 µl); primer 2, 50 pmol (1.0 µl); TaqMan® probe, 5 pmol (1 µl).

  ii) Add 22 µl PCR reaction mix to a well of a MicroAmp® optical reaction plate for each sample to be assayed.

  iii) Add 3 µl of extracted sample template or blank extraction control and securely cover each well with a cap.

  iv) Spin the plate for 1 minute in a suitable centrifuge to mix the contents of each well.

  v) Place the plate in a TaqMan® Sequence Detection System for PCR amplification and run the following programme:

    One cycle at 50°C for 2 minutes,
    One cycle at 95°C for 10 minutes,
    Forty cycles at 95°C for 15 seconds, 58°C for 1 minute.

    Note: If a TaqMan® thermal cycler is not available, an ordinary thermal cycler can be used and the PCR products analysed by end-point fluorescence readers or alternatively by electrophoresis on a 1.5% agarose gel. A product of 250 bp is expected.

  vi) Reading the results: Assign a threshold cycle (C_T) value to each PCR reaction from a scan of all amplification plots (a plot of the fluorescence signal versus cycle number). Negative test samples, uninfected negative or extraction blank controls should have a C_T value >40.0. Positive test samples and controls should have a C_T value < 40.0 (strongly positive samples have a C_T value <30.0).

d) Pig inoculation

In the past, pig inoculation has been used to differentiate between CSF and ASF, as these diseases produce indistinguishable clinical signs. Two groups of pigs, one unvaccinated and the other vaccinated against CSF have been used in the in vivo experiments. Pig inoculation is now unlikely to be necessary because there are alternative laboratory tests that give reliable results for both ASF and CSF. The pig inoculation test is slow, expensive and difficult to perform and results in acute distress for the animals involved, which raises serious animal welfare concerns. It is therefore no longer recommended for use.

2. Serological tests

Antibodies persist in recovered pigs for long periods after infection, sometimes for life, and a number of tests are available for detecting these antibodies, although only a few of them have been developed for routine use in diagnostic laboratories (3, 11, 19, 21, 25). The most commonly used is the ELISA (27, 29), which is suitable for
examining either serum or fluid from the tissues. Confirmatory testing of ELISA-positive samples should be carried out in critical cases using an alternative test, such as the IFA test (24), immunoperoxidase staining or immunoblotting (11, 22). Antibody is usually not detected in pigs infected with virulent ASFV as they die before it is produced. Antibodies are produced in pigs infected with low or moderately virulent ASF viruses, but these are not fully neutralising antibodies.

Where ASF is endemic, confirmation of suspected cases of disease is best done using a standard serological test (ELISA), combined with an alternative serological test (IFA) or an antigen-detection test. In some countries, over 95% of positive cases have been identified using a combination of IFA tests and FAT (26).

It should be noted that when pigs have been infected with avirulent isolates or those of low virulence, serological tests may be the only way of detecting infected animals.

Both the counter immunoelectrophoresis test and ELISA can be used for the large-scale screening of sera, although the ELISA is more sensitive for detecting individual positive sera and has been used extensively as part of eradication programmes (5).

The method used depends on the staff and facilities available.

a) **Enzyme-linked immunosorbent assay (the prescribed test for international trade)**

The ELISA (3, 21) is a direct test that can detect antibodies to ASFV in pigs that have been infected by viruses of low or moderate virulence. Commercial ELISA kits are available that show high levels of specificity and sensitivity. A cheaper alternative is to prepare a soluble antigen for use in an indirect ELISA, and the procedure using this soluble antigen is described below.

ELISAs show a decreased sensitivity when the serum samples to be tested are poorly preserved. To solve this problem, several new ELISAs based on the use of ASFV recombinant proteins are now being validated (13).

Carrying out a second confirmatory test such as the immunoblotting test or the IFA test described below is recommended in the case of a doubtful result or a positive result when sera are suspected to be poorly preserved.

- **Antigen preparation for ELISA**

  The ELISA antigen is prepared from infected cells grown in the presence of pig serum (12).

  i) Infect MS (monkey stable) cells at multiplicity of infection of 10 with adapted virus, and incubate in medium containing 2% pig serum.

  ii) Harvest the cells at 36–48 hours post-infection, when the CPE is extensive. Wash in PBS, sediment at 650 \( g \) for 5 minutes, wash the cell pellet in 0.34 M sucrose in 5 mM Tris/HCl, pH 8.0, and centrifuge to pellet cells.

  Carry out steps (iii) to (v) on ice:

  iii) Resuspend the cell pellet in 67 mM sucrose in 5 mM Tris/HCl, pH 8.0 (1.8 ml per 175 cm\(^2\) flask), and leave for 10 minutes with agitation after 5 minutes.

  iv) Add nonionic detergent Nonidet P-40 to a final concentration of 1% (w/v), and leave for 10 minutes (with agitation after 5 minutes) to lyse the cells.

  v) Add sucrose to a final concentration of 64% (w/w) in 0.4 M Tris/HCl, pH 8.0, and centrifuge at 1000 \( g \) for 10 minutes to pellet nuclei.

  vi) Collect the supernatant and add EDTA (2 mM final concentration), beta-mercaptoethanol (50 mM final concentration) and NaCl (0.5 M final concentration) in 0.25 mM Tris/HCl, pH 8.0, and incubate for 15 minutes at 25°C.

  vii) Centrifuge at 100,000 \( g \) for 1 hour at 4°C over a layer of 20% (w/w) sucrose in 50 mM Tris/HCl, pH 8.0.

  Remove the band immediately above the sucrose layer and use as the ELISA antigen. Store at –20°C.

- **Test procedure (3, 21)**

  i) Coat ELISA microlitre plate(s) with antigen by adding 100 µl of the recommended or pretitrated dilution of antigen in 0.05 M carbonate/bicarbonate buffer, pH 9.6, to each well.
ii) Incubate at 4°C for 16 hours (overnight) and then wash five times with 0.05% Tween 20 in PBS, pH 7.2.

iii) Dilute the test sera and positive and negative control sera 1/30 in 0.05% Tween 20 in PBS, pH 7.2, and add 100 µl of each diluted serum to duplicate wells of the antigen-coated plate(s).

If four pairs of each positive and negative control serum are added to wells in different parts of the plate, 40 sera can be tested in duplicate on one plate, as shown on the plate plan below.

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iv) Incubate plates at 37°C for 1 hour (optionally on a plate shaker), and then wash five times with 0.05% Tween 20 in PBS.

v) To each well add 100 µl of protein-A/horseradish-peroxidase conjugate (Pierce) at the recommended or pretitrated dilution in 0.05% Tween 20 in PBS.

vi) Incubate the plates at 37°C for 1 hour, and then wash five times with 0.05% Tween 20 in PBS.

vii) **Substrate:** Add hydrogen peroxide to the substrate solution (0.04% orthophenylenediamine (OPD) in phosphate/citrate buffer, pH 5.0) at the rate of 10 µl/25 ml, and add 100 µl of substrate to each well.

Alternatively, DMAB/MBTH substrate solution can be used instead of OPD: Add 200 µl of substrate to each well (10 ml DMAB 80.6 mM Solution + 10 ml of MBTH 1.56 mM solution + 5 µl H₂O₂).

**DMAB/MBTH substrate preparation:**

DMAB – 3-Dimethylaminobenzoic acid (SIGMA D-1643); MBTH – 3-Methyl-2-benzothiazolinone hydrazone hydrochloride monohydrate (SIGMA M-8006).

**DMAB 80.6 mM Solution:** Dissolve 13.315 g of DAMB acid in 1000 ml of 0.1 M phosphate buffer, pH 7 (5.3 g KH₂PO₄, 8.65 g Na₂HPO₄ made up to 1000 ml in distilled water) by continuous agitation for 1 hour at room temperature, adjusting the pH to 7 with NaOH (5 M). Filter through a funnel.

**MBTH 1.56 mM solution:** Dissolve 0.3646 g of MBTH in 1000 ml of 0.1 M phosphate buffer, pH 7 (5.3 g KH₂PO₄, 8.65 g Na₂HPO₄ made up to 1000 ml in distilled water) by continuous agitation for 1 hour, adjusting the pH to 6.25 with concentrated Hydrochloric acid. Filter through a funnel.

The volume required per plate is: 10 ml DMAB + 10 ml MBTH + 5 µl H₂O₂ 30%

Substrate can be prepared as stock solutions, aliquoted and kept at −20°C. Mix the DAMB and the MBTH solutions (1:1) just before use and add the required quantity of 30% H₂O₂.

viii) Incubate at room temperature for approximately 6–10 minutes (before the negative control begins to be coloured). The time necessary for the colour to develop will depend on both the temperature of the substrate when added to the wells, and the room temperature.

ix) Stop the reaction by adding 100 µl of 1.25 M sulphuric acid to each well.

x) **Reading the results:** Positive sera have a clear colour (yellow in case of OPD substrate, blue in case of DMAB/MBTH substrate) and can be read by eye, but to ensure that all positive sera are identified, it is necessary to read the absorbance in each well spectrophotometrically, at 492 nm (OPD substrate) or at 600–620 nm (in case of DMAB/MBTH), in an ELISA reader. Using OPD substrate any serum is considered to be positive if it has an absorbance value of more than twice the mean absorbance value of the control negative sera on that plate. Using DMAB/MBTH substrate the cut off point has to be established by the following equation:

\[
\text{CUT OFF} = \text{Optical Density negative serum} \times 1 + \text{Optical Density Positive serum} \times 0.2.
\]
b) **Indirect fluorescent antibody test**

This test (20) should be used as a confirmatory test for sera from areas that are free from ASF and are positive in the ELISA, and for sera from endemic areas that give an inconclusive result in the ELISA.

- **Test procedure**
  i) Prepare a suspension of ASFV-infected pig kidney or monkey cells at a concentration of $5 \times 10^5$ cells/ml, spread small drops on glass slides, air dry and fix with acetone at room temperature for 10 minutes. Note that slides can be stored at $-20^\circ C$ until ready for use.
  ii) Heat inactivate test sera at 56°C for 30 minutes.
  iii) Add appropriate dilutions of test sera and positive and negative control sera in buffered saline to slides of both infected and uninfected control cells, and incubate for 1 hour at 37°C in a humid chamber.
  iv) Wash the slides by immersing four times in fresh clean PBS and then distilled water.
  v) Add predetermined or recommended dilutions of anti-pig immunoglobulin/FITC or protein-A/FITC conjugate to all slides, and incubate for 1 hour at 37°C in a humid chamber.
  vi) Wash the slides by immersing four times in fresh clean PBS and then distilled water, mount in PBS/glycerol, and examine under an ultraviolet light microscope with suitable barrier and exciter filters.
  vii) **Reading the results:** The control positive serum on infected cells must be positive and all other controls must be negative before the test can be read. Sera are positive if infected cultures show specific fluorescence.

c) **Immunoblotting test (11, 22)**

This test should be used as an alternative to the IFA test to confirm equivocal results with individual sera. The immunoblotting test is very specific and enables easier and more objective interpretation of the results and a better recognition of weak-positive samples.

Viral proteins that induce specific antibodies in pigs have been determined. These polypeptides have been placed on antigen strips and have been shown in the immunoblotting test to react with specific antibodies from 9 days post-infection.

- **Preparation of antigen strips**
  i) Prepare cytoplasmic soluble virus proteins as described for the preparation of ELISA antigen in Section B.2.a.
  ii) Electrophorese through 17% acryl-amide/N,N'-diallyltartardiamide (DATD) gels with appropriate molecular weight standards.
  iii) Transfer the proteins on to a 14 × 14 cm² nitrocellulose membrane by electrophoresis at a constant current of 5 mA/cm in transfer buffer (20% methanol in 196 mM glycine, 25 mM Tris/HCl, pH 8.3).
  iv) Dry the membrane and label the side on to which the proteins were electrophoresed.
  v) Cut one strip from the edge of the filter and carry out the immunoblotting procedure described below. Identify the region containing proteins of 23–35 kDa by comparison with the molecular weight standards run in parallel, and cut this region into 0.5 cm wide strips. Label each strip on the side on to which the proteins were electrophoresed.

These strips (approximately 4 cm long) constitute the antigen strips used for immunoblotting and contain proteins with which antibodies in both acute and convalescent pig sera will react. These antibodies persist for life in some pigs.

- **Preparation of chloranaphthol substrate solution**
  This solution must be prepared immediately before use.
  i) Dissolve 6 mg of 4-chloro-1-naphthol in 2 ml of methanol and add this solution slowly to 10 ml of PBS while it is being stirred.
  ii) Remove the white precipitate that is formed by filtration through Whatman No.1 filter paper (optional).
  iii) Add 4 µl of 30% hydrogen peroxide.

- **Test procedure**
  The antigen strips must be kept with the labelled side uppermost during the immunoreaction procedure.
i) Incubate the antigen strips in blocking buffer (2% nonfat dried milk in PBS) at 37°C for 30 minutes with continuous agitation.

ii) Prepare 1/40 dilutions of test sera and positive and negative control sera in blocking buffer.

iii) Incubate the antigen strips in the appropriate serum at 37°C for 45 minutes with continuous agitation. Incubate one antigen strip in positive control serum and one in negative control serum. These two strips are controls. Wash four times in blocking buffer; the final wash should be for 5 minutes with continuous agitation.

iv) Add protein-A/horseradish-peroxidase conjugate at the recommended or pretitrated dilution (usually at 1/1000 dilution) in blocking buffer to all antigen strips. Incubate at 37°C for 45 minutes with continuous agitation. Wash four times in blocking buffer; the final wash should be for 5 minutes with continuous agitation.

v) Prepare the substrate solution, add to the antigen strips, and incubate at room temperature for 5–15 minutes with continuous agitation.

vi) Stop the reaction with distilled water when the protein bands are suitably dark.

vii) **Reading the results:** Positive sera react with more than one virus protein in the antigen strip; they must give a similar protein pattern and have the same intensity of colour as the antigen strips stained with positive control serum.

d) **Counter immunoelectrophoresis (immunoelectro-osmophoresis) test**

This test (19) can be carried out rapidly and specific antibody can be detected in some sera 30 minutes after the test is set up. It requires the use of electrophoresis equipment (electrophoresis chamber, slide frames, gel cutter) and a 500-volt constant-current power supply. Due to its low sensitivity, this test is recommended for screening groups of pigs, but not individual animals.

- **Test procedure**
  
  i) Place the required number of 2.5 × 10 cm glass slides in the slide frame on a level table and cover with the recommended volume of 0.6% agarose in veronal/acetate buffer, pH 8.6 (ionic strength 0.025) containing 0.1% sodium azide, and allow to set.

  ii) Cut four pairs of wells, 3 mm in diameter, 10 mm apart in the gel on each slide as shown below.

```
  +  -
S  Ag  S  Ag
  ○  ○  ○  ○
  ○  ○  ○  ○
```

  
  
  S = serum; Ag = antigen; + = positive electrode; − = negative electrode

  iii) Fill the wells with the appropriate reagents, including control positive and negative antigens and sera, using capillary (haematocrit) tubes.

  iv) Place the frames in the electrophoresis chamber and run for 30 minutes with a constant voltage of 19 volts/cm.

  v) After electrophoresis, examine the slides over an indirect light source for specific lines of precipitation.

  vi) Wash the slides in 2% NaCl solution overnight and for 2 hours in several changes of distilled water before drying.

  vii) Stain dried slides with 0.075% amido black in equal volumes of methanol, 12% acetic acid and 1.6% sodium acetate, containing 0.007% glycerol, for 5–10 minutes and destain with three 10-minute washes in an aqueous solution of 45% methanol and 10% glacial acetic acid.

  viii) **Reading the results:** On the stained slides, the lines of precipitation observed between the antigen and unknown serum wells of a positive sample should be similar to that formed between the positive antigen and serum control wells.

C. **REQUIREMENTS FOR VACCINES AND DIAGNOSTIC BIOLOGICALS**

At present there is no vaccine for ASF.
REFERENCES


* * *

**NB:** There are OIE Reference Laboratories for African swine fever (see Table in Part 3 of this *Terrestrial Manual* or consult the OIE Web site for the most up-to-date list: www.oie.int).
CHAPTER 2.8.2.

ATROPHIC RHINITIS OF SWINE

SUMMARY

Definition of the disease: Atrophic rhinitis is an infectious disease of swine characterised by serous to mucopurulent nasal discharge, shortening or twisting of the snout, atrophy of the turbinate (conchal) bones and reduced productivity. It may occur enzootically or more sporadically, depending on a variety of factors including herd immunity. The most severe progressive form is caused by infection with toxigenic strains of Pasteurella multocida alone or in combination with Bordetella bronchiseptica. Infections with B. bronchiseptica alone can cause a mild to moderate form with nonprogressive turbinate bone atrophy. Turbinate atrophy may only be obvious at slaughter or may be detected in the live animal by use of radiography or tomography. Environmental and management factors also contribute to the severity and incidence of this disease. A large proportion of apparently normal pig herds may be infected with B. bronchiseptica or nontoxigenic P. multocida and show a mild degree or low prevalence of turbinate atrophy.

Identification of the agents: The diagnosis of atrophic rhinitis depends on clinical and post-mortem observations in affected swine assisted by the recovery and characterisation of P. multocida and B. bronchiseptica. The isolation of both organisms is often complicated by the more profuse growth of other bacteria. Isolation rates are improved by preservation of the nasal or tonsillar swab at 4–8°C in a non-nutritive transport medium and by using a selective culture medium.

Pasteurella multocida and B. bronchiseptica can be identified by traditional biochemical tests. Pasteurella multocida isolates may be further characterised by their capsular and somatic antigens. Capsular type D is most prevalent in many areas of the world, but in some regions type A predominates. Capsular antigens may be distinguished serologically by indirect haemagglutination or immunofluorescence, chemically by flocculation in acriflavine, or by susceptibility to hyaluronidase. Somatic antigen types can be distinguished by a gel diffusion precipitation test, with type 3 found most frequently in swine. Toxigenicity of P. multocida isolates can be demonstrated by testing for cytotoxicity in cultured cells. A commercially available toxin-specific enzyme-linked immunosorbent assay (ELISA) is now widely used in some areas of the world to differentiate toxigenic from nontoxigenic isolates. In addition, it is suitable for detection of toxin production by bacteria from primary culture plates without the need for prior isolation and identification of individual colonies.

Recently developed assays based on the use of DNA probes or polymerase chain reaction (PCR) provide rapid, sensitive and highly specific detection of B. bronchiseptica and both toxigenic and nontoxigenic P. multocida for those laboratories with the capability to perform them. A multiplex PCR useful for capsular typing of P. multocida has also been described.

Serological tests: Detection of antibodies to P. multocida and B. bronchiseptica is of little value as nontoxigenic strains of P. multocida share cross-reactive antigens with toxigenic strains and B. bronchiseptica can be isolated from many swine herds. A test based on detection of antibodies to the P. multocida toxin is commercially available but its usefulness is limited as not all infected swine develop such antibodies. Widespread vaccination with P. multocida toxoid induces antibodies of vaccinal origin, complicating interpretation of results.

Requirements for vaccines and diagnostic biologicals: Several vaccines are available commercially that contain bacterins of B. bronchiseptica and a mixture of toxigenic and/or
nontoxigenic strains of *P. multocida*, or a toxoid derived from *P. multocida* or from a recombinant
Escherichia coli.

### A. INTRODUCTION

Atrophic rhinitis is an infectious disease of swine. The initial clinical signs are sneezing, snuffling and eye
discharge with resultant dark tear-staining and subsequent nasal discharge, which can vary from serous to
mucopurulent; in some cases pigs may show epistaxis. Atrophy of the nasal turbinate (conchal bone) and septal
deviation may lead to shortening or twisting of the snout and, in severe cases, difficulty in eating. Two forms have
been recognised (9):

- **a)** A severe progressive form caused by toxigenic isolates of *Pasteurella multocida*, most commonly capsular
types D or A, alone or in combination with *Bordetella bronchiseptica*.

- **b)** A less severe form with mild to moderate turbinate atrophy, often without significant snout changes, caused
by *B. bronchiseptica*.

Increased severity is associated with overstocking and poor management, housing and environmental
conditions. Reduced productivity is generally associated with moderate to severe atrophic rhinitis, although the
precise relationship between infection with these bacteria and reduced weight gains has not been thoroughly
elucidated. *Bordetella bronchiseptica* and toxigenic *P. multocida* are commonly found in many domesticated and
wild animal species that could potentially transmit the bacteria to swine herds.

*B. bronchiseptica* or toxigenic *P. multocida* may be present in a herd without clinical evidence of disease,
especially when other respiratory pathogens are absent and environmental and management conditions are
optimal. Such carrier herds pose a risk of transmitting these agents to other herds in which progression to severe
disease may occur.

The diagnosis of atrophic rhinitis depends on clinical, pathological and microbiological investigations, with the
latter being particularly important for herds infected subclinically. It is generally accepted that a herd in which
toxigenic *P. multocida* is present be defined as affected with progressive atrophic rhinitis, whether or not clinical
signs of the disease are evident (26). Control in many countries has, therefore, centred on detection of infection,
even in asymptomatic animals considered to be potential carriers.

Turbinate atrophy may only be seen at slaughter when snout sections at the level of the first/second premolar
tooth are examined. Subjective assessment of turbinate atrophy is convenient and often useful for monitoring
herds (9), but objective scales of measurement (16) are best suited for studies requiring data analysis. Radiography (12)
and tomography (24) can provide objective observations from live animals; tomography reveals not only severe lesions but more subtle changes that may not be apparent by radiography. However, these
techniques are of limited use due to the equipment and expertise required. Diagnosis is assisted by detection of
characteristic histopathological features including fibrous replacement of the bony plates of the ventral conchae
with varying degrees of inflammatory and reparative changes.

### B. DIAGNOSTIC TECHNIQUES

#### 1. Identification of the agents

- **a) Culture**

  As *P. multocida* preferentially colonises the tonsil, tonsillar swabs or biopsies will provide the highest
isolation rates (1). Nasal swabs are preferred for isolation of *B. bronchiseptica*. When sampling the tonsil is
not practical, nasal swabs suffice for isolation of both organisms. Swabs with flexible shafts should be used;
sample collection in young pigs is facilitated by the use of mini-tipped swabs. A single swab is used to
sample both sides of the nasal cavity and should then be placed in a non-nutritive transport medium (e.g.
phosphate buffered saline) and kept at 4–8°C during transit to avoid overgrowth by other faster-growing
bacteria. Transit time should not exceed 24 hours.

  Although both *P. multocida* and *B. bronchiseptica* grow readily on blood agar, a selective medium is
preferred as overgrowth of other bacteria that are present in higher numbers often interferes with their
detection. An additional difficulty related to *B. bronchiseptica* is that this organism grows more slowly than
most other bacteria present in clinical samples. Various formulations of media containing antibiotics have
been used for isolation of *P. multocida*, but comparison of studies in the literature indicates that the highest
isolation rates are obtained with modified Knight medium (bovine blood agar containing 5 µg/ml
clindamycin, 0.75 µg/ml gentamicin) (22) or KPMD (bovine blood agar containing 3.75 U/ml bacitracin, 5 µg/ml clindamycin, 0.75 µg/ml gentamicin, and 2.25 µg/ml amphotericin B) (1). MacConkey agar with 1% glucose and 20 µg/ml furaltadone is used by many laboratories for selective growth of *B. bronchiseptica* from nasal swabs, but a modified Smith-Baskerville medium (a peptone agar formulation containing 20 µg/ml penicillin, 20 µg/ml furaltadone, and 0.5 µg/ml gentamicin) appears superior, especially when the number of *B. bronchiseptica* present is low (22, 35). A further improvement in isolation rate was reported using blood agar containing 40 µg/ml cephalixin (22). A selective blood agar medium for simultaneous isolation of both *P. multocida* and *B. bronchiseptica*, containing 5 mg/litre clindamycin-HCl, 0.75 mg/litre gentamicin sulphate, 2.5 mg/litre K-tellurite, 5 mg/litre amphotericin-B and 15 mg/litre bacitracin, has also been described (10). However, it should be noted that K-tellurite has sometimes been found to be inhibitory to the growth of type D *P. multocida* (22).

b) **Biochemical characteristics**

*P. multocida* is a Gram-negative, bipolar, pleomorphic rod and forms nonhaemolytic, greyish colonies on blood agar with a characteristic, ‘sweetish’ odour (29). It fails to grow on MacConkey agar but yields positive oxidase and catalase reactions and produces indole.

*B. bronchiseptica* is also a Gram-negative rod, forming convex colonies 1–2 mm in size, usually haemolytic, on blood agar or Bordet-Gengou medium after 48 hours of growth (30). It is nonfermentative, but positive for oxidase, catalase, citrate and urea and grows in 6.5% NaCl.

Agglutination tests using specific antisera have been described for confirming the identity of presumptive *B. bronchiseptica* isolates but appropriate sera are not widely available for use.

- **Capsular typing of *P. multocida***
  
  Capsular typing of *P. multocida* is useful for epidemiological purposes. Serotyping by indirect haemagglutination has traditionally been used (3) but only a few laboratories throughout the world make and maintain the antisera required. However, simpler chemical methods can usually distinguish most swine isolates. Those producing a type D capsule form a heavy flocculate in 1/1000 aqueous acriflavine (5), while capsular type A strains can be identified by inhibition of growth in the presence of hyaluronidase (4). A small proportion of swine isolates are noncapsulated.

- **Acriflavine test procedure for capsular type D *P. multocida***
  
  i) Inoculate a tube containing 3 ml of brain–heart infusion broth, using a freshly grown bovine blood agar culture, for each *P. multocida* isolate to be tested. Include a known type D strain and a known type A strain as positive and negative controls.
  
  ii) Incubate inoculated tubes at 37°C for 18–24 hours.
  
  iii) Pellet bacteria by centrifugation and remove 2.5 ml of the supernatant.
  
  iv) Add 0.5 ml of a 1/1000 aqueous solution of acriflavine neutral. Acriflavine solution should be freshly prepared each week and stored at 4°C, protected from light.
  
  v) Mix to resuspend the bacterial pellet and incubate the tube at room temperature, without shaking.
  
  vi) Observe at 5 minutes for the presence of a heavy flocculent precipitate.

- **Hyaluronidase test procedure for capsular type A *P. multocida***
  
  i) Prepare fresh bovine blood agar cultures of the isolates to be tested. Include a known type A strain and a known type D strain as positive and negative controls.
  
  ii) Inoculate each strain to be examined on a separate trypticase soy blood agar plate with 6% bovine blood by streaking several parallel lines of growth, approximately 3–5 mm apart, across the diameter of the plate. For maximum production of hyaluronic acid it is important that the plates be fresh and not dehydrated.
  
  iii) Heavily streak a hyaluronidase-producing strain of *Staphylococcus aureus* at right angles to the lines of *P. multocida* growth.
  
  iv) Incubate the plates at 37°C, in a humidified atmosphere, and observe periodically for up to 24 hours. Type A strains will exhibit a marked inhibition of growth in the region adjacent to the growth lines of *S. aureus*.

- **Somatic antigen typing of *P. multocida***
  
  Differences in the cell wall lipopolysaccharide among *P. multocida* strains provide the basis for somatic antigen typing. Sixteen types can be distinguished by a gel diffusion precipitation test (18), with type 3 found
most frequently in swine. Although the required antisera are not widely available, many reference laboratories and some diagnostic laboratories offer somatic antigen typing.

- **Detection of the *P. multocida* toxin**
  Diagnosis of progressive atrophic rhinitis depends upon characterisation of *P. multocida* isolates as toxigenic. The heat-labile toxin of *P. multocida* produces dermonecrosis in guinea pigs and is lethal in mice following intraperitoneal injection. Toxigenicity can also be demonstrated *in vitro* by testing for cytopathic effects on monolayers of embryonic bovine lung (EBL) cells (34), African green monkey kidney (Vero) cells (28) or bovine turbinate cells (13). The bacteria are grown in brain–heart infusion broth incubated at 37°C for 24 hours and then pelleted by centrifugation. The supernatant is sterilised by filtration and titrated in monolayer cultures prepared in microtitre plates. Following incubation at 37°C for 2–3 days, the monolayers are stained with crystal violet and examined microscopically to detect cytopathic effects. A rapid cell culture test, in which the suspect colonies are grown on an agar overlay of EBL cells (6), permits more efficient analysis of large numbers of isolates.

An enzyme-linked immunosorbent assay (ELISA) using monoclonal antibodies can detect the toxin in mixtures of bacteria recovered from primary isolation media (14). This is an important advantage as swine may be colonised simultaneously with a mixture of toxigenic and nontoxigenic strains (1, 9). Cell culture methods would require every colony of *P. multocida* in the sample to be tested, which is clearly impractical, to achieve the same level of sensitivity as the ELISA.

This ELISA is commercially available throughout Europe and in a few other selected regions of the world¹ (though not in the United States of America) and has been widely adopted in many areas as the preferred test for identification of carriers and for control of progressive atrophic rhinitis. Though highly specific, a positive result without previous history of disease or suspicious signs should be thoroughly investigated to recover toxigenic isolates from the animals sampled.

c) **Nucleic acid recognition methods**
  Colony morphology and biochemical testing remain the basis for identification of toxigenic *P. multocida* and *B. bronchiseptica* in many laboratories. However, a number of recently described assays based on the use of DNA probes (20, 32) or polymerase chain reaction (PCR) (19, 23, 25, 31) for detection of toxigenic *P. multocida* and/or *B. bronchiseptica* from swine show promise as faster, more specific, and more sensitive diagnostic tools. Diagnostic laboratories are increasingly using PCR for identification of these agents as the equipment and expertise become more readily available. Proper in-house validation with known controls as well as standardised, ongoing quality control measures are essential (see Chapter 1.1.5 Validation and quality control of polymerase chain reaction methods used for the diagnosis of infectious diseases).

A multiplex PCR assay for capsular typing of *P. multocida*, that appears to provide more reliable results than phenotypic methods, has recently been described (37) and may be of use in suitably equipped laboratories.

Various DNA fingerprinting techniques, including restriction endonuclease analysis (REA), ribotyping, pulsed-field gel electrophoresis, and PCR-based methods have been evaluated by numerous groups for the purpose of differentiating *P. multocida* isolates. Few direct comparisons between methods have been carried out using strains from pigs with atrophic rhinitis, but REA currently appears to be the method of choice for epidemiologic investigations as it provides a high level of discrimination without the need for specialized equipment or reagents (11, 15, 17).

2. **Serological tests**
  At present there are no satisfactory serological tests that can be relied on to detect those animals infected with toxigenic *P. multocida* and capable of developing or spreading the disease. Detection of antibodies to *P. multocida* is not helpful, as nontoxicogenic strains share many cross-reacting antigens with toxigenic strains. An ELISA for detection of antibodies to the *P. multocida* toxin has been described (14) that is commercially available in Europe and a few other areas of the world (see footnote 1). However, many animals infected with toxigenic *P. multocida* fail to produce antibodies to the toxin, and widespread use of toxoid-containing vaccines limits the diagnostic value of this ELISA to herds with no history of vaccination or to detection of vaccine response in vaccinated herds.

¹ For availability/purchase information contact DakoCytomation Denmark A/S, Produktionsvej 42, DK-2600 Glostrup, Denmark; http://www.dakocytomation.com.
Infection with *B. bronchiseptica* can be detected serologically by agglutination testing with formalin-treated bacteria or with a more sensitive ELISA (38). Unless monitoring the status of a negative herd, *B. bronchiseptica* serology may be of little value as the organism is present in many apparently normal pig herds.

C. REQUIREMENTS FOR VACCINES AND DIAGNOSTIC BIOLOGICALS

There are several commercially available vaccines that contain whole-cell bacterins of *B. bronchiseptica* and a mixture of toxigenic and nontoxigenic *P. multocida*, or a *P. multocida* toxoid. Live, attenuated *B. bronchiseptica* vaccines are also available. Vaccines containing only *B. bronchiseptica* are not suitable for control of progressive atrophic rhinitis, but may be of benefit in herds with the nonprogressive form. *Pasteurella multocida* and *B. bronchiseptica* vaccines appear to reduce the level of colonisation by these bacteria, but do not eliminate them or prevent infection.

The *P. multocida* toxin is the single most important protective antigen with respect to progressive atrophic rhinitis. Vaccines based on a *P. multocida* toxoid offer specific protection against the action of the toxin, which, by itself, can be used to reproduce all of the major signs of this disease (see ref. 14 for review). The level of toxin produced by *P. multocida* is relatively low and the toxin-specific antibody response induced by bacterin-only vaccines may not be optimal. The difficulty and expense of large-scale purification prevent routine incorporation of purified toxoid into vaccines. Field studies have shown that a recombinant *P. multocida* toxin derivative, missing a segment of the amino-terminal portion of the protein, is non-toxic but immunogenic and has superior efficacy in swine (2, 27). More recently, full-length recombinant toxoid engineered to contain two amino acid substitutions that eliminate toxigenicity was also found to be highly efficacious (36). A DNA vaccine encoding a full-length but enzymatically inactive toxoid was shown to be highly immunogenic in pigs but has so far not been evaluated for efficacy against challenge (33).

*Bordetella bronchiseptica* produces a variety of toxins and adhesins that are potential virulence factors in swine. Only one, the outer membrane protein pertactin, has been shown to protect against disease in pigs (21). Despite this fact, a dermonecrotic toxin produced by *B. bronchiseptica*, unique from the toxin produced by *P. multocida*, has traditionally been regarded as the primary virulence factor and protective immunogen in swine (9). Several studies strongly implicate the toxin as a virulence factor and it undoubtedly plays a role in pathogenesis and, perhaps, in protection. However, the role of pertactin and several additional virulence factors in protective immunity is most likely equal to, or perhaps exceeds, that of the toxin.

*Bordetella bronchiseptica* is subject to phenotypic variation under certain growth conditions (e.g. temperatures below 37°C or the presence of chemical modulators such as MgSO₄ or nicotinic acid), in which production of most virulence factors is reversibly turned off. Spontaneously occurring mutants, permanently unable to produce most virulence factors, also arise with a low frequency during culture. Careful attention to colony morphology, on an area of the plate with well-separated colonies, is essential to retain cultures in the phase I (also known as Bvg*+*), or virulent, mode. Phase I colonies are small (1–2 mm in diameter), domed, and haemolytic on blood agar. Loss of haemolysis and the appearance of larger, flat colonies indicate conversion to the avirulent form. Whenever possible, cultures should be propagated using single haemolytic colonies to minimise the slow accumulation of avirulent clones within the culture.

Precise details of standards for the production of effective commercial vaccines are not available, but they are known to contain 10¹⁰ cells of formalin-killed *B. bronchiseptica* and 10 µg of *P. multocida* toxoid per dose. It is also clear that purified toxoid (inactivated by formaldehyde) is more immunogenic than crude toxoid, and that the immunogenicity of the inactivated form is not affected by mixture with a *B. bronchiseptica* bacterin. The truncated, recombinant *P. multocida* toxin derivative has been inactivated by deletion of a portion of the gene that does not appear to compromise protective immunogenicity. All commercially available vaccines contain either an oil adjuvant or aluminium hydroxide gel.

*Bordetella bronchiseptica* used for vaccine production should be a phase I virulent culture and the *P. multocida* isolates used should be toxigenic. Seeds of *B. bronchiseptica* and toxigenic *P. multocida* of established identity and passage history should be stored by conventional means. A defined number of passages should be used to give the production culture. *Bordetella bronchiseptica* should be inactivated by formaldehyde. As the toxin of *P. multocida* has an intracellular location and is released on cell lysis during the stationary phase, the culture supernatant should be harvested approximately 48 hours after the end of the exponential phase of growth.

1. Seed management

a) Characteristics of the seed

The seed-lot system should be employed for the bacterial strains used to prepare whole-cell bacterins, as well as for the strains from which purified antigens are derived.
In the case of whole-cell bacterins, the origin and history of both the *P. multocida* and *B. bronchiseptica* strains should be described and the full characterisation of the master seeds should be laid down in a master seed batch protocol.

Working seeds used for vaccine production should be derived from the master seed and checked for all relevant properties as described in the master seed batch protocol.

b) **Method of culture**

All bacterial strains should be cultivated in suitable media that efficiently support growth and the expression of relevant antigens.

c) **Validation as a vaccine**

i) **Purity**

Both the master seed and the working seed must be pure cultures, free from bacterial, mycotic, mycoplasmal and viral contamination.

Identity of the bacterial species and the production of relevant antigens should be confirmed.

ii) **Safety**

Although inactivation of the bacterial cultures by a validated method is a standard procedure, both bacterial species produce dermonecrotic toxins; detoxification of these toxins should be confirmed when toxoids are used as vaccine components. Standard safety tests for inactivated vaccines should be carried out (7, 8).

iii) **Efficacy**

The efficacy of a trial vaccine should be measured by vaccinating groups of pregnant sows. Their progeny should be challenged by virulent cultures of *B. bronchiseptica* and toxin-producing *P. multocida*. Significant protection should be obtained against the clinical signs of the progressive form of atrophic rhinitis, i.e. turbinate atrophy. The clinical signs induced in the controls and vaccinates may be compared according to the scoring system of Done (12).

2. **Method of manufacture**

Both *B. bronchiseptica* and *P. multocida* cultures should be propagated in media that supports efficient growth and allows optimal expression of the antigens that are relevant for the induction of protective antibodies. *Bordetella bronchiseptica* should be confirmed to be a phase I culture and, for *P. multocida*, it should be confirmed that the culture contains sufficient levels of toxin.

*Bordetella bronchiseptica* cells, and either *P. multocida* cells and/or toxin, are inactivated, detoxified and formulated with an adjuvant. Commonly used adjuvants are aluminium salts or oil emulsions.

3. **In-process control**

During the manufacturing process, the following in-process controls are carried out.

a) **Purity and identity of the seed cultures**

Cultures are inoculated on blood agar plates and incubated. No nonspecific colonies should grow on these plates.

b) **Purity and identity of the production cultures**

Cultures are inoculated on blood agar plates and incubated. No nonspecific colonies should grow on these plates.

c) **Inactivation of cultures before further processing**

Cultures are inactivated with formaldehyde. Tests are performed to check the effectiveness of the inactivation process and to test for residual formaldehyde.

d) **Quantification of antigens**

This is carried out by performing a total cell count using a bacterial counting chamber for enumerating whole cells or an antigenic mass determination for defined antigens, e.g. *P. multocida* toxin, by quantitative enzyme immunoassay.
4. Batch control

a) Sterility
Every batch of vaccine should be tested for sterility according to standard methods (see Chapter 1.1.9 Tests for sterility and freedom from contamination of biological materials) described in the European Pharmacopoeia or the United States Code of Federal Regulations.

b) Safety
Every batch of vaccine should be tested for safety in the target animal, by giving a double dose by the recommended route of vaccination, and a second, single dose 2 weeks later. No abnormal local or systemic reactions should occur.

c) Potency
Every batch of vaccine should be tested for potency using a validated serological test that correlates with the protection obtained in the efficacy experiment, as described under Section C.1.c.iii. The potency test is not necessarily carried out in the target animal — mice or rabbits can be used. In these latter cases, correlation has to be shown with protective antibody levels in the target animal.

d) Duration of immunity
Normally the vaccine is applied during the late stage of pregnancy, so that progeny will be protected by the uptake of colostral antibodies.

When the vaccine may be applied irrespective of the stage of pregnancy, duration of immunity should be at least 6 months, so that booster vaccinations twice a year should maintain effective antibody levels.

e) Stability
Every batch of vaccine should be subjected to an accelerated shelf-life test, which has been correlated with real-time shelf-life testing.

f) Preservatives
When a preservative is used, the concentration should be measured for each batch. It must not exceed the maximum permitted level.

g) Precautions
When an oil emulsion is used as the adjuvant, accidental injection of the operator can cause a severe local reaction. Medical attention should be sought immediately, treating the wound as a grease-gun injury.

5. Tests on the final product

a) Safety
Every batch of vaccine should be tested for safety, as described in Section C.4.b.

b) Potency
Every batch of vaccine should be tested for potency, as described in Section C.4.c.

REFERENCES


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CHAPTER 2.8.3.

CLASSICAL SWINE FEVER
(hog cholera)

SUMMARY

Classical swine fever (CSF), also known as hog cholera, is a contagious viral disease of pigs. The causative virus is a member of the genus Pestivirus of the family Flaviviridae, and is closely related to the viruses of bovine viral diarrhoea and Border disease. There is only one serotype of CSF virus (CSFV).

The disease may run an acute, subacute, chronic, late onset, or inapparent course, depending on a variety of viral and host factors of which the age of the animals, the virulence of the virus and the time of infection (pre- or post-natal) are of greatest importance. Adult pigs usually display less severe signs of disease than young animals and stand a better chance of survival. In pregnant sows, the virus may cross the placental barrier and reach the fetuses. In-utero infection with strains of the virus of moderate or low virulence can result in what is referred to as the ‘carrier sow’ syndrome followed by prenatal or early post-natal death, the birth of diseased piglets or an apparently ‘healthy’ but persistently infected litter. An outbreak of CSF has serious consequences for trade in pigs and pig products.

The highly variable clinical picture of CSF often precludes a diagnosis on clinical and pathological grounds alone. Laboratory methods are therefore essential for an unambiguous diagnosis. Detection of virus or viral nucleic acid in whole blood and of antibodies in serum are the methods of choice for diagnosing CSF in live pigs, whereas detection of virus, viral nucleic acid or antigen in organ samples is most suitable when the pig is dead.

Identification of the agent: The direct fluorescent antibody test (FAT) on cryostat sections of organs from affected pigs can be used for the detection of CSF antigen. A panel of monoclonal antibodies (MAbs) is used to determine whether the fluorescence is due to CSF or non-CSF Pestivirus antigens. For the detection of CSF genome, polymerase chain reaction (PCR) is commonly used. The isolation of CSFV should be attempted in the pig kidney (PK-15) cell line, or other suitable cell lines. The cultures are examined for virus growth by immunofluorescence or immunoperoxidase staining; positive isolates are further characterised by the use of MAbs and by partial genetic sequencing. Polymerase chain reaction protocols for the identification of CSFV nucleic acid have now gained international acceptance and are being used in several laboratories, both for detection of the agent and differentiation from ruminant pestiviruses. Antigen-capture enzyme-linked immunosorbent assays (ELISAs) are also useful for herd screening, but should not be used on a single animal basis.

Serological tests: Detection of virus-specific antibodies is particularly useful in herds suspected of being infected at least 21 days previously with CSFV. Serological methods are also valuable for monitoring and for prevalence studies, and are essential if a country wishes to be internationally recognised as being free from the disease in the absence of vaccination.

As CSFV cross-reactive antibodies against ruminant Pestivirus are occasionally observed in breeding pigs, screening tests have to be followed by confirmatory tests that are CSFV-specific. Certain ELISAs are relatively CSFV-specific, but the definitive method for differentiation is the comparative neutralisation test, which compares the level of antibodies to different Pestivirus species.

Requirements for vaccines and diagnostic biologicals: Vaccines against CSF are based on live virus that has been attenuated by passage through cell cultures or through a suitable host
species that is not of the family Suidae. The production of these modified live virus (MLV) vaccines is based on a seed-lot system that has been validated with respect to virus identity, sterility, purity, safety, nontransmissibility, stability and immunogenicity. If CSFV is used in the production of vaccine or in challenge studies, the facility should meet the OIE requirements for Containment Group 4 pathogens.

Effective inactivated, conventional whole virus vaccines are not available. Subunit ‘marker vaccines’ are now available, which, in contrast to MLV vaccines, induce antibodies that can be distinguished from antibodies induced by field virus using an accompanying diagnostic test. The presently registered ‘marker vaccines’ are based on the major envelope glycoprotein (E2-subunit) of CSFV, and are produced in insect cells using recombinant DNA technology.

A. INTRODUCTION

The viruses that cause classical swine fever (CSF), bovine viral diarrhoea (BVD) and Border disease (BD) are members of the family Flaviviridae, genus Pestivirus, and are closely related, both antigenically and structurally. Clinical signs and lesions seen at post-mortem in pigs affected with CSF are highly variable due to both viral and host factors. Furthermore, congenital infections with ruminant pestiviruses in pigs can give rise to a clinical disease that is indistinguishable from CSF (31, 33, 35).

Spread of disease in all age groups, accompanied by pyrexia, huddling, inappetance, dullness, weakness, conjunctivitis, constipation followed by diarrhoea, and an unsteady gait are the prevailing signs. Several days after the onset of clinical signs, the ears, abdomen and inner thighs may show a purple discoloration. Animals with acute disease die within 1–3 weeks. Sudden death in the absence of clinical illness is not symptomatic of CSF.

Under certain circumstances related to the animals’ age and condition, as well as to the virus strain involved, subacute or chronic clinical illness may develop, which can be protracted for 2–4 weeks or even months. Chronic illness leads to a stunting of growth, anorexia, intermittent pyrexia and diarrhoea. Congenital persistent infections may go undetected for months and may be confined to only a few piglets in the herd or may affect larger numbers. The clinical signs are nonspecific: wasting in the absence of pyrexia. Chronic, persistent infections always lead to the death of the animal. Herd mortality rates may be slightly above the expected level. CSF affects the immune system, a main characteristic being generalised leukopenia, which can often be detected before the onset of fever. Immunosuppression may lead to concurrent infections.

In acute cases, gross pathological lesions might be inconspicuous or absent. In typical cases, the lymph nodes are swollen and marbled red, and haemorrhages occur on serosal and mucosal membranes of the intestinal organs. Spleenic infarctions may occur. In subacute and chronic cases, necrotic or ‘button’ ulcers may be observed in the mucosa of the gastrointestinal tract, epiglottis and larynx, in addition to the above lesions.

Histopathological findings are not pathognomonic. Lesions may include parenchymatous degeneration of lymphatic tissue, cellular proliferation of vascular interstitial tissue, and a nonsuppurative meningoencephalomyelitis, with or without vascular cuffing.

A useful critique of diagnostics and vaccination for CSF, from an authoritative source, has recently been published (3), which, as well as general guidance, also provides sources of information on validation and scientific opinion on the applicability of certain commercial products in these areas.

B. DIAGNOSTIC TECHNIQUES

The variability of the clinical signs and post-mortem lesions do not provide firm evidence for unequivocal diagnosis. Other viral diseases, such as African swine fever, porcine dermatitis and nephropathy syndrome (PDNS), and post-weaning multisystemic wasting syndrome (PMWS), thrombocytopenic purpura and various septicemic conditions including salmonellosis (especially due to Salmonella choleraesuis), erysipelas, pasteurellosis, actinobacillosis (due to Actinobacillus suis) and Haemophilus parasuis infections may be confused with acute CSF. In fact, these bacteria often cause concurrent infections, and isolating these pathogens may obscure the real cause of disease, the CSF virus (CSFV). Similarly concurrent PDNS can lead to oversight of an underlying CSF infection.

A tentative diagnosis based on clinical signs and post-mortem lesions must therefore be confirmed by laboratory investigations. As pyrexia is one of the first signs of CSF and is accompanied by a viraemia (7), detection of virus or viral nucleic acid in whole blood, collected in heparin or ethylene diamine tetra-acetic acid (EDTA), or in
tissues, collected from a few febrile animals, is the method of choice for detecting infected herds at an early stage. This is all the more necessary in view of the serious consequences of an outbreak of CSF for trade in pigs and pig products.

Laboratory methods for diagnosis of CSF are aimed at detection of the virus, viral nucleic acid or viral antigens, or detection of specific antibodies. For a correct interpretation of the test results the inspecting veterinarian should pay particular attention to the simultaneous and clustered occurrence of two or more of the prevailing signs of disease listed above. Random sampling is unsuitable for CSF diagnosis. Additionally, whole blood samples for virus detection and reverse-transcription polymerase chain reaction (RT-PCR) analyses can be collected from a larger group of pigs.

CSF is subject to official control and the virus has a high risk of spread from the laboratory: consequently, a risk analysis should be carried out to determine the level of biosecurity needed for the diagnosis and characterisation of the virus. The facility should meet the requirements for the appropriateContainment Group as determined by the risk assessment and as outlined in Chapter 1.1.2 Biosafety and biosecurity in the veterinary microbiology laboratory and animal facilities. Countries lacking access to such a specialised national or regional laboratory should send specimens to an OIE Reference Laboratory.

Antibodies develop in the third week of illness and persist in the surviving animal for life. Samples for antibody detection are collected in ordinary (nonheparinised) tubes from convalescent pigs and from contact herds when more than 3 weeks have elapsed since the suspected contact with a confirmed outbreak took place.

1. Identification of the agent

a) Immunological methods

   • Fluorescent antibody test

   The fluorescent antibody test (FAT) is a rapid test that can be used to detect CSFV antigen in cryostat sections of tonsils, spleen, kidney, lymph nodes or distal portions of the ileum. Tissues should be collected from several (febrile and/or diseased) animals (4) and transported without preservatives under cool conditions, but not frozen. Cryostat sections are stained directly with anti-CSF immunoglobulin conjugated to fluorescein isothiocyanate (FITC) or indirectly using a secondary FITC conjugate and examined by fluorescence microscopy. During the first stage of the infection, tonsillar tissue is the most suitable, as this is the first to become affected by the virus irrespective of the route of infection (25). In subacute and chronic cases, the ileum is frequently positive and occasionally may be the only tissue to display fluorescence. A negative FAT result does not completely rule out CSF infection. When suspicion of CSF continues, further samples should be obtained or attempts made at virus isolation in cell culture (e.g. pig kidney [PK-15]) or another cell line of pig origin that is as sensitive and known to be free from Pestivirus contamination.

   There is a relatively high risk of false (positive and negative) results when FAT is used by laboratories not thoroughly acquainted with the method. Thus FAT should only be used by laboratories that have experience of using the technique, perform the technique on a routine basis and have had training in interpreting the fluorescence.

   • Test procedure

   Include positive and negative control sections in each series of organ samples to be examined.

   i) Cut out a piece of tonsil, spleen, kidney and ileum of approximately 1 × 1 × 0.5 cm, and mount it with a cryo-embedding compound or distilled water on a cryostat table.

   ii) Freeze the piece of organ on to the cryostat table.

   iii) Cut sections not more than 4–8 µm thick and mount these on to 10 × 32 mm grease-free cover-slips with one corner cut-off. All sections are mounted with this corner in the same position (e.g. top right).

   iv) After drying, fix the mounted sections for 10 minutes at room temperature in acetone (analytical grade) or air-dry for 20 minutes at 37°C.

   v) Immerse the sections briefly in phosphate buffered saline (PBS), remove excess fluid with tissue paper and place them (cut off corner top right) on a frame in an incubation chamber humidified with a small volume of water placed in the bottom of the chamber.

   vi) Dispense the anti-CSF immunoglobulin at working dilution on to the entire section and incubate in the closed chamber for 30 minutes at 37°C. If a secondary FITC conjugate is required, wash the section five times for 2 minutes each in PBS at room temperature, then add the FITC conjugate at working dilution and incubate as previously described.

   vii) Wash the sections five times for 2 minutes each in PBS at room temperature.
viii) Remove the remaining PBS by touching the cover-slip against tissue paper and mount the cover-slip (with the section between cover-slip and slide) with mounting buffer on to a microscope slide.

ix) Remove excess mounting fluid with tissue paper and examine the sections for fluorescence using a UV microscope. A CSF-positive section shows brilliant green fluorescing cells. In the tonsils, fluorescence in the epithelial lining of the crypts is particularly evident. In kidney sections, fluorescence is most abundant in the proximal and distal tubules of the renal cortex and the collecting ducts in the medulla. In the ileum, fluorescence is most prominent in the epithelial cells of the Lieberkühn glands, whereas in the spleen reactivity is more diffuse, with concentrations of lymphoid cells in the periarterial lymphoid sheath (PALS).

The FAT involves the use of an anti-CSF immunoglobulin prepared from a polyclonal antibody to CSFV that will not distinguish between the antigens of different pestiviruses. Conjugates used for the FAT on cryostat sections or inoculated cell cultures should be prepared from anti-CSFV gamma-globulins raised in specific pathogen free pigs. The working dilution of the conjugates (at least 1/30) should combine a maximum brilliance with a minimum of background. The test should only be performed on samples from freshly dead animals, as autolysis and bacterial contamination can often result in high background staining.

Strains of modified live virus (MLV) vaccine multiply mainly in the regional lymph nodes and in the crypt epithelium of the tonsils. Pigs vaccinated with MLV strains may yield a positive FAT for 2 weeks after vaccination (22, 28). Rabbit inoculation is used to differentiate between lapinised and field strains of CSFV. In contrast to field strains, lapinised strains given intravenously cause a febrile reaction and induce an immune response in rabbits. As nucleic acid sequencing has become available and more reliable, animal inoculation is no longer necessary to differentiate between field strains and vaccine strains of CSFV.

Pigs infected with ruminant pestiviruses can give false-positive FAT reactions. Congenital infections with ruminant pestiviruses can cause clinical signs and pathological lesions indistinguishable from those in chronic CSF (31, 33, 35). Infections by CSFV or ruminant pestiviruses can be differentiated by testing sera from the dam and litter mates, or from other contacts of an FAT-positive piglet, for neutralising antibodies to each virus. If the virus was isolated, or viral nucleic acid can be detected, using RT-PCR, subsequent sequencing provides a rapid and accurate tool to distinguish ruminant pestiviruses from CSFV. Another method of differentiating these viruses is by the inoculation of seronegative piglets with a suspension of suspect material, followed at least 4 weeks by virus neutralisation (VN) tests on their sera for the respective antibodies. However, VN tests may take several days, and animal inoculation methods take several weeks.

• Immunoperoxidase procedure for differentiation of pestiviruses by monoclonal antibodies

The use of a panel of three monoclonal antibodies (MAbs), either horseradish peroxidase (HRPO) or FITC-conjugated, or used in conjunction with an anti-mouse conjugate and specifically detecting all field strains of CSFV, vaccine strains of CSFV and ruminant pestiviruses, respectively, would allow an unambiguous differentiation between field and vaccine strains of CSFV on the one hand, and between CSFV and other pestiviruses on the other (11, 36, 38). A prerequisite is that the MAb against CSFV recognises all field strains and that the anti-vaccine MAb recognises all vaccine strains used in the country. No single MAb selectively reacts with all ruminant pestiviruses (11). The use of an MAb to differentiate a CSF vaccine strain can be omitted in nonvaccination areas. A polyclonal anti-CSF immunoglobulin conjugated to HRPO serves as a positive control. Caution should be exercised when using evidence of a single MAb as sole confirmation of an isolate as CSF.

• Test procedure

i) Cut eight or more cryostat sections (4–8 µm) of the FAT-positive tonsil, or another positive organ if the tonsil is not available.

ii) Fix the sections on to flying cover-slips for 10 minutes in acetone (analytical grade) and allow to air dry.

iii) Prepare working dilutions of the respective MAb-peroxidase conjugates in PBS + 0.01% Tween 80 + 5% horse serum, pH 7.6. (FITC–MAb can also be used, as well as unconjugated MAb provided that a secondary conjugate is used.)

iv) After rinsing with PBS, overlay two sections with the working dilution of the respective monoclonal conjugates, and two sections with the working dilution of the polyclonal conjugate (controls).

v) Incubate for 1 hour at 37°C in a humid chamber.

vi) Wash the sections six times for 10 seconds each in PBS.
vii) Stain the sections with freshly prepared chromogen–substrate solution* for 5–15 minutes at room temperature.

viii) Rinse the sections in 0.05 M sodium acetate, pH 5.0, in distilled water and mount them on microscope slides.

ix) Examine sections with a light microscope. Dark red staining of the cytoplasm of the epithelial cells lining the tonsillar crypts indicates recognition of the virus isolate by the respective conjugate, and is considered to be positive.

x) Interpretation of the test:

<table>
<thead>
<tr>
<th>Polyclonal antibody</th>
<th>Monoclonal antibody specific for</th>
<th>Interpretation</th>
</tr>
</thead>
<tbody>
<tr>
<td>CSF strain</td>
<td>CSF vaccine strain</td>
<td>BVD/BD strain</td>
</tr>
<tr>
<td>+</td>
<td>+</td>
<td>–</td>
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</table>

†The existence of novel strains of CSF should always be considered and any isolate from cases where CSF is still suspected should be sent to an OIE Reference Laboratory.

* Antigen-capture assay

For rapid diagnosis of CSF in live pigs, antigen-capture enzyme-linked immunosorbent assays (ELISAs) have been developed for screening herds suspected of having been recently infected. The ELISAs are of the double-antibody sandwich type, using monoclonal and/or polyclonal antibodies against a variety of viral proteins in either serum, the blood leukocyte fraction or anticoagulated whole blood; in addition, some test kits can be used to test clarified tissue homogenates (8). The technique is relatively simple to perform, does not require tissue culture facilities, is suitable for automation and can provide results within half a day. The disadvantage of being less sensitive than virus isolation, especially in adult pigs and mild or subclinical cases, may be compensated by testing all pigs of the suspect herd showing pyrexia. However, the lowered specificity of these tests should also be taken into consideration. The test is not suitable for the diagnosis of CSF in a single animal.

b) Isolation of virus

Isolation of virus in cell cultures is a more sensitive but slower method for diagnosis of CSF than immunofluorescence on frozen sections. Isolation is best performed in rapidly dividing PK-15 cells seeded on to cover-slips simultaneously with a 2% suspension of the tonsil in growth medium. Other pig cell lines may be used, but should be demonstrably at least as sensitive as PK-15 cells for isolation of CSFV. The cultures are examined for fluorescent foci by FAT after 24–72 hours or after 4–5 days incubation are fixed for immunoperoxidase staining.

The tonsil is the most suitable organ for virus isolation from pigs that died or were killed for diagnostic purposes. Alternatively, spleen, kidney, ileum or lymph nodes can also be used.

A detailed procedure for virus isolation is as follows:

i) Prepare a 100-fold strength glutamine–antibiotic stock solution: dissolve glutamine (2.92 g) in 50 ml distilled water (solution A) and sterilise by filtration. Dissolve each of the following antibiotics in 5–10 ml sterile distilled water: penicillin (10⁶ International Units [IU]); streptomycin (1 g); mycostatin (5 × 10⁵ U); polymixin B (15 × 10⁴ U); and kanamycin (1 g). Pool these solutions (solution B). Mix aseptically solutions A and B, make up to 100 ml with sterile distilled water, and store in 5 ml aliquots at –20°C. Exact antibiotic constitution is not critical, provided sterility is achieved and cells are not affected.

* Chromogen–substrate solution
A. Stock solution of chromogen: 0.4% 3-amino-9-ethyl carbazole; N,N-dimethyl-formamide (1 ml).
   Caution TOXIC compound. Both chemicals are carcinogens and irritants to eyes, skin and respiratory tract.
B. 0.05 M sodium acetate, pH 5.0; 19 ml (sterile filtered through a membrane).
C. Stock solution of substrate (30% hydrogen peroxide).
Keep stock solutions A and C at 4°C in the dark and solution B at room temperature. Stock solution A can be kept at 4°C for at least 6 months and solution C for 1 year. Immediately before use, dilute 1 ml of solution A in 19 ml of solution B. Then add 10 µl of stock solution C. Mix well and stain the sections.
ii) Cut 1–2 g of tissue into small pieces and, using a mortar and pestle or other device, grind in a small amount of cell culture medium with sterile sand into a homogeneous paste. Alternatively, use an appropriate crushing machine at 4°C.

iii) Make a 20% (w/v) suspension by adding Hanks’ balanced salts solution (BSS) or Hanks’ minimal essential medium (MEM); 1 ml of the glutamine–antibiotic stock is added for each 10 ml of suspension. This mixture is held at room temperature for up to 1 hour.

iv) Centrifuge at 1000 g for 15 minutes.

v) A PK-15 monolayer is trypsinised, the cell suspension is centrifuged at 160 g for 10 minutes, and resuspended to contain approximately $2 \times 10^6$ cells/ml in growth medium (Eagle’s MEM with Earle’s salts; 5% fetal bovine serum free from ruminant pestiviruses and pestivirus antibodies; and 0.2 ml of the glutamine–antibiotic stock solution per 10 ml cell suspension). As a guide, one 75 cm² flask will give approximately 50 ml of cell suspension at the appropriate concentration.

vi) Either:

- **Suspension inoculation:** mix nine parts of cell suspension (from step v) and one part of supernatant fluid (from step iv) and inoculate 1.0–1.5 ml into 6–8 Leighton tubes with cover-slips or other appropriate cell culture flasks. Three tubes are inoculated with 1.0–1.5 ml of cell suspension alone as controls. After completion of the sample inoculations, three tubes are inoculated with CSFV as positive controls. Careful precautions must be taken to avoid cross-contamination with this known positive virus suspension. Negative cultures should also be prepared. Incubate at 37°C.

- **Or:**

  **Pre-formed monolayer inoculation:** for each tissue, inoculate 1.0–1.5 ml of cell suspension (prepared as in step v) into 6–8 Leighton tubes with cover-slips or other appropriate cell culture flasks. Incubate at 37°C for a minimum of 4 hours and a maximum of 36 hours. Then drain the medium and inoculate 0.2 ml of supernatant fluid (from step iv), incubate for 1 hour at 37°C, rinse and overlay with 1 ml of growth medium and incubate at 37°C.

vii) At 1, 2 and 3 days after inoculation, two cultures, together with a positive and negative control culture are washed twice for 5 minutes each in Hanks’ BSS, Hanks’ MEM or PBS, fixed with cold acetone (analytical grade) for 10 minutes, and stained with a direct anti-CSFV conjugate at its appropriate working dilution or indirectly, as described in Section B.1.a.

If the 2% tonsil suspension proves to be toxic for the cells, then the test should be repeated using a higher dilution or another organ. Use of the method employing pre-formed monolayers (above) will help to avoid such.

viii) After washing in PBS three times for 5 minutes each, the cover-slip cultures are mounted in 90% carbonate/bicarbonate buffered glycerol, pH>8.0, and examined for fluorescent foci.

Instead of Leighton tubes, 6-well plates with cover-slips can be used. Alternatively, cultures growing on flat-bottomed microtitre plates or M24-plates can also be used for virus isolation. In such case, plates are fixed and stained as described later for the neutralising peroxidase-linked assay (NPLA).

Whole blood (heparin or EDTA treated) from clinically diseased pigs is a suitable sample for early CSF diagnosis. The leukocyte fraction or other components may be used, but for reasons of sensitivity and simplicity whole blood is preferred (10). The procedure is as follows:

i) Freeze a sample of whole blood at –20°C and thaw in a waterbath at 37°C.

ii) Inoculate 300 µl haemolysed blood on to a PK-15 monolayer grown to approximately 75% confluence* in an M24-plate, and allow adsorption for 1 hour at 37°C.

iii) Remove inoculum, wash the monolayer once with Hanks’ BSS or Hanks’ MEM, and add growth medium.

iv) After a further incubation period of 3–4 days, the plates are washed, fixed and stained, as described later for the NPLA, using in each step a volume of 300 µl to compensate for the larger cell surface.

Note: this method is less sensitive than conventional virus isolation for the detection of acute CSF.

- **Reverse-transcription polymerase chain reaction**

Many methods for RT-PCR have been described and are still being developed. (20). This internationally accepted method is rapid and more sensitive than antigen-capture ELISAs or virus isolation making it particularly suited to preclinical diagnosis. Several conventional and real-time PCR protocols have been

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* Simultaneous inoculation, though slightly more sensitive, is less suitable as the anticoagulant may interfere with the adhesion of cells on to the surface.
described (14, 20, 24, 26, 27) and a suitable protocol may be obtained from the literature or from the OIE Reference Laboratories for CSF (see Table given in Part 3 of this Terrestrial Manual). Due to its speed and sensitivity, RT-PCR is a suitable approach to screening suspect cases of disease and is now accepted by a number of countries and the European Union (EU) (1). However, it has to be kept in mind that false positive results due to laboratory contamination can occur as well as false negative results due to inhibitors contained in the sample. Any positive results from primary outbreaks should always be confirmed by other tests. It is mandatory to include an adequate number of positive and negative controls in each run; it is also recommended that internal controls be included. See Chapter 1.1.5 Validation and quality control of polymerase chain reaction methods used for the diagnosis of infectious diseases, for further details on PCR techniques. The test can be applied to individual or pooled blood samples as well as solid organs and has been used successfully to control outbreaks.

The molecular epidemiology of CSF is based on the comparison of genetic differences between virus isolates. RT-PCR amplification of CSFV RNA followed by nucleotide sequencing is the simplest way of obtaining the sequence data to make these comparisons. A number of different regions of the CSFV genome may be targeted for molecular epidemiological studies (23). Two regions have been extensively studied and provide large sets of sequence data with which new isolates can be compared. One of these regions lies within the 5'-noncoding region (5'NCR) of the genome (150 nucleotides) and the other lies within the E2 major glycoprotein gene (190 nucleotides). In brief, the method used is to extract virus RNA from infected PK-15 cell cultures, perform RT-PCR to amplify one or both targets within the 5'NCR or the E2 gene, and then determine the nucleotide sequence of the products and compare with stored sequence information held in the databases. A database of these sequences is available from the OIE Reference Laboratory for CSF (Hanover, Germany). Recent findings on analysing ruminant pestivirus sequences highlight the need for analysis of multiple regions in order to accurately type strains by this method (15). CSFV isolates from primary outbreaks should be sent to an OIE Reference Laboratory for investigation of molecular epidemiology. An import permit should be obtained prior to dispatch.

2. Serological tests

Detection of virus-specific antibodies is particularly useful on premises suspected of having infections with CSF strains of low virulence. Due to the immunosuppressive effect of CSFV, antibodies cannot be detected with certainty until 21 days post-infection. Serological investigations aimed at detecting residual foci of infection, especially in breeding herds, may also be useful in a terminal phase of CSF eradication.

As the incidence of infection with ruminant pestiviruses may be high in breeding stock, only tests that will discriminate between CSF and BVD/BD antibodies are useful. VN and the ELISA using MAbs satisfy the requirements for sensitivity, but positive results should be confirmed by comparative VN testing.

Neutralisation tests are performed in cell cultures using a constant-virus/varying-serum method. As CSFV is noncytopathic, any non-neutralised virus must be detected, after multiplication, by an indicator system. The NPLA (29) and the fluorescent antibody virus neutralisation (FAVN) test (18) are the most commonly used techniques. Both tests can be carried out in microtitre plates. The NPLA system is now favoured, being easier to read and having the advantage that the results can be determined by use of an inverted light microscope, though a crude assessment of titre can be made with the naked eye.

a) Neutralising peroxidase-linked assay (a prescribed test for international trade)

The NPLA is carried out in flat-bottomed microtitre plates. Sera are first inactivated for 30 minutes at 56°C. For international trade purposes, it is best to test with an initial serum dilution of 1/5 (1/10 final dilution). For surveillance schemes within a country, a screening dilution of 1/10 may suffice. Appropriate controls to ensure specificity and sensitivity of reactions are incorporated into each test.

- Test procedure
  i) Dispense dilutions of serum in growth medium (Eagle’s MEM, 5% fetal bovine serum and antibiotics) in 50 µl volumes into duplicate wells of a microtitre plate. The fetal bovine serum must be free from both BVDV and antibodies to it. A third well may be included for each sample. This well contains serum and not virus and is used as a serum control (for cytotoxicity and/or nonspecific staining).
  ii) Add 50 µl of virus suspension to the wells, diluted in growth medium to contain approximately 100 TCID<sub>50</sub>/50 µl, and mix the contents on a microplate shaker for 20 seconds.
  iii) Incubate the plates in a CO<sub>2</sub> incubator for 1 hour at 37°C.
  iv) Add to all wells 50 µl of growth medium containing 2 × 10<sup>5</sup> cells/ml.
  v) Allow the cells to grow at 37°C in 5% CO<sub>2</sub> to become confluent, usually within 3–4 days.
  vi) Discard the growth medium and rinse the plates once in 0.15 M NaCl.
vii) Drain the plates by blotting on a towel.

viii) The cell monolayers may be fixed in one of several ways:

- The plates are incubated for 45 minutes at 37°C, and then for at least a further 45 minutes at –20°C. The plates are removed from the freezer, the wells are filled with 100 µl 4% paraformaldehyde in PBS and reincubated for 5–10 minutes at room temperature. The paraformaldehyde is discarded and the plates are rinsed with 0.15 M NaCl; or
- The plates are incubated at 70–80°C for 1–2 hours; or
- The plates are fixed with 80% acetone and incubating at 70–80°C for 1 hour; or
- The plates are fixed in 20% acetone in PBS for 10 minutes followed by thorough drying at 25–30°C for 4 hours. (This can be done quickly with the aid of a hair-dryer – after 3–5 minutes complete dryness is obtained as observed by the whitish colour of the cell monolayer.)

ix) Add to each well 50 µl of a hyperimmune porcine CSF antiserum or monoclonal antibody, diluted in 0.5 M NaCl containing 1% Tween 80 + 0.1% sodium azide, pH 7.6. Incubate at 37°C for 15 minutes. The working dilution of the antiserum should be determined by prior titration: i.e. a serum with an NPLA titre of 1/30,000 could be used at 1/100.

x) Wash the plates five times in 0.15 M NaCl containing 1% Tween 80, pH 7.6.

xi) Add to each well 50 µl of an anti-porcine or anti-murine (as appropriate) IgG-HRPO conjugate, diluted to its working concentration in 0.5 M NaCl with 1% Tween 80, pH 7.6, and then incubate for 10 minutes at 37°C.

xii) Wash the plates five times in 0.15 M NaCl containing 1% Tween 80, pH 7.6.

xiii) Add 50 µl of chromogen–substrate solution to each well and stain for 15–30 minutes at room temperature. This solution is described in Section B.1.a ‘Immunoperoxidase procedure for differentiation of pestiviruses by monoclonal antibodies’.

xiv) The test is read visually. Infected cell sheets are completely or partially stained reddish brown. The monolayer should be examined by low-power microscopy to determine the end-point of the titration. The cytoplasm of infected cells is stained dark red.

xv) The following controls are included in the test: cell control, positive serum and back titration of test virus. The back-titration should confirm that virus has been used at a concentration of between 30 and 300 TCID₅₀/50 µl.

NOTE: The incubation times given above are for guidance only. Longer incubation times, with reagent dilutions optimised to such times, may be used, in order to conserve reagents.

b) Fluorescent antibody virus neutralisation test (a prescribed test for international trade)

Leighton tube method:

i) Seed a suspension of PK-15 cells at a concentration of 2 × 10⁵ cells/ml into Leighton tubes with a cover-slip.

ii) Incubate the cultures for 1–2 days at 37°C until they reach 70–80% confluency.

iii) Inactivate the sera for 30 minutes at 56°C. For international trade purposes, it is best to test with an initial serum dilution of 1/5 (1/10 final dilution).

iv) Incubate equal volumes of diluted serum and virus suspension containing 200 TCID₅₀ (50% tissue culture infective dose) per 0.1 ml for 1–2 hours at 37°C. Thus a constant amount of CSFV of 100 TCID₅₀ is used for each reaction well.

v) Remove the cover-slips from the Leighton tubes, wash briefly in serum-free medium, overlay the cell sheet with the serum/virus mixture (from step iv) and incubate for 1 hour at 37°C in a humid atmosphere.

vi) Place the cover-slip in a clean Leighton tube and incubate the cultures in maintenance medium for 2 more days.

vii) Remove the cover-slips from the Leighton tubes, wash the monolayers twice for 5 minutes each in PBS, pH 7.2, fix in pure acetone for 10 minutes and stain with the working dilution of the conjugate for 30 minutes at 37°C before washing.

viii) Mount the cover-slips on grease-free microscope slides with 90% carbonate/bicarbonate buffered glycerol, pH>8.0, and examine for fluorescence.
When the FAVN test is performed in microtitre plates, the procedure for the NPLA (see below) can be followed up to step viii. The plates are then stained with the working dilution of the conjugate for 30 minutes at 37°C and examined for fluorescence. Note: When detecting fluorescence, microplates are best examined from above, using a long focal-length objective.

Occasionally, sera from pigs infected with BVDV or BDV react in the FAVN or NPLA at low dilution as if they were infected with CSFV. The extent of cross-reactivity depends on the strain of ruminant pestivirus involved and the interval between infection and time of sampling (37). The usually high antibody levels reached after exposure to CSF infection, including strains of low virulence, allow the use of comparatively high initial dilutions in NPLA tests for CSF antibody, thus avoiding most, but not all, cross-reactions (29, 30). In case of continued doubt, comparative tests using a strain of CSFV, a strain of BVDV and a strain of BDV, that are representative for the country or region, have proved useful. Comparative neutralisation tests are end-point titrations in which the same series of twofold dilutions of the suspected serum sample is tested in duplicate against 100 TCID$_{50}$ of each selected virus strain. The comparative tests are performed according to the protocols described for the FAVN or NPLA; the cell lines used must be suitable for BVDV and BDV. Neutralisation titres are expressed as the reciprocal of the highest serum dilution that prevents virus growth in 50% of two replicate wells. A three-fold difference or more between end-points of two titrations should be considered decisive for an infection by the virus species yielding the highest titre. It may be necessary to use different strains of the same genotype, and/or to test several pigs from an infected herd to obtain a definitive result.

c) Enzyme-linked immunosorbent assay (a prescribed test for international trade)

Competitive, blocking and indirect techniques may be used on any suitable support and a number have been described (e.g. 5, 13, 17, 21, 34). The tests used should minimise cross-reactions with BVDV and other pestiviruses. However, the test system must ensure identification of all CSF infections, and at all stages of the immune response to infection.

Antigen: The antigen should be derived from or correspond to viral proteins of one of the recommended CSFV strains. Cells used to prepare antigen must be free from any other Pestivirus infection.

Antisera: Polyclonal antisera for competitive or blocking assays should be raised in pigs or rabbits by infection with one of the recommended CSFV strains or with the lapinised C strain. MAbs should be directed against or correspond to an immunodominant viral protein of CSFV. Indirect assays should use an anti-porcine immunoglobulin reagent that detects both IgG and IgM.

The sensitivity of the ELISA should be high enough to score positive any serum from convalescent animals, i.e. at least 21 days post-inoculation that reacts in the neutralisation test. The ELISA may only be used with serum or plasma samples derived from individual pigs. If the ELISA procedure used is not CSF-specific, then positive samples should be further examined by differential tests to distinguish between CSF and other pestiviruses.

The complex-trapping blocking ELISA (5) is a one-step method and is suitable for use in automated ELISA systems e.g. robots. The sera are tested undiluted. The test is fast and easy to perform, and detects antibodies against low virulence strains of CSFV at an early stage after infection. As the MAbs are specific for CSFV, the complex-trapping blocking ELISA will only rarely detect antibodies against BVDV, although BD antibodies can be more problematic. Positive sera are retested for confirmation by the NPLA.

Recently, a novel ELISA has been described that uses fused protein derived from viral peptides (19). The test claims to provide greater sensitivity and earlier detection of antibody than is obtained by conventional ELISAs, but, at this time, its reactivity with antibody induced by diverse strains of CSF is not known.

More information on commercial kits for diagnosis can be obtained from the OIE Reference Laboratories.

C. REQUIREMENTS FOR VACCINES AND DIAGNOSTIC BIOLOGICALS

Effective inactivated whole virus vaccines against CSF are not available.

C1. Modified live virus vaccines

MLV vaccines are produced from CSFV strains that have been attenuated by passage either in cell cultures or in a suitable host species not belonging to the family Suidae. Production is carried out in cell cultures, or in non-Suidae, based on a seed-lot system. This must be validated with respect to identity, sterility, purity, safety, nontransmissibility, stability and immunogenicity.
Chapter 2.8.3. – Classical swine fever (hog cholera)

Guidelines for the production of veterinary vaccines are given in Chapter 1.1.8 Principles of veterinary vaccine production. The guidelines given here and in Chapter 1.1.8 are intended to be general in nature and may be supplemented by national and regional requirements.

The vaccine production facility should operate under the appropriate biosecurity procedures and practices. If CSFV is used for vaccine production or for vaccine–challenge studies, that part of the facility where this work is done should meet the requirements for Containment Group 4 pathogens as outlined in Chapter 1.1.2.

To produce a seed lot and a final vaccine of high quality, the optimal conditions for virus yield must be determined. For vaccines produced in cell cultures, growth curve experiments must be done to study the effect of composition of the medium, regulation of pH and atmospheric CO₂ content, starting concentration of seeded cells, ratio between cell sheet surface and medium volume, phase of the cell growth at the time of viral infection, stationary or rolling conditions during viral replication, etc. For vaccines produced in animals, their age, breed, weight, the size of inoculum (number of animal ID₅₀ [50% infectious dose]), pathogenesis of the infection, and the clinical signs are factors that must be investigated to determine the peak of virus growth and the tissues to be harvested.

Regardless of the production method, the substrate should be harvested under aseptic conditions and be subjected to a freeze–thaw cycle to release cell-associated virus. Coarse cell or tissue elements are removed by filtration or low-speed centrifugation. A stabiliser is added, such as lactose at a final concentration of 5%. The vaccine is homogenised before lyophilisation to ensure a uniform batch.

The vaccine virus in the final product should not differ by more than five passages from the material used for validating the seed lot. The commercial vaccine should be produced in batches in lyophilised form as a homogeneous product.

1. Seed management

a) Characteristics of the seed

To validate a seed lot for an MLV CSF vaccine, samples of the seed lot must first pass pilot experiments. Except for tests to confirm identity, sterility, purity and stability of attenuation, pilot experiments may also be performed with representative samples of the final commercial product. These samples must originate from the same seed lot as tested above.

Except where otherwise specified, all pigs used in pilot tests are 6–8 weeks of age, healthy, free from antibodies against CSFV and BVDV, of the same breed and origin, grouped at random if necessary, and kept under the same conditions. Pregnant sows must be of equal parity.

The seed virus must be sterile and induce specific neutralising antibodies against a virulent strain of CSFV in pigs.

b) Method of culture

Production is performed in cell cultures or in a suitable host of a species not of the family Suidae.

c) Validation as a vaccine

i) Purity

Vaccine must be virologically pure.

Each of three seronegative pigs is inoculated intramuscularly with an amount of seed-lot virus equivalent to tenfold the amount of virus contained in one dose of vaccine. This is repeated 3 weeks later using the same dose and route of administration. Serum samples are collected 2 weeks after the last inoculation, and tested by the most sensitive method for freedom from antibodies to the viruses of African swine fever, Aujeszky’s disease, BVD, foot and mouth disease (all types), transmissible gastroenteritis, swine vesicular disease, porcine reproductive and respiratory syndrome, and porcine influenza (types H1N1 and H3N2); and for porcine adenoviruses, porcine teschoviruses (types 1 and 2), porcine parvovirus and porcine circoviruses (types 1 and 2).

ii) Safety

Vaccines should be tested for any pathogenic effects on healthy pigs, and also on pigs that might be immunosuppressed due to the presence of concurrent infection or medication, as well as testing to ensure that the vaccine does not cross the placenta of pregnant sows.

For tests of safety in conventional pigs, each of ten seronegative pigs is inoculated intramuscularly with ten vaccine doses. Ten other pigs serve as controls. All pigs are observed for 3 weeks thereafter.
Body temperatures are recorded and blood samples are collected daily, with an anticoagulant, for the first week. Body weights are recorded at inoculation and 2 weeks later. No animal should die or show signs of illness caused by the vaccine (seed-lot) virus. A daily group average body temperature should not reach 40.5°C or more throughout the trial period. The average weight gain should not fall significantly (p <0.05) below that of the controls. Leukopenia (white blood cell [WBC] count <7 × 10^6 cells/ml) may be disregarded if it is only in one pig for 1 day.

To ensure attenuation, even in immunosuppressed pigs, each of ten pigs is immunosuppressed by daily injections, each of 2 mg prednisolone/kg body weight, for 5 consecutive days. On day 3, each animal is inoculated with the equivalent of one dose of vaccine, and kept under observation for 3 weeks thereafter. No animal should die or become ill due to the vaccine virus.

To ensure safety in pregnant animals, each of ten sows, 25–35 days pregnant, is inoculated intramuscularly with the equivalent of one dose of vaccine. A further ten animals of the same parity and gestation serve as controls. The vaccination should not interfere with normal gestation to term, and the number of live piglets born from the test group should not be significantly fewer (p <0.05) than that for the control pigs.

For field trials, a minimum of 200 pigs is used, farrowed and reared by at least 20 dams, and seronegative for CSF and BVD. The litters are equally distributed over at least two farms. Half of the piglets in each litter are inoculated intramuscularly at 7–14 days of age with the equivalent of one dose of vaccine. The uninoculated littermates are controls. All piglets are weighed at inoculation and 2 weeks later, and are kept under observation for 3 weeks. A mortality rate that exceeds 5% due to causes other than vaccination invalidates the trial. No animal should die or show signs of disease due to the vaccine virus. The average weight gain of the inoculated pigs in the litters should not be more than 20% below that of the controls during the 2 weeks post-inoculation.

iii) Nontransmissibility

To confirm nontransmissibility, 24 seronegative pigs are divided into four groups of equal size. Five pigs in each group are inoculated intramuscularly with the equivalent of one dose of vaccine. The remaining pigs represent in-contact controls. All pigs are challenged 6 weeks later with at least 10^5 PID<sub>50</sub> (50% pig infectious dose) of a virulent strain of CSFV. All in-contact animals should be serologically negative at the time of challenge, and then die within 3 weeks. All vaccinated pigs should remain healthy and survive.

iv) Stability of attenuation

To confirm stability of virus attenuation, two pigs are each inoculated intramuscularly with an amount of seed-lot virus equivalent to 100 doses of vaccine, and then killed 6–7 days later. The tonsils of both pigs are pooled and made into a 10% suspension in PBS, pH 7.2. This is used to inoculate two further pigs intramuscularly with 2 ml, and these are then killed 6–7 days later. This protocol is repeated five times. During these passages, the tonsillar tissue may be stored at 4°C, if storage is to be under 24 hours, or at –70°C for longer periods. At the same time, the presence of CSF antigen is confirmed at each passage by the direct FAT in cryostat sections of the tonsils, or by virus isolation in a suitable substrate. If CSFV or antigen cannot be demonstrated after a certain passage, a second series of passages is performed to show infection, commencing with the last two pigs of the previous series.

Five pigs are inoculated intramuscularly with the sixth pig passage of the seed-lot virus, equivalent to one vaccine dose or, if this passage has not been reached, the highest passage of the two series where virus or viral antigen was detected. Five further pigs are similarly inoculated with one dose of the seed-lot virus, equivalent to one vaccine dose. All pigs are weighed at the time of inoculation and again 2 weeks later. Blood is collected daily into anticoagulant during the first week, and all pigs are kept under observation for 3 weeks. No animal should die or become ill from the vaccine virus. The average weight gain of the two groups during the first 2 weeks should not differ significantly (p <0.05). Leukopenia (WBC count of <7 × 10^6/ml) is permitted, at the most, in one pig of either group for 1 day.

v) Immunogenicity

To demonstrate adequate immunogenicity, ten pigs are each inoculated with an amount of virus equivalent to one dose of vaccine, and two other pigs are housed separately as uninoculated controls. All pigs are challenged 7 days later with 10^5 PID<sub>50</sub> of a virulent strain of CSFV. Only the controls should die.

In a test for duration of immunity, ten pigs are each inoculated with one dose, and two others are maintained separately as controls. Six months later, the sera of the inoculated pigs are tested for CSF antibodies; at least eight pigs should be positive. All pigs are then challenged with at least 10^5 PID<sub>50</sub> of a virulent strain of CSFV, and observed for 3 weeks. Only the controls should die.

To test protection of the development of the carrier sow syndrome, 20 pregnant sows at the same stage of gestation are randomly divided into two groups. The sows of one group are vaccinated once...
or twice with an amount of virus equivalent to one dose of vaccine, and intranasally challenged 4 weeks after the last vaccination with a field strain of low virulence, together with the unvaccinated control sows. All sows are killed 4 weeks after challenge and the fetuses are examined for the presence of CSFV or viral antigen. Vaccination should significantly reduce transplacental transmission of the virus.

Under the storage conditions prescribed by the manufacturer for the final product, a volume of virus equivalent to one dose of vaccine must maintain its immunogenicity at least until the end of the stated shelf life.

2. Method of manufacture

Each batch of MLV CSF vaccine must be derived from the same seed lot that has been used for the pilot tests. Also, each batch must be prepared according to the production protocol and under the conditions laid down for the registration of the final product. The properties of each batch and those of the seed lot must be verified as uniform.

3. In-process control

The protocol for production will depend on the vaccine strain, the production system (animals or cell cultures), and available facilities. The norms for cell culture vaccines may vary according to the production system, namely, primary cultures, cell lines, monolayers or suspension cultures.

4. Batch control

All pigs used in batch control tests must be 6–8 weeks of age and free from antibodies to CSFV and BVDV. They must be uniform in origin, breed, husbandry, and randomly distributed into any groups where necessary.

a) Identity

The vaccine must induce specific neutralising antibodies against a virulent strain of CSFV.

b) Sterility

Tests for sterility and freedom from contamination of biological material may be found in Chapter 1.1.9.

c) Safety

Each of three pigs is inoculated intramuscularly with ten doses of the reconstituted vaccine as a single injection. The pigs are observed for 3 weeks thereafter and body temperatures are taken daily for the first week. No pig should die or show signs of disease attributable to the vaccine, the average daily body temperature must at no time reach 40.5°C or more, and the pigs should grow normally.

d) Purity

The batch must be virologically pure. To test for this, three pigs are each inoculated intramuscularly with ten vaccine doses. Serum samples are collected at the time of inoculation and again 5 weeks later. These are tested for antibodies to BVD (neutralisation for 1 hour at 37°C) and porcine parvovirus (haemagglutination inhibition using four haemagglutinating units). All three pigs must remain disease free. Tests for virological purity need not be carried out when using vaccines produced in rabbits.

e) Potency

Potency is expressed as the number of 50% protective doses (PD$_{50}$) contained in one vaccine dose. One vaccine dose is at least 100 PD$_{50}$.

Two groups of five 6–8-week-old piglets are inoculated intramuscularly with a 1/40 and a 1/160 dilution of the reconstituted vaccine, respectively, using buffered salt solution, pH 7.2. The vaccinated pigs together with two controls are challenged intramuscularly with 10$^3$ PID$_{50}$ of a virulent strain of CSFV 2 weeks later. The pigs are observed for 2 weeks thereafter, during which time the controls should die. From the pigs that survive without showing any signs of CSF, the number of PD$_{50}$ contained in the vaccine is calculated using the usual statistical methods.

This potency test may be replaced by an infectivity assay, provided that the manufacturer can show that there is a distinct and reproducible relationship between the virus content of the vaccine and the protection it will confer on pigs against challenge.
Chapter 2.8.3. – Classical swine fever (hog cholera)

f) **Stability**

The period of validity of a batch of lyophilised CSF vaccine should not be under 1 year.

C2. **Marker vaccines**

Despite the existence of safe and effective MLV vaccines against CSF, their use has been discouraged in the EU and some other CSF-free or near free countries, because antibodies provoked by such vaccines cannot be distinguished from antibodies induced by the wild-type virus. A ‘marker vaccine’, which allows discrimination of infected from vaccinated animals (DIVA), does not have this disadvantage: it can elicit a protective immune response that can be distinguished from the immune response induced by field virus. An additional prerequisite of any DIVA strategy is the availability of a companion serological test that is highly discriminatory, for demonstrating absence of infection and in tracing residual infections.

The minimum demands for CSF marker vaccines and the companion discriminatory tests have been formulated as follows (6):

a) **Vaccine**

The vaccine should provide protection against any natural-contact challenge, i.e. it should prevent clinical signs and re-excretion of the virus. The efficacy of vaccination should be shown experimentally by studies in which transmission of wild-type virus in vaccinated groups of pigs is studied. The protective effect of vaccination should be achieved within the shortest possible period and ideally less than 2 weeks. A fast and reliable protection should preferably be obtained after one single application. Furthermore, it should be ensured that infection of vaccinated pregnant sows does not lead to transplacental infection and the birth of litters congenitally infected with CSFV. Duration of immunity should be at least 6 months.

Many different marker vaccines for CSF are under development, and two have been registered in the EU. Both are subunit vaccines that employ the E2 glycoprotein of CSFV as an immunogen and have been subject to independent assessment (9, 32). The E2 subunit is produced by insect cells that are infected by genetically modified baculovirus, which contains the E2 gene of CSFV. The vaccines, therefore, do not contain any CSFV, while the baculo (vector) virus is chemically inactivated. The final preparations are adjuvanted with mineral oils to form a double (water/oil/water), or a single (water in oil) emulsion. Several studies of the ability of these E2 DIVA vaccines to prevent horizontal and vertical transmission have given conflicting results (3).

b) **Companion discriminatory test**

The companion discriminatory serological test should be very sensitive because vaccination will reduce the prevalence of the disease. It should ideally provide discrimination within the same time-frame as a development of antibody to the immunising protein and should be used primarily as a herd test. If a high sensitivity reduces the specificity of the test, already compromised by the presence of antibodies to other pestiviruses, good and fast confirmatory assays should be available to discriminate positive from false-positive results.

The existing accompanying DIVA tests for E2 subunit vaccines are ELISAs that rely on the detection of antibody to the Erns protein (12, 16). Such tests have recently been approved by the European Commission (2), for use in determining whether herds vaccinated with an E2 subunit vaccine may also have been exposed to field virus. An assessment of their performance (12) has revealed that neither discriminatory ELISA consistently detected individual marker-vaccinated, CSF-challenged weaner pigs, hence the recommendation only to employ such a strategy at the herd level.

**REFERENCES**


Chapter 2.8.3. — Classical swine fever (hog cholera)


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**NB:** There are OIE Reference Laboratories for Classical swine fever (see Table in Part 3 of this *Terrestrial Manual* or consult the OIE Web site for the most up-to-date list: www.oie.int).
CHAPTER 2.8.4.

NIPAH VIRUS ENCEPHALITIS

See Chapter 2.9.6. Hendra and Nipah virus diseases.

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CHAPTER 2.8.5.
PORCINE BRUCELLOSIS

SUMMARY

Brucellosis in pigs is caused by Brucella suis, a bacterial infection that, after an initial bacteraemia, causes chronic inflammatory lesions in the reproductive organs of both sexes, with occasional localisation and lesions in other tissues. The species Brucella suis consists of five biovars, but the infection in pigs is caused by B. suis biovars 1, 2 or 3. The disease caused by biovars 1 and 3 is similar, while that caused by biovar 2 differs from 1 and 3 in its host range, its limited geographical distribution and its pathology. Biovar 2 is rarely pathogenic for humans, whereas biovars 1 and 3 are highly pathogenic causing severe disease. Porcine brucellosis is of widespread occurrence; generally, however, the prevalence is low, with the exception of South America and South-East Asia where the prevalence is higher. In some areas, B. suis infection has become established in wild or feral pigs – diagnostic methods recommended for wild and feral pigs are the same as for domestic pigs. Various biovars of B. suis cause infections in animals other than pigs, such as reindeer, caribou, hares and various murine species, and occasionally in cattle and dogs. Brucella suis infections in animals other than pigs are dealt with in an Appendix at the end of this chapter.

Signs of disease in sows include abortion at any stage of gestation, and birth of dead or weak piglets. In boars, the most prominent sign is orchitis, and the secondary sex organs may be affected. Brucella suis may be present in the semen, sometimes in the absence of clinical signs. Transmission during copulation is more common than is the case with brucellosis in ruminants. In both sexes, bones and especially joints and tendon sheaths may be affected, causing lameness and sometimes paralysis. Pigs are susceptible to artificial infection with B. abortus and B. melitensis, but reports of natural disease in pigs being caused by either of these organisms are rare. In humans, the infection is usually confined to those who are occupationally exposed to pigs, and to laboratory workers. The capability of B. suis to colonise the bovine udder with subsequent shedding in milk, has the potential to be a serious human health risk.

Identification of the agent: Brucella suis is readily isolated from live pigs by culture of birth products, and from carcasses by culture of lymph nodes and organs. Selective media are available for culture of contaminated samples. In nature, B. suis occurs invariably in the smooth phase – the appearance on solid medium is typical of smooth brucellae. Biovars of porcine origin agglutinate with monospecific A antiserum, and not with M antiserum. Definite identification of species and biovars may be effected by phage typing and biochemical tests, preferably carried out in specialised laboratories.

Serological tests: To date, none of the conventional serological tests has been shown to be entirely reliable in routine diagnosis in individual pigs. Their preferred use is for the identification of infected herds. The indirect enzyme-linked immunosorbent assay (ELISA) and competitive ELISA are the preferred tests for international trade purposes. The buffered Brucella antigen tests (BBATs), i.e. the buffered plate agglutination test (BPAT) and the rose bengal test (RBT), are suggested as alternative tests for screening purposes or complete herd tests. The procedures for the BBATs are the same as those described in Chapter 2.4.3 Bovine brucellosis. A fluorescence polarisation assay has also been developed. The allergic skin test is also useful for identifying infected herds.

Requirements for vaccines and diagnostic biologicals: Brucella suis strain 2 vaccine has been used for immunising pigs in China (People’s Rep. of). Confirmation of the results obtained in China is required before strain 2 vaccine can be recommended for general use. In other countries, experimental work has shown that B. melitensis Rev.1 vaccine is superior to B. suis strain 2 in protecting sheep against B. melitensis. Sufficient data is not available to conclude if B. abortus
strain RB51 vaccine is efficacious in protecting swine against exposure to B. suis. In practice, no product has yet found general acceptance. Preparation, testing and use of an established allergen, brucellysate (or brucellin fraction F) are described.

### A. INTRODUCTION

Porcine brucellosis is an infection caused by biovars 1, 2 or 3 of *Brucella suis*. It occurs in many countries where pigs are raised. Generally, the prevalence is low, but in some areas, such as South America and South-East Asia, the prevalence is much higher. Porcine brucellosis may be a serious, but presently unrecognised, problem in some countries. *Brucella suis* biovar 1 infections have been reported from feral pigs in some of the southern States of the United States of America (USA), and in Queensland, Australia. In both countries, a number of human infections have been reported from people who hunt and handle material taken from feral pigs (22, 25).

The disease is generally transmitted by consumption of feed contaminated by birth and/or abortion products and uterine discharges. Pigs will readily eat aborted fetuses and membranes. Transmission during copulation also occurs frequently, and this has implications for those practising artificial insemination.

In pigs, as in ruminants, after the initial bacteraemia, *B. suis* colonises cells of the reproductive tract of either sex. In females, placentas and fetuses are invaded, while in males, invasion occurs in one or more of the following: testis, prostate, epididymis, seminal vesicles, and/or bulb-urethral glands. In males the lesions, which are most often unilateral, start with a hyperplasia that may progress to abscess formation; the final stage is characterised by sclerosis and atrophy. Arthritis may occur in various joints, and sometimes spondylitis occurs.

The most common manifestation of brucellosis in female pigs is abortion, occurring very early or at any time during gestation. Vaginal discharge is not often evident, and the problem may appear to be infertility rather than abortion. In males, brucellosis is more likely to be persistent, with lesions in the genital tract often leading to interference with sexual activity, which can be temporary or permanent. The boar may excrete *brucellae* in the semen without any apparent abnormality in the sex organs or interference with sexual activity.

In both sexes, there may be swollen joints and tendon sheaths, lameness and, occasionally, posterior paralysis. A significant proportion of both male and female pigs will recover from the infection, often within 6 months, but many will remain permanently infected.

Brucellosis caused by *B. suis* biovar 2 differs from infection caused by biovars 1 and 3 in its host range, its distribution, and in its pathology. In general, the geographical distribution of biovar 2 has historically been in a broad range between Scandinavia and the Balkans (2). The prevalence in wild boars appears to be high throughout Europe (1, 4, 11). In recent outbreaks in Europe, wild pigs have been implicated as the source of transmission of biovar 2 to outdoor reared pigs (11). In addition to wild swine, the European hare (*Lepus capensis*) is also a reservoir for *B. suis* biovar 2 and has been implicated as a possible source of transmission to domestic livestock (2, 13). *Brucella suis* biovar 2 causes miliary lesions in tissues, particularly reproductive tissues, that often become purulent. To date, biovar 2 has rarely been reported as the cause of human brucellosis. However, biovar 2 infection has been reported in two immuno-compromised hunters, who had been extensively exposed through gutting or skinning boars or hares (12).

The common biovars of *B. suis* (1 and 3) are serious human pathogens and precautions are needed when handling and disposing of potentially infective material. This is especially so in the laboratory after culture has greatly increased the number of organisms present. Laboratory manipulation of the cultures or contaminated material from infected animals must be done under strict biosecurity conditions to safely handle this dangerous zoonotic agent. Biosecurity containment level 3 is recommended (see Chapter 1.1.2 Biosafety and biosecurity in the veterinary microbiology laboratory and animal facilities).

### B. DIAGNOSTIC TECHNIQUES

As far as biovars 1 and 3 are concerned, culture methods are at least as sensitive as serology (6). Biovar 2 appears to be highly sensitive to selective media and could be more difficult to isolate (Garin-Bastuji & Blasco, unpublished data). As the produce of almost all pig-raising enterprises passes through abattoirs, surveillance methods (serology and culture) can be applied effectively at this point. In many areas, traditional village pig breeding is now accompanied by the development of larger commercial units, thereby increasing the use of artificial insemination. Whereas artificial insemination using brucellosis-free boars can be a valuable aid in the control of porcine brucellosis, the inadvertent use of infected semen could, obviously, cause incalculable damage.

#### 1. Identification of the agent

Optimal samples for bacteriologic culture and methods for processing of samples are similar to those described for bovine brucellosis in Chapter 2.4.3 Bovine brucellosis. Standard and selective media used for other species
of brucellae are suitable for \textit{B. suis} (see Chapter 2.4.3 Bovine brucellosis). The addition of serum is not essential, but basal medium containing 5% serum is a satisfactory medium, both for isolation, maintenance of cultures and typing. The addition of CO\textsubscript{2} to the atmosphere is not required.

In nature, \textit{B. suis} invariably occurs in the smooth form and colonies are indistinguishable from other smooth brucellae, described in Chapter 2.4.3 Bovine brucellosis.

Biovars 1, 2 and 3 of \textit{B. suis} are all A surface antigen dominant, and growth may be presumptively identified by slide agglutination with monospecific A antiserum. Confirmatory identification of species and biovar should be performed in a specialised reference laboratory. The OIE Reference Laboratories for brucellosis are listed in the Table given in Part 3 of this Terrestrial Manual.

Confirmation of species and biovar depends on phage tests, production of H\textsubscript{2}S (only biovar 1 produces H\textsubscript{2}S), and growth in the presence of dyes. Some strains of \textit{B. suis} biovar 1 are atypical in that they grow on media containing 20 µg/ml of basic fuchsin. Most strains of \textit{B. suis} are inhibited by safranin O at a concentration of 1/10,000, whereas \textit{B. suis} reacts more rapidly in the urease test than either \textit{B. abortus} or \textit{B. melitensis}. Oxidative metabolic tests are supplemental tests that can be used for distinguishing \textit{B. suis} from other smooth \textit{Brucella} species.

Molecular genetic techniques using the polymerase chain reaction (PCR) and specific primers are available that can distinguish \textit{B. suis} from other smooth species of \textit{Brucella} (3, 24). However, these PCR techniques cannot distinguish biovars within \textit{B. suis}, and these techniques have not been fully evaluated and standardised. The 3.3 Mb complete genomic sequence of \textit{B. suis} strain 1330 has been determined, and is similar in chromosome structure, organization, and gene content to that of \textit{B. melitensis} strain 16M (20) and \textit{B. abortus} strain 9-941 (15). The \textit{B. suis} sequence has been beneficial in basic research on taxonomy, metabolic pathways, and genes mediating virulence in \textit{B. suis}, and may prove beneficial in developing new diagnostic tests or vaccines.

2. Serological tests

None of the conventional serological tests used for the diagnosis of porcine brucellosis are reliable for diagnosis in individual pigs. A significant problem is the fact that weaners up to 2–3 months of age are susceptible to infection with \textit{B. suis}, but their agglutinating antibody response to the infection is very limited.

These conventional tests use antigens that are dependent on smooth lipopolysaccharide (LPS) for their activity. Due to the sharing of an ‘O’ chain polysaccharide, such antigens react equally with the LPS of \textit{Yersinia enterocolitica} serotype O:9 and are not, therefore, able to distinguish between antibodies to these two infections. \textit{Yersinia enterocolitica} infection in pigs is not uncommon in some areas (1, 28). Studies have suggested that the sensitivities and specificities of the buffered acidified plate antigen assay, the 2-mercaptoethanol test, the indirect enzyme-linked immunosorbent assay (I-ELISA), a competitive ELISA (c-ELISA), and the fluorescent polarisation assay (FPA) are similar (19). Use of the FPA (17) or cELISA has been reported to eliminate cross-reactivity with \textit{Y. enterocolitica} but this should be confirmed in additional field studies performed in various epidemiological situations. Swine serum may sometimes also contain nonspecific antibody, thought to be IgM, further reducing the specificity of conventional serology, especially the serum agglutination test (SAT). Also, swine complement interacts with guinea-pig complement to produce a pro-complementary activity that reduces the sensitivity of the complement fixation (CF) test. Sensitivity levels as low as 38% (21) and 49% (23) have been reported for the CF test; therefore this test cannot be recommended for the diagnosis of brucellosis in individual pigs. For international and other trade, e.g. purchasing boars, the disease status of the herd and of the area in which the herd is situated are of more importance than tests on individual animals. Although swine brucellosis serological tests are best used on a herd basis, regulations in some countries require that only pigs whose serum shows an agglutination titre <30 International Units (IU) per ml and a CF test of less than 20 ICFTU (international CF test units) be allowed to cross international borders.

- Reference sera

Primary reference standards are those against which all other standards are compared and calibrated. These reference standards are currently being developed and will be available to national reference laboratories when completed.

a) Enzyme-linked immunosorbent assay (prescribed tests for international trade)

- Indirect ELISA

Indirect and competitive ELISAs have been developed for the diagnosis of brucellosis in individual pigs and for screening large numbers of sera. These techniques promise to be more efficient than any of the tests mentioned above, and the C-ELISA appears to be better at distinguishing antibody reactions that are due to \textit{Y. enterocolitica} serotype O:9 from those that are due to \textit{Brucella}. A method for the 1-ELISA is described in detail in Chapter 2.4.3 Bovine brucellosis, however, monoclonal antibody specific for porcine IgG conjugated with horseradish peroxidase (HRPO) can be used.
• Competitive ELISA

C-ELISA procedures for detection of porcine antibody to *Brucella* sp. (17) are identical to the procedures used for bovine antibody to *B. abortus* described in Chapter 2.4.3. This assay is capable of eliminating most reactions due to *Y. enterocolitica* serotype O:9 and in some situations other cross-reacting antibody, such as IgM, will not compete well. The C-ELISA is recommended as a confirmatory test as its sensitivity and specificity exceeds those of the agglutination tests.

b) Fluorescence polarisation assay (an alternative test for international trade)

The FPA for detection of porcine antibody to *Brucella* sp. is essentially the same as that described for cattle (for more details see Chapter 2.4.3); an example serum dilution used is 1/25 for the tube test and 1/10 for the plate test (17). It is a simple technique for measuring antigen/antibody interaction and may be performed in the laboratory or in the field. This assay may assist in eliminating much of the reactivity resulting from exposure to *Y. enterocolitica* serotype O:9 and other cross-reacting antibody. Lyophilised porcine sera tend to increase background activity in this assay. The FPA may be used as a screening and/or confirmatory test.

c) Buffered *Brucella* antigen tests (an alternative test for international trade)

For screening purposes or complete herd tests, the buffered *Brucella* antigen tests (BBAT), i.e. the card test, the rose bengal plate agglutination test (RBT) or the buffered plate agglutination test (BPAT), are recommended as alternative tests. The preparation and standardisation of BBAT antigens and the methods of performing the tests are described in Chapter 2.4.3 Bovine brucellosis. All biovars of *B. suis* affecting pigs have the same immunodominant A antigen as do most of the *B. abortus* biovars, which makes the *B. abortus* antigens appropriate for testing swine sera.

3. Other Tests

a) Allergic (hypersensitivity) tests

Brucelmin-INRA is an LPS extract from rough *B. melitensis* B115. This preparation does not stimulate the formation of antibodies that would be reactive in BBAT, CF test or ELISA. The product has been developed for use in ruminants, but is also effective for confirming the disease at the herd level in pigs. A rough strain is used in its preparation, thereby avoiding the presence of smooth LPS. The preparation, standardisation and testing, of Brucellmin-INRA is described in detail in Chapter 2.4.3 Bovine brucellosis. As a diagnostic agent in pigs 0.1 ml of the allergen is injected intradermally into the skin at the base of the ear or preferably on one side of the tail. The latter appears more practical and less hazardous. The reaction is read after 48 hours. A positive reaction shows erythema of non-pigmented skin and an oedematous swelling. In severe reactions, there may also be some necrosis.

C. REQUIREMENTS FOR VACCINES AND DIAGNOSTIC BIOLOGICALS

Numerous attempts have been made to develop a vaccine to immunise pigs against *B. suis*. Only one product has found any acceptance for field use – *B. suis* strain 2 (S2) vaccine used extensively in south China (People’s Rep. of) (16, 29)1. To date, it does not appear to have been used elsewhere in pigs, probably because it has been shown to confer less protection in sheep against *B. melitensis* than the Rev.1 vaccine (27). Sufficient data are not available to conclude if *B. abortus* strain RB51 vaccine is efficacious in protecting swine against exposure to *B. suis*.

APPENDIX: *BRUCELLA SUIS* INFECTIONS IN ANIMALS OTHER THAN PIGS

1. Rangiferine brucellosis

*Brucella suis* biovar 4 causes serious disease in reindeer or caribou (*Rangifer tarandus* and its various subspecies) throughout the Arctic region, Siberia, Canada and Alaska (18). Some of these animals are domesticated, others are wild and migratory. *Rangifer tarandus* is very susceptible to *B. suis* infection, which causes fever, depression and various local signs, such as abortion, retained placentas, metritis, sometimes with blood-stained discharge, mastitis, bursitis and orchitis. In the Arctic region, *B. suis* biovar 4 constitutes a serious zoonosis (7). Transmission to humans may be by direct contact or through consumption of milk and other

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1 Obtainable from the National Institute for the Control of Veterinary Products and Pharmaceuticals, Ministry of Agriculture, 30 Baishiqiao Road, Beijing 100081, China (People’s Rep. of), or from VLA Weybridge, New Haw, Addlestone, Surrey KT15 3NB, United Kingdom. Supply from the Weybridge laboratory needs the prior permission of the World Health Organization.
inadequately heated products from reindeer. Bone marrow, which is considered to be a special delicacy in this region, is also a source of human infection.

The methods already described for isolating and identifying B. suis in samples taken from pigs are equally applicable to B. suis biovar 4 in samples taken from reindeer. Biovar 4 grows well on all the usual media used for the culture of Brucella. It reacts positively with both A and M monospecific sera. For serology, the tube agglutination test has been reported to be satisfactory, with titres from 1/20 being considered to be diagnostic. The CF test has also been used but the clinical interpretation of these tests in reindeer has not been established.

Vaccination of reindeer with B. abortus S19 vaccine, or alternatively with B. abortus 45/20 adjuvant vaccine, has been tried experimentally without any clear-cut result. In the case of S19, the reaction to vaccination was rather severe and immunity in the vaccinated animals could only be demonstrated against challenge with very small doses of B. suis biovar 4. Gall et al. (8) compared several serological tests and found that the specificity values for the BPAT and CFT using reindeer/caribou sera was lower than the I-ELISA, C-ELISA and the FPA, while sensitivity values were similar for all tests.

2. Brucella suis infection in other nonporcine species

There are two different types of epidemiological situation with regard to B. suis infection in other nonporcine species. In the first case, B. suis infection occurs in animals that are not the natural host of the particular infection through the ingestion of contaminated materials or by co-habitation with infected natural hosts. For example, Arctic foxes and wolves may contract B. suis biovar 4 from reindeer; dogs and rodents, such as rats and mice, may acquire other B. suis biovars by cohabitation with infected hosts. Cattle and horses may become infected by cohabitation or interaction with infected swine (5). The infecting bacteria are invariably the well defined biovars of the natural host species.

In the second case, wildlife species that are natural hosts for B. suis or B.-suis-like infections become infected. One example is the so-called murine brucellosis of the former USSR, where small rodents are infected with B. suis biovar 5. Other similar situations have been reported from Queensland, Australia and from Kenya. In all three cases, B. suis strains with different characteristics were involved, and at least one of them was difficult to classify.

Brucellosis caused by B. suis biovar 2 is perhaps a special case. Biovar 2 infection historically has been confined to an area between Scandinavia and the Balkans. The reservoir of infection is in wild pigs (Sus scrofa) living in the same area (1, 5, 11, 13, 14), or in the European hare (Lepus capensis) (26), or in both. Domestic swine reared outdoors in this area are at highest risk for transmission of biovar 2 from wildlife vectors. After invading domestic pig herds, biovar 2 is likely to spread as rapidly as biovars 1 and 3. The disease in hares is characterised by the formation of nodules, varying in size from that of a millet seed to a cherry or even larger; these often become purulent. Such nodules may occur in almost any location, sometimes subcutaneously or intramuscularly, in the spleen, liver or lung and in the reproductive organs of either sex. The bodily condition of the hare may be surprisingly unaffected. Other species may also become infected by cohabitation with infected swine, wild boars or hares. Gutting or skinning wild boars in cattle sheds could be a method of transmission to cattle (10).

Serological investigations in nonporcine species are usually carried out for screening purposes. In these particular circumstances, specificity is more important than sensitivity. Here the CF test is recommended, although the buffered Brucella plate agglutination test may be useful because of its simplicity. In many previous investigations, the tube agglutination test was used, apparently with satisfaction. The indirect ELISA appears to be very useful for epidemiological sero-surveys in wild boars as it is more sensitive and specific than the RBT and CF test. The test has also been used successfully on blood samples that are in poor condition such as wildlife samples. When poor quality samples are tested on other tests, the results may be uninterpretable. Another advantage of the ELISA is that if serum is not available, it is possible to test meat samples (9). However, in nonporcine species the interpretation of serological results may be problematic. Where supposedly positive samples are encountered, serological screening should be followed by bacteriological investigation.

For bacteriological investigations in situations such as these, where the infecting organisms may have unusual characteristics, it is advisable to duplicate the culture on selective media by culture on plain medium supplemented with 5% serum, and to broaden the investigation by incubating the cultures in an atmosphere containing 10% CO₂. Colonies resembling Brucella can be tentatively identified by Gram staining, by slide agglutination tests with monospecific A and M sera, and by anti-rough Brucella serum (Chapter 2.4.3 Bovine brucellosis) Brucella suis biovar 5 is unusual in that it reacts with monospecific M serum, and not with monospecific A serum. Further identification is best carried out in a specialised laboratory.

REFERENCES

Chapter 2.8.5. — Porcine brucellosis


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**NB:** There are OIE Reference Laboratories for Porcine brucellosis (see Table in Part 3 of this *Terrestrial Manual* or consult the OIE Web site for the most up-to-date list: www.oie.int).
CHAPTER 2.8.6.

PORCINE CYSTICERCOSIS

See Chapter 2.9.5. Cysticercosis.

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CHAPTER 2.8.7.

PORCINE REPRODUCTIVE AND RESPIRATORY SYNDROME

SUMMARY

Porcine reproductive and respiratory syndrome (PRRS) is characterised by reproductive failure of sows and respiratory problems of piglets and growing pigs. The disease is caused by the PRRS virus, a virus currently classified as a member of the order Nidovirales, family Arteriviridae, genus Arterivirus. The primary target cell of the virus is the alveolar macrophage of the pig. Two major antigenic types of the virus exist, the European and the American type. The virus is primarily transmitted via infected pigs but also by faeces, urine, semen and fomites.

PRRS occurs in most major pig-producing areas throughout the world. The reproductive failure is characterised by infertility, late fetal mummification, abortions, stillbirths, and the birth of weak piglets that often die soon after birth from respiratory disease and secondary infections. Older pigs may demonstrate mild signs of respiratory disease, usually complicated by secondary infections. No other species are known to be naturally infected with PRRSV.

Identification of the agent: Virological diagnosis of PRRS virus infection is difficult; the virus can be isolated from tissues such as serum, ascitic fluids, or organ samples, such as lungs, tonsil, lymph nodes and spleen of affected pigs. As porcine alveolar macrophages are the most susceptible culture system for virus of both antigenic types, these cells are recommended for virus isolation. MARC-145 (MA-104 clone) cells are also suitable. There is variability between batches of macrophages in their susceptibility to PRRS virus. Thus, it is necessary to identify a batch with high susceptibility, and maintain this stock under liquid nitrogen until required. The virus is identified and characterised by immunostaining with specific antisera. Additional techniques, such as immunohistochemistry and in situ hybridisation on fixed tissues and reverse-transcription polymerase chain reaction, have been developed for laboratory confirmation of PRRS virus infection.

Serological tests: A wide range of serological tests is currently available for the detection of serum antibodies to PRRS virus. The immunoperoxidase monolayer assay uses alveolar macrophages and the indirect immunofluorescence assay uses MARC-145 cells that are usually infected using either the European or the American antigenic type of the virus, respectively. Both assays can however be designed with both cell and PRRS virus types. Commercial or in-house enzyme linked immunosorbent assays (ELISA) are now often used. One commercial ELISA is specific for both the European and American types of the virus. An indirect ELISA, a blocking ELISA and a double ELISA that can distinguish between serological reactions to the European and the American types have been described.

Requirements for vaccines and diagnostic biologicals: Vaccines can be of value as an aid in the prevention of reproductive and respiratory forms of PRRS. Modified live vaccines are not suitable for use in pregnant sows and gilts and in boars. Vaccination may result in shedding of vaccinal virus in semen. Modified live virus vaccines can persist in vaccinated animals, and transmission to nonvaccinated animals and subsequent vaccine-virus-induced disease have been reported.

A. INTRODUCTION

Porcine reproductive and respiratory syndrome (PRRS) is characterised by reproductive failure of sows and respiratory disease in pigs. (2). The disease was first recognised in 1987 in the United States of America, and
within a few years it became a pandemic. PRRS is caused by the PRRS virus (PRRSV). It was discovered in 1991 in The Netherlands (33) and is classified as a member of the order Nidovirales, family Arteriviridae, genus Arterivirus (4). PRRSV is a single-stranded positive-sense RNA virus and the biology of the virus has been well characterised. Soon after the discovery of the virus it became apparent the North American (NA) and European (EU) PRRSV isolates represented two genotypes with antigenic differences (20, 25, 32). Additional investigations have demonstrated regional differences within each continent. These differences are now becoming blurred as NA-like PRRSV has been introduced into Europe (through the use of a modified-live vaccine made from a NA isolate) and EU-like virus has been discovered in North America. Most PRRSV isolates from South America and Asia are NA-like and it is assumed these viruses were introduced through the movement of swine and/or semen.

There is an increasing diversity among strains of the two genotypes, which has been attributed to the high error-rate inherent in PRRSV replication (5) and recombination between strains (28). There have also been recent descriptions of stains with a high degree of polymorphism, providing further insights into the emergence of the relatively new pathogen of pigs (26). The effects of such diversity on diagnostics and vaccines are largely unknown, but do raise concerns and should be considered.

The reproductive syndrome is recognised by late-gestation abortions and early or delayed farrowings that contain dead and mummified fetuses, stillborn pigs, and weak-born pigs. An increase in repeat breeders during the acute phase of the epizootic is commonly reported. Infrrequently, there are reports of early- to mid-gestation reproductive failure. In boars and unbred replacement gilts and sows, transient fever and anorexia may be observed. The respiratory syndrome is recognised by dyspnoea (thumping), fever, anorexia, and listlessness. Younger pigs are more affected than older animals with boars and sows (unbred) frequently having subclinical infection. An increase in secondary infections is common and mortality can be high. In PRRSV-infected boars and boars that have been vaccinated with live attenuated vaccine, PRRSV can be shed in semen, and changes in sperm morphology and function have been described (7). The virus is primarily transmitted directly via infected pigs and also by faeces, urine and semen. It can also be spread indirectly, presumably via aerosol routes and possibly by mechanical vectors. Gross and microscopic lesions consistent with PRRSV infection have been well described (13). In general, the lesions are more severe in younger animals than older ones. Differences in virulence between PRRSV isolates within a genotype and between genotypes are believed to exist based on field observations and some experimental studies (13). Although there is now an extensive body of research completed since the discovery of PRRSV, there are still many gaps in the knowledge base about the apparent link between PRRSV and other diseases as well as understanding the PRRSV immune response.

### B. DIAGNOSTIC TECHNIQUES

#### 1. Identification of the agent

Identification of PRRSV can be accomplished by virus isolation, the detection of nucleic acids, and the detection of viral proteins. Isolation of PRRSV can be difficult since all virus isolates (especially EU viruses) may not easily infect a cell line derived from the MA-104 monkey kidney cell line (16). Interestingly, this continuous cell culture system has been the only one reported to sustain a PRRSV infection. Porcine alveolar macrophages (PAM) will support replication of most, if not all PRRSV isolates. However, the collection of PAM is not an easy task as only pigs of high health status and less than 8 weeks of age should be used as the source of PAM (33). Different batches of PAM are not always equally susceptible to PRRSV; thus it is necessary to test each batch before use. PAM can be stored in liquid nitrogen until needed as described below. Isolation of PRRSV using PAM is a technique that can be performed in most diagnostic laboratories. This technique should be sensitive for isolation of all PRRSV strains and will be explained in detail. Detection of PRRSV nucleic acid can be accomplished with reverse-transcription polymerase chain reaction (RT-PCR), nested set RT-PCR, and real-time RT-PCR (17, 18, 22, 30, 31). These tests are commonly used to detect nucleic acid in tissues and serum. They are also useful when virus isolation is problematic, such as when testing semen (7) and when testing tissues partially degraded by autolysis or by heat during transport of specimens for virus isolation. A multiplex PCR assay has been designed to differentiate North American and European PRRSV isolates (11). Restriction fragment length polymorphism analysis of PCR-amplified products has been developed for the differentiation of field and vaccine PRRSV isolates (34) and recently molecular epidemiological studies of PRRSV strains have been performed using phylogenetic analyses of specific structural gene sequences. All of these nucleic acid tests are more rapid than virus isolation and do not require cell culture infrastructure. Although seldom used for diagnostic purposes, in-situ hybridisation is capable of detecting and differentiating North American and European PRRSV genotypes in formalin-fixed tissues (20). Immunohistochemistry can be used to identify viral proteins (12, 19) and when performed on formalin-fixed tissues enables the visualisation of antigen together with histological lesions.

#### a) Harvesting of alveolar macrophages from lungs

Lungs should preferably be obtained from SPF pigs or from a herd of pigs that is proven to be free from PRRSV infection. Best results are obtained with pigs that are under 8 weeks of age. The macrophages should be harvested from the lung on the same day that the pig is slaughtered. The lungs should be washed three or four times with a total volume of approximately 200 ml sterile phosphate buffered saline
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(PBS). The harvested wash fluid is then centrifuged for 10 minutes at 1000 g. The resulting pellet of macrophages is resuspended in PBS and centrifuged (washed) twice more. The final pellet is resuspended in 50 ml PBS, and the number of macrophages is counted to determine the cell concentration. The macrophages can then be used fresh, or can be stored in liquid nitrogen according to standard procedures at a final concentration of approximately $4 \times 10^7$ macrophages/1.5 ml. Macrophage batches should not be mixed.

b) Batch testing of alveolar macrophages

Before a batch of macrophages can be used it should be validated. This should be done by titrating a standard PRRSV with known titre in the new macrophages, and by performing an immunoperoxidase monolayer assay (IPMA) with known positive and negative sera on plates seeded with the new macrophages. The cells are suitable for use only if the standard PRRSV grows to its specified titre, (TCID$_{50}$ or 50% tissue culture infective dose). It is recommended that alveolar macrophages and fetal bovine serum (FBS) to supplement culture medium be pestivirus free.

c) Virus isolation on alveolar macrophages

Alveolar macrophages are seeded in the wells of flat-bottomed tissue-culture grade microtitre plates. After attachment, the macrophages are infected with the sample. Samples can be sera or ascitic fluids, or 10% suspensions of tissues, such as tonsils, lung, lymph nodes, and spleen. In general, the PRRSV gives a cytopathic effect (CPE) in macrophages after 1–2 days of culture, but sometimes viruses are found that give little CPE or give a CPE only after repeat passage. After a period of 1–2 days or once CPE has been observed, the presence of PRRSV needs to be confirmed by immunostaining with a specific antiserum or monoclonal antibody (MAb).

i) Seeding macrophages in the microtitre plates

Defrost one vial containing $6 \times 10^7$ macrophages/1.5 ml. Wash the cells once with 50 ml PBS and centrifuge the cell suspension for 10 minutes at 300 g (room temperature). Collect the cells in 40 ml RPMI (Rose-Peake Memorial Institute) 1640 medium supplemented with 5% FBS and 10% antibiotic mixture (growth medium). Dispense 100 µl of the cell suspension into each well of a microtitre plate (with one vial of cells, four plates can be seeded at a concentration of $10^5$ cells in each well of the plates).

ii) Preparation of sample (serum, ascitic fluid, 10% tissue suspension) dilutions in a dummy plate

Dispense 90 µl of growth medium into each well of a microtitre plate. Add 10 µl samples to the wells of rows A and E (duplicate 1/10 dilution). Shake the plates and transfer 10 µl from rows A and E to rows B and F (1/100 dilution). Shake the plates and transfer 10 µl from rows B and F to rows C and G (1/1000 dilution). Shake the plates and transfer 10 µl from rows C and G to rows D and H (1/10,000 dilution). Shake the plates.

iii) Incubation of samples

Transfer 50 µl of the sample dilutions from the dilution plates to the corresponding wells of the plate with macrophages (first passage). Incubate for 2–5 days and observe daily for a CPE. At day 2, seed macrophages in new microtitre plates (see above). Transfer 25 µl of the supernatants from the plates of the first passage to the corresponding wells of the freshly seeded plates (second passage). Incubate for 2–5 days and observe daily for a CPE.

iv) Reading and interpreting the results

Wells in which macrophages show CPE in the first passage only are considered to be false positive because of the toxicity of the sample. Wells in which macrophages show CPE in both passages or in the second passage only are considered to be suspect positive. All wells with macrophage monolayers that do not show CPE need to be identified as PRRSV negative by immunostaining with a PRRSV-positive antiserum or MAb. CPE-positive samples need to be identified as PRRSV positive by culturing CPE-positive supernatant samples, or the original sample dilutions, for both 24 and 48 hours in macrophages, followed by immunostaining with a PRRSV-positive antiserum or MAb.

v) Immunostaining with a PRRSV-positive antiserum or MAb

Infect macrophages with 50 µl of supernatant or tissue sample as described in Section B.2.a, and grow the infected cells for 24 and 48 hours. Prepare an appropriate dilution of a PRRSV-positive serum in dilution buffer, and immunostain the macrophages as described in Section B.2.a or B.2.b.

2. Serological tests

A variety of assays for the detection of serum antibodies to PRRSV have been described. Serological diagnosis is, in general, easy to perform, with good specificity and sensitivity, especially on a herd basis. Sera of individual
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Pigs sometimes cause difficulties because of nonspecific reactions, but this problem may be solved by resampling the pig after 2–3 weeks. Serology is generally performed with a binding assay, such as the IPMA, immunofluorescence assay, or the enzyme-linked immunosorbent assay (ELISA) — of which many varieties are described (1, 6, 8, 14, 23, 25, 33, 35). These tests are often performed with viral antigen of one antigenic type, which means that antibodies directed against the other, heterologous, antigenic type may be detected with less sensitivity. A blocking ELISA has been used extensively in Denmark and has been described as a double ELISA set-up using both European and American virus as antigen and thus it can distinguish between serological reaction to the European and the American types (25). The first live attenuated vaccine for PRRS based on the American type virus has been observed to spread to nonvaccinated animals (3, 27), and subsequent development in herds of vaccine-virus-induced PRRS reproductive failures has been reported in Denmark (3, 21). Reaction to American-type vaccine-like PRRSV can be anticipated in countries using or having used this vaccine; European countries may therefore observe reactions and isolation of both antigenic types (3, 21). The identification of European-type strains of PRRSV in the USA and Canada has also been reported (10), but the prevalence of infection by such strains is not well documented.

Antibodies to the virus can be detected by antibody-binding assays as early as 7–14 days after infection, and antibody levels reach maximal titres by 30–50 days. Some pigs may become seronegative within 3–6 months, but others remain seropositive for much longer. Neutralising antibodies develop slowly and do not reach high titres. They can be detected from 3 to 4 weeks after infection and they can persist for 1 year or more. The use of complement to make the serum virus neutralisation test more sensitive has been reported (15). Extensive research into the duration of antibody titres after infection has not yet been done, and the results probably depend on the test used. Maternal antibodies have a half-life of 12–14 days, and maternal antibody titre can, in general, be detected until 4–8 weeks after birth, depending on the antibody titre of the sow at birth and the test used. In an infected environment, pigs born from seropositive females can seroconvert actively from the age of 3–6 weeks.

This chapter describes the IPMA in detail as this test can easily be performed in laboratories where virus isolation procedures using macrophages have been established, and can be used with virus of both antigenic types. This assay can also be adapted to the MARC-145 cell line for both the European and American types (25). An indirect immunofluorescence assay (IFA) using MARC-145 cells can also be performed for PRRSV serology and is included in the present chapter. Commercial ELISAs with good sensitivity and specificity are available and have been compared (9).

### a) Detection of antibodies with the immunoperoxidase monolayer assay

Alveolar macrophages are seeded in the wells of microtitre plates. After attachment, the macrophages are infected with PRRSV. The object is to infect approximately 30–50% of the macrophages in a well so as to be able to distinguish nonspecific sera. After an incubation period, the macrophages are fixed and used as a cell substrate for serology. An alternative method is to use MARC 145 cells instead of macrophage cells. On each plate, 11 sera can be tested in duplicate. Test sera are diluted and incubated on the cell substrate. If antibodies are present in the test serum, they will bind to the antigen in the cytoplasm of the macrophages. In the next incubation step, the bound antibodies will be detected by an anti-species horseradish-peroxidase (HRPO) conjugate. Finally, the cell substrate is incubated with a chromogen/substrate solution. Reading of the test is done with an inverted microscope.

- **Seeding macrophages in the microtitre plates**
  1. Defrost one vial containing $6 \times 10^7$ macrophages/1.5 ml.
  2. Wash the cells once with 50 ml of PBS and centrifuge the cell suspension for 10 minutes at 300 g (room temperature).
  3. Collect the cells in 40 ml RPMI 1640 medium supplemented with 5% FBS, 100 IU (International Units) penicillin and 100 µg streptomycin (growth medium).
  4. Dispense 100 µl of the cell suspension into each well of a microtitre plate (with one vial of cells, four plates can be seeded at a concentration of $10^6$ cells in each well of the plates).

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1 Preparation of chromogen solution

Stock solution of chromogen (3-amin-9-ethyl-carbazole [AEC]): (a) 4 mg AEC; (b) 1 ml N,N-dimethyl-formamide.

Dissolve (a) in (b) and store the AEC stock solution at 4°C in the dark.

**Preparation of chromogen/substrate solution (prepare shortly before use)**

Prepare 0.05 M sodium acetate buffer, pH 5.0, as follows: Dissolve 4.1 g sodium acetate in 1 litre distilled water. Adjust the pH to 5.0 with 100% acetic acid.

Add 1 ml AEC stock solution to 19 ml of 0.05 M sodium acetate buffer.

Add 10 µl 30% H₂O₂ for each 20 ml of chromogen/substrate solution.

Filter the solution through a 5 µm filter.
v) Incubate the plates for 18–24 hours at 37°C in a 5% CO₂ cabinet, under humid conditions. Alternatively, use HEPES buffer (N-2-hydroxyethylpiperazine, N-2-ethanesulphonic acid) in the medium.

- **Infection of cells with PRRSV**
  i) Add to each well 50 µl of a virus suspension containing 10⁵ TCID₅₀/ml, but leave two wells uninfected to act as controls.
  ii) Incubate the plates for 18–24 hours at 37°C in a 5% CO₂ cabinet.

- **Fixation of the cells**
  i) Discard the growth medium and rinse the plates once in saline.
  ii) Knock the plates gently on a towel to remove excess liquid and then dry them (without lid) for 45 minutes at 37°C.
  iii) Freeze the plates (without a lid) for 45 minutes at –20°C. (Plates that are not used immediately for testing must be sealed and stored at –20°C.)
  iv) Incubate the cells for 10 minutes at room temperature with cold 4% paraformaldehyde (in PBS). Alternatively the cells could be fixed in ice-cold absolute ethanol for 45 minutes at 5°C or in ice-cold 80% acetone for 45 minutes.
  v) Discard the paraformaldehyde and rinse the plates once in saline.

- **Preparation of serum dilutions in a dilution plate**
  i) Dispense 180 µl of 0.5 M NaCl with 4% horse serum and 0.5% Tween 80, pH 7.2 (dilution buffer), to the wells of rows A and E of the dummy plate(s).
  ii) Dispense 120 µl of dilution buffer to all other wells.
  iii) Add 20 µl of the test serum or control sera to the wells of rows A and E (= 1/10 dilution), and shake.
  iv) Dilute the sera four-fold by transferring 40 µl from rows A and E to rows B and F, and so on to provide further dilutions of 1/40, 1/160 and 1/640.

- **Incubation of sera in the plate with fixed macrophages**
  i) Transfer 50 µl from each of the wells of the dummy plate(s) to the corresponding wells of the plate with the fixed macrophages. Seal the plate(s) and incubate for 1 hour at 37°C.
  ii) Discard the serum dilutions and rinse the plate(s) three times in 0.15 M NaCl + 0.5% Tween 80.

- **Incubation with conjugate**
  i) Dilute the rabbit-anti-swine (or anti-mouse, if staining isolation plate with MAb) HRPO conjugate to a predetermined dilution in 0.15 M NaCl + 0.5% Tween 80. Add 50 µl of the conjugate dilution to all wells of the plate(s). Seal the plate(s) and incubate for 1 hour at 37°C. Rinse the plates three times.

- **Staining procedure**
  i) Dispense 50 µl of the filtered chromogen/substrate (AEC) solution to all wells of the plate(s) (see footnote 1).
  ii) Incubate the AEC for at least 30 minutes at room temperature.
  iii) Replace the AEC with 50 µl of 0.05 M sodium acetate, pH 5.0 (see footnote 1).

- **Reading and interpreting the results**

  If antibodies are present in the test serum, the cytoplasm of approximately 30–50% of the cells in a well are stained deeply red by the chromogen. A negative test serum is recognised by cytoplasm that remains unstained. A serum that reacts nonspecifically might stain all cells in a well (compared with a positive control serum). The titre of a serum is expressed as the reciprocal of the highest dilution that stains 50% or more of the wells. A serum with a titre of <10 is considered to be negative. A serum with a titre of 10 or 40 is considered to be a weak positive. Often nonspecific staining is detected in these dilutions. A serum with a titre of ≥160 is considered to be positive.

b) **Detection of antibodies with the indirect immunofluorescence assay**

  Although there is no single standard accepted immunofluorescence assay in use at this time, several protocols have been developed and are used by different laboratories in North America. The IFA can be
performed in microtitre plates or eight-chamber slides using the MARC-145 cell line and a MARC-145 cell-line-adapted PRRSV isolate. To prevent cross-reactivity with pestivirus, it is recommended that cells and FBS, to supplement culture medium, be pestivirus free. After an incubation period, PRRSV-infected cells are fixed and used as a cell substrate for serology. Serum samples are tested at a single screening dilution of 1/20 and samples are reported as being negative or positive at this dilution. Each porcine serum to be tested is added to wells or chambers containing PRRSV-infected cells. Antibodies to PRRSV, if present in the serum, will bind to antigens in the cytoplasm of infected cells. Following this step, an anti-porcine-IgG conjugated to fluorescein is added, which will bind to the porcine antibodies that have bound to PRRSV antigens in the infected cells. The results are read using a fluorescence microscope. Microtitre plates may also be prepared for serum titration purposes (see Section b1 below).

**Seeding and infection of MARC-145 cells in microtitre plates**

i) Add 50 µl of cell culture medium (e.g. Minimal Essential Medium [MEM] containing 2 mM L-glutamine, 1 mM sodium pyruvate, 100 IU penicillin and 100 µg streptomycin) without FBS to each well of columns 2, 4, 6, 8, 10 and 12 of a 96-well plate using a multichannel pipettor.

ii) Trypsinise confluent MARC-145 cells (grown in culture flasks) to be used for seeding 96-well microtitre plates and resuspend cells in cell culture medium containing 8% FBS at a concentration of 100,000–125,000 cells/ml. The MARC-145 cells are trypsinised from culture flasks for IFA once a week using trypsin/EDTA (ethylene diamine tetra-acetic acid) and are seeded in culture flasks at a concentration of 250,000 cells/ml. After 4 days in culture flasks, new cell culture medium containing 2% FBS is added for 3 additional days.

iii) Using a multichannel pipettor, add 150 µl of the cell suspension to each well of the 96-well plate.

iv) Dilute PRRSV preparation in MEM without FBS to $10^{2.2}$ TCID$_{50}$/50 µl and distribute 50 µl in each well of columns 1, 3, 5, 7, 9 and 11.

v) Incubate the plates for approximately 48–72 hours at 37°C in a humidified 5% CO$_2$ cabinet to obtain a monolayer with approximately 40–50% of the cells infected as determined by indirect immunofluorescence. Alternatively, microtitre plates may first be seeded with MARC-145 cell suspensions (e.g. concentration of 100,000 cells/ml in medium supplemented with 5–10% FBS) and incubated for up to 72 hours until they are confluent. Then volumes of 50 µl of PRRSV preparations (e.g. $10^5$ TCID$_{50}$/ml) are added per well and the plates are incubated for an additional 48–72 hours prior to fixation. The use of organic buffers such as HEPES in medium has been suggested to stabilise the pH when CO$_2$ incubators are not available.

**Seeding and infection of MARC-145 cells in eight-chamber glass slides**

i) Add 500 µl of a MARC-145 cell suspension (e.g. in MEM supplemented with 10% FBS) at a concentration of 100,000 cells/ml to each chamber of eight-chamber glass slides.

ii) Incubate the cells for approximately 48–72 hours at 37°C in a humidified 5% CO$_2$ cabinet until they are confluent.

iii) Add to each chamber 50 µl of PRRSV suspension containing $10^5$ TCID$_{50}$/ml and further incubate cells for approximately 18 hours at 37°C in a humidified 5% CO$_2$ cabinet. At this time 15–20 infected cells per field of view may be observed by indirect immunofluorescence.

**Fixation of the cells**

i) Discard the medium, rinse once with PBS and discard the PBS. For chamber slides, remove the plastic chamber walls, leaving the gasket intact.

ii) Add volumes of 150 µl cold (4°C) acetone (80% in water) to each well of the 96-well plate. Incubate the plates at 4°C for 30 minutes. For chamber slides, acetone (80–100%) at room temperature is used to fix the cells for 10–15 minutes at room temperature. Some manufactured brands of acetone will degrade the chamber slide gasket leaving a film on the slide. It is recommended to check the acetone before using for routine fixation.

iii) Discard the acetone and dry the plates and slides at room temperature.

iv) The plates can then be placed in a plastic bag, sealed and stored at −70°C until use. Chamber slides can be kept similarly in slide cases.

**Preparation of serum dilutions**

i) Dilute serum samples to a 1/20 dilution in PBS (0.01 M; pH 7.2) in separate 96-well plates (e.g. add 190 µl of PBS using a multichannel pipettor followed by 10 µl of the sera to be tested).

ii) Include as controls reference PRRSV antibody positive and negative sera of known titre.
• **Incubation of sera with fixed MARC-145 cells**
  i) Stored plates are removed from the –70°C freezer and when the plates reach room temperature rehydrate the cells with 150 µl PBS for a few minutes. Discard the PBS by inverting the plates and blotting dry on paper towels. Cells of eight-chamber slides are not rehydrated.
  ii) Add volumes of 50 µl of each diluted serum to one well containing the fixed noninfected cells and to one well containing the fixed infected cells. Add similar volumes for each serum to a single chamber.
  iii) Add volumes of 50 µl of the negative control serum and positive control serum dilutions in the same manner.
  iv) Incubate the plates with their lids on at 37°C for 30 minutes in a humid atmosphere. Slides should be incubated similarly in boxes or slide trays with a cover.
  v) Remove the serum samples and blot the plates dry on paper towels. A total of six washes using 200 µl of PBS are performed. The PBS is added to each well, followed by inversion of the plates to remove the PBS. After removing serum samples, slides are rinsed in PBS followed by a 10-minute wash.

• **Incubation with conjugate**
  i) Add volumes of 50 µl of appropriately diluted (in freshly prepared PBS) rabbit or goat anti-swine IgG (heavy and light chains) conjugated with FITC (fluorescein isothiocyanate) to each well using a multichannel pipettor. Similar volumes are added to individual chambers.
  ii) Incubate plates or slides with their lids on at 37°C for 30 minutes in a humid atmosphere.
  iii) Remove the conjugate from the plates and blot the plates dry on paper towels. A total of four washes using PBS are performed as described above. Discard the conjugate from the slides, rinse in PBS, wash for 10 minutes in PBS and rinse in distilled water. Tap the slides on an absorbent pad to remove excessive water.
  iv) The plates and the slides are read using a fluorescence microscope.

• **Reading and interpreting the results**
  The presence of a green cytoplasmic fluorescence in infected cells combined with the absence of such a signal in noninfected cells is indicative of the presence of antibodies to PRRSV in the serum at the dilution tested. The degree of intensity of fluorescence may vary according to the amount of PRRSV-specific antibody present in the serum tested.

  Absence of specific green fluorescence in both infected and noninfected cells is interpreted as absence of antibody to PRRSV in that serum at the dilution tested. The test should be repeated if the fluorescence is not seen with the use of the positive control sera on infected cells or if fluorescence is seen using the negative control serum on infected cells. No fluorescence should be seen on noninfected cells with any of the control sera. Any test serum giving suspicious results should be retested at a 1/20 dilution and if results are still unclear, a new serum sample from the same animal is requested for further testing.

b1) **Evaluation by IFA of sera for antibody titres**

  Microtitre plates and IFA may also be used for serum titration purposes. Up to 16 sera may be titred per 96-well microtitre plate.
  i) Seed 96-well microtitre plates with MARC-145 cells and incubate at 37°C in a humidified 5% CO₂ cabinet until they are confluent.
  ii) Inoculate all wells with the PRRSV preparation except the wells of columns 1, 6 and 11, and incubate the plates at 37°C in a humidified 5% CO₂ cabinet for 48–72 hours.
  iii) Discard culture medium and rinse the monolayers once with PBS (0.01 M, pH 7.2). Fix the monolayers with cold acetone (80% aqueous solution) for 10 minutes at ambient temperature. Discard the acetone, air-dry the plates and keep the plates with lids at –20°C for short-term storage or –70°C for long-term storage, until use.
  iv) Serially dilute sera including a PRRSV-positive control serum using a four-fold dilution in PBS, beginning at 1/16 or 1/20. Dilute a negative control serum at 1/16 or 1/20 dilution. Dispense 50 µl of each dilution (1/16, 1/64, 1/256, 1/1024 or 1/20, 1/80, 1/320, 1/1280) in wells containing viral antigen of columns 2, 3, 4, 5 or 7, 8, 9, 10. For each serum, also dispense 50 µl of dilution 1/16 or 1/20 in control wells of columns 1 and 6. Similarly dispense dilutions of positive and negative control sera in wells of columns 11 and 12.
v) Incubate the plates at 37°C for 30 minutes in a humid chamber. Discard the sera and rinse the plates three times using PBS.

vi) Add 50 µl of appropriately diluted anti-swine IgG conjugated with FITC and incubate plates at 37°C for 30 minutes in a humid chamber. Discard conjugate, rinse plates several times and tap the plates on absorbent material to remove excessive liquid.

- Reading and interpreting the results

Following examination with a fluorescence microscope, the titre of a serum is recorded as the reciprocal of the highest serum dilution in which typical cytoplasmic fluorescence is observed. For paired serum samples, a four-fold increase in titre with a 2-week interval is indicative of active infection in an individual animal. No specific fluorescence should be observed with test sera or positive and negative control sera on noninfected control cells. No fluorescence should be seen on infected cells with negative control serum. Specific fluorescence should be observed on infected cells with positive control serum at appropriate dilutions. The IFA end-point may vary among laboratories. Test results may also vary depending on the PRRSV isolate used in the test because of antigenic diversity.

c) Detection of antibodies with the enzyme-linked immunosorbent assay

Several laboratories have developed ELISAs (indirect or blocking) for serological testing (1, 6, 8, 14, 25). A double-blocking ELISA format that can distinguish between serological reactions to the European and the American antigenic type has been described (25). ELISA kits are available commercially to determine the serological status of swine towards PRRSV. These kits use as antigens either the European or the American PRRSV types separately or as combined antigens. Their main advantage is the rapid handling of a large number of samples. Commercial ELISAs using recombinant proteins of both PRRSV types as antigens have also been developed and are available.

C. REQUIREMENTS FOR VACCINES AND DIAGNOSTIC BIOLOGICALS

Modified-live (MLV) PRRSV vaccines are commercially available in many countries for the control of the reproductive and/or respiratory forms of PRRS. In the USA and Europe, a killed virus vaccine is licensed as an aid in the reduction of abortions and weak piglets caused by the reproductive form of PRRS. All PRRS vaccines currently licensed in the USA contain the NA antigenic type. In Europe, two MLV vaccines are licensed and available commercially, one is based on the NA antigenic type and the other is the EU antigenic type. It is assumed the most benefit from vaccination occurs when the vaccine virus is more closely related antigenically to the field virus (29). Although vaccination of pigs does not prevent PRRSV infection, it may be helpful in herds experiencing problems with PRRS or herds at high risk of PRRSV infection. MLV vaccines are not intended to be used in naive herds, pregnant sows or gilts or boars of breeding age. MLV vaccines are intended to be used in sows and gilts 3–6 weeks prior to breeding and in piglets from 3 weeks of age or older as an aid in the reduction of diseases caused by PRRS. Vaccine virus can persist in boars and be disseminated through semen (7). MLV vaccine virus may be shed and transmitted to nonvaccinated contact pigs (27). Guidelines for the production of veterinary vaccines are given in Chapter 1.1.8 Principles of veterinary vaccine production. The guidelines given here and in Chapter 1.1.8 are intended to be general in nature and may be supplemented by national and regional requirements.

1. Seed management

a) Characteristics of the seed

The isolate of PRRSV used for vaccine production must be accompanied by a history describing its origin and passage history. The isolate must be safe in swine at the intended age of vaccination and provide protection against challenge. Isolates for a MLV vaccine must be shown not to revert to virulence after passage in host animals.

b) Method of culture

The PRRSV is propagated in a continuous African green monkey kidney cell line, such as MA-104 or Vero. Viral propagation should not exceed five passages from the master seed virus (MSV) unless further passages prove to provide protection in swine.

c) Validation as a vaccine

The MSV should be free from bacteria, fungi and mycoplasma. The MSV must be tested for and free from extraneous viruses, including transmissible gastroenteritis virus, porcine adenovirus, porcine circovirus type 1 and 2, porcine haemagglutinating encephalitis virus, porcine parvovirus, all pestiviruses, reovirus, and
rabies virus by the fluorescent antibody technique. The MSV must be free from extraneous virus by CPE and haemadsorption on the Vero cell line and an embryonic swine cell type.

Attenuated PRRSV isolates are known to cause viraemia and will transmit to susceptible animals. The MSV should be shown to be nonvirulent in piglets and pregnant animals by five serial passages (up to ten passages depending on country) of the MSV through susceptible swine using the most natural route of infection.

In an immunogenicity trial, the MSV at the highest passage level intended for production must protect susceptible swine against a virulent, heterologous challenge strain. For the respiratory form, 3-week-old piglets are vaccinated with the highest passage level of MSV. The piglets are challenged with a virulent isolate of PRRSV 2–16 weeks later to determine protection from respiratory clinical signs of PRRS. To determine protection from the losses caused by the reproductive form of PRRS, vaccinated animals are challenged at approximately 85 days of gestation. A significant number of the vaccinates must be protected from the clinical signs of reproductive disease, including fetal mummification, stillborn piglets and/or weak piglets, when compared with the controls. Field trial studies should be conducted to determine the safety of the vaccine. Nonvaccinated sentinel pigs should be included at each site for monitoring the shed of the attenuated virus.

2. Method of manufacture

The African green monkey kidney cell line is seeded into suitable vessels. MEM supplemented with FBS is used as the medium for production; the FBS must be free from pestivirus or antibodies to pestivirus and free from bovine spongiform encephalopathy risk. Incubation is at 37°C.

Cell cultures are inoculated directly with PRRS working virus stock, which is generally from 1 to 4 passages from the MSV. Inoculated cultures are incubated for 1–8 days before harvesting the culture medium. During incubation, the cultures are observed daily for CPE and bacterial contamination.

Killed virus vaccines are chemically inactivated with either formalin or binary ethylenimine and mixed with a suitable adjuvant. MLV vaccines are generally mixed with a stabiliser before bottling and lyophilisation. If formalin is used as an inactivant, the final product should be tested for residual formaldehyde concentration and should not exceed 0.74 g/litre.

3. In-process control

Production lots of PRRSV must be titrated in tissue culture for standardisation of the product. Low-titred lots may be concentrated or blended with higher-titred lots to achieve the correct titre.

4. Batch control

Final container samples are tested for purity, safety and potency. MLV vials are also tested for the maximum allowable moisture content.

a) Purity

Samples are examined for bacterial, fungal and pestivirus contamination. To test for bacteria, ten vessels, each containing 120 ml of soybean casein digest medium, are inoculated with 0.2 ml from ten final-container samples. The ten vessels are incubated at 30–35°C for 14 days and observed for bacterial growth. To test for fungi, ten vessels, each containing 40 ml of soybean casein digest medium, are inoculated with 0.2 ml from ten final-container samples. The vessels are incubated at 20–25°C for 14 days and observed for fungal growth.

b) Safety

Safety tests can be conducted in a combination of guinea-pigs, mice or pigs.

c) Potency

Final container samples of an MLV vaccine are titrated (log10) in microtitre plates for determination of the titre.

• Test procedure

i) Prepare tenfold dilutions from 10−1 through 10−5 by using 0.2 ml of rehydrated test vaccine and 1.8 ml of MEM. An internal positive control PRRSV should be titrated in the appropriate range.
ii) Inoculate 0.1 ml/well from each dilution into five wells of a 96-well plate containing African green monkey kidney monolayers.

iii) Incubate the plate at 37°C in a CO₂ atmosphere for 5–7 days.

iv) Read the plates microscopically for CPE. The internal positive control PRRSV should give a titre within 0.3 log₁₀ TCID₅₀ from its predetermined mean.

v) Determine the TCID₅₀/dose by the Spearman–Kärber method. The release titre must be at least 1.2 logs higher than the titre used in the immunogenicity trial. The 1.2 logs include 0.5 logs for stability throughout the shelf life of the product and 0.7 logs for potency test variability.

Killed virus vaccines may use host animal or laboratory animal vaccination/serology tests or vaccination/challenge tests to determine potency of the final product. Parallel-line assays using ELISA antigen-quantifying techniques to compare a standard with the final product are acceptable in determining the relative potency of a product. The standard should be shown to be protective in the host animal.

d) Duration of immunity

Duration of immunity studies are conducted before the vaccine receives final approval. For the respiratory form of PRRS, duration should be shown up to the market age in pigs. Duration of immunity for the reproductive form should be shown through weaning of the piglets.

e) Stability

All vaccines are initially given a shelf life of 24 months before expiry. Real-time stability studies are then conducted to confirm the appropriateness of the expiry date.

f) Preservatives

Antibiotics are added during production, generally gentamicin sulphate or neomycin not to exceed 30 µg/ml.

g) Precautions (hazards)

There is no inherent risk of reversion to virulence when using properly inactivated vaccines. However, this is not the case when using MLV vaccines. To mitigate this potential risk, MLV vaccination is only recommended for use in PRRSV-positive herds as an aid in the reduction of disease associated with the respiratory and/or reproductive form of PRRS. MLV vaccine is not recommended for use in pregnant sows and gilts, or in breeding boars. The first MLV vaccine for PRRS based on the American-type virus has been reported to spread to nonvaccinated animals (3, 27) and subsequently cause vaccine-virus-induced PRRS reproductive failures in the affected herds (3, 21). There is mounting evidence that MLV vaccines, of the European type, can also spread to nonvaccinated animals and that they can also cross the placenta and give rise to congenitally infected piglets (24).

5. Tests on the final product

a) Safety

See Section C.4.b.

b) Potency

See Section C.4.c.

REFERENCES


* * *

NB: There is an OIE Reference Laboratory for Porcine reproductive and respiratory syndrome (see Table in Part 3 of this Terrestrial Manual or consult the OIE Web site for the most up-to-date list: www.oie.int).
CHAPTER 2.8.8.

SWINE INFLUENZA

SUMMARY

Swine influenza is a highly contagious viral infection of pigs. Swine influenza virus (SIV) infections cause respiratory disease characterised by coughing, sneezing, nasal discharge, elevated rectal temperatures, lethargy, breathing difficulty, and depressed appetite. In some instances, SIV infections are associated with reproductive disorders such as abortion. Clinical signs and nasal shedding of virus can occur within 24 hours of infection. Morbidity rates can reach 100% with SIV infections, while mortality rates are generally low. Secondary bacterial infections can exacerbate the clinical signs following infection with SIV. Transmission is through contact with SIV-containing secretions such as nasal discharges and aerosols created by coughing or sneezing.

Identification of the agent: Virus identification is best accomplished by collection of samples within 24–48 hours after development of clinical signs. The animal of choice is an untreated, acutely ill pig with an elevated rectal temperature. Virus can readily be detected in lung tissue and nasal swabs. Virus isolation can be conducted in embryonated chicken eggs and on continuous cell lines. Isolated viruses can be subtyped using the haemagglutination inhibition (HI) and the neuraminidase inhibition tests, or by reverse transcription-polymerase chain reaction assays. Immunohistochemistry can be conducted on formalin-fixed tissue and a fluorescent antibody test can be conducted on fresh tissue. Enzyme-linked immunosorbent assays (ELISA) are commercially available for detection of type A influenza viruses.

Serological tests: The primary serological test for detection of SIV antibodies is the HI test conducted on paired sera. The HI test is subtype specific. The sera are generally collected 10–21 days apart. A four-fold or greater increase in titre between the first and second sample is suggestive of a recent SIV infection. Additional serological tests that have been described are the agar gel immunodiffusion test, indirect fluorescent antibody test, virus neutralisation, and ELISA.

Requirements for vaccines and diagnostic biologicals: Inactivated, adjuvanted SIV vaccines are commercially available. Vaccines may be in the form of a single SIV subtype or may contain multiple SIV subtypes. Vaccines should reflect the current antigenic profile of field viruses, containing subtypes and strains that are changed as needed to assure protection. The finished vaccine must be shown to be pure, safe, potent, and efficacious.

A. INTRODUCTION

Swine influenza is a highly contagious viral infection of pigs that can have significant economic impact on an affected herd (26, 32). The swine influenza virus (SIV) is a type A orthomyxovirus with a segmented RNA genome. The type A swine influenza viruses are further subdivided based on their haemagglutinin and neuraminidase proteins. Subtypes of SIV that are most frequently identified in pigs include classical and avian H1N1, reassortant (r) H3N2, and rH1N2 (5, 9, 10, 21, 25, 29). Other subtypes that have been identified in pigs include rH1N7, rH3N1, avian (av) H4N6, avH3N3, and avH9N2 (2, 13, 16, 26, 28). The H1N1, H1N2 and H3N2 viruses found in Europe are antigenically and genetically different from those found in America (1, 3, 4, 7, 13–16, 23, 24, 31, 37–39). Pigs have receptors in their respiratory tract that will bind swine, human, and avian influenza viruses. Consequently, pigs have been called ‘mixing vessels' for the development of new influenza viruses when swine, avian, and/or human influenza viruses undergo genetic reassortment in pigs (12). SIV infections are described as causing respiratory disease characterised by coughing, sneezing, nasal discharge, elevated rectal temperatures, lethargy, breathing difficulty and depressed appetite. Other agents that may cause respiratory disease in pigs include porcine reproductive and respiratory syndrome virus, Aujeszky’s disease (pseudorabies) virus, porcine respiratory coronavirus, Actinobacillus pleuropneumoniae and other bacterial agents, and Mycoplasma hyopneumoniae. However, most of these have other signs that do not mimic swine influenza (20). Actinobacillus pleuropneumoniae, in the acute form of the infection, has clinical signs most similar to swine...
influenza, such as dyspnoea, tachypnoea, abdominal breathing, coughing, fever, depression and anorexia. Clinical signs and nasal shedding of SIV can occur within 24 hours of infection, and shedding typically ceases by day 7–10 after infection. Two forms of the disease occur in swine, epidemic or endemic. In the epidemic form, the virus quickly moves through all phases of a swine unit with rapid recovery, provided there are not complicating factors such as secondary bacterial infections. In the endemic form, clinical signs may be less obvious and not all pigs may demonstrate traditional clinical signs of infection. Morbidity rates can reach 100% with SIV infections, while mortality rates are generally low. The primary economic impact is related to retarded weight gain resulting in an increase in the number of days to reach market weight. Transmission is through contact with SIV containing secretions such as nasal discharges and aerosols created by coughing or sneezing. Human infections with SIV can occur and a limited number of deaths have been reported (9, 10, 22, 25). Precautions should be taken to prevent human infection as described in Chapter 1.1.2 Biosafety and biosecurity in the veterinary microbiological laboratory and animal facilities. Conversely, human influenza viruses can be transmitted from animal caretakers to pigs. Similarly, influenza virus can also be transmitted from poultry to pigs as well as from pigs to poultry.

B. DIAGNOSTIC TECHNIQUES

1. Identification of the agent

Because SIV is a potential human pathogen, all work with infectious tissues, swabs, embryonated eggs, and cell cultures should be done in a class II biological safety cabinet.

a) Culture

• Sample processing

Lung tissue can be processed for virus isolation in a variety of ways, for example with a mortar and pestle, stomacher, homogeniser, or mincing with a scalpel blade or scissors. Processing of the tissue is done in cell culture medium with antibiotic supplement (e.g. 10 × working strength), at a final concentration of 10–20% weight to volume. Nasal swabs should be collected in cell culture medium or phosphate buffered saline (PBS), supplemented with antibiotics and bovine serum albumin (5 mg/ml). Fetal bovine serum should not be included. Samples should ideally be shipped to a diagnostic laboratory overnight on wet ice, not frozen. Upon receipt at the laboratory, the nasal swabs are vigorously agitated by hand or on a vortex mixer. The nasal swab and lung materials are centrifuged at 1500–1900 g for 15–30 minutes at 4°C. The supernatant is collected and maintained at 4°C until inoculated. If supernatant is to be held for longer than 24 hours before inoculation, it should be stored at –70°C. Lung supernatant is inoculated without further dilution. Nasal swab supernatant can also be inoculated without dilution or diluted 1/3 in cell culture medium. Antibiotics are added to the cell culture medium used for processing and/or the supernatant can be filtered to reduce bacterial contamination, but this may decrease virus titre. As an alternative, the virus preparation may be treated with antibiotics such as gentamicin (100 µg/ml) or penicillin (10,000 units/ml); streptomycin (10,000 units/ml) and 2% fungizone (250 mg/ml) for 30–60 minutes at 4°C prior to inoculating the embryos or cell culture.

• Cell culture virus isolation

i) Virus isolation can be conducted in cell lines and primary cells susceptible to SIV infection. Madin–Darby canine kidney (MDCK) is the preferred cell line, but primary swine kidney, swine testicle, swine lung, or swine tracheal cells can be used.

ii) Wash confluent cell monolayers (48–72 hours post-seeding) three times with cell culture medium containing a final concentration of 1 µg/ml of TPCK1-treated trypsin; however, the concentration will depend on the type of trypsin and the cells used (0.3–10 µg/ml may be used). The cell culture medium can be supplemented with antibiotics, but is not supplemented with fetal bovine serum.

iii) Inoculate cell cultures with an appropriate amount of tissue suspension or swab supernatant. Note: The volume of inoculum will vary with the size of the cell culture container. In general, 100–200 µl are inoculated in each well of a 24-well culture plate, 1 ml in each Leighton tube, and 1–2 ml into a 25 cm² flask.

iv) Incubate inoculated cell cultures for 1–2 hours at 37°C with occasional rocking. When using cell culture containers that are open to the environment, such as culture plates, incubation should be done in a humidified incubator with 5% CO₂.

v) Remove the inoculum and wash the cell monolayer three times with the cell culture medium containing trypsin.

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1 TPCK: tosylphenylalanychloromethane
vi) Add an appropriate volume of the cell culture maintenance medium to all containers and incubate at 37°C for 5–7 days with periodic examination for cytopathic effect (CPE). If CPE is not observed at the end of the incubation period, the cell culture container can be frozen at −70°C, thawed, and blind passaged as described above (step iii). If CPE is observed, an aliquot of the cell culture medium can be tested for haemagglutinating viruses or by reverse transcription-polymerase chain reaction (RT-PCR) for conserved influenza virus genes such as nucleoprotein or matrix, and can be collected and used as inoculum for confirmation by the fluorescent antibody technique (see Section B.1.e below). Cover-slips (Leighton tube, 24-well cell culture plate) or chamber slides with MDCK (or other appropriate cell) monolayer can be inoculated for this purpose. The isolation procedure is as described above (step iii). In some instances, it may be necessary to make tenfold dilutions of the cell culture virus in order to have appropriate CPE on the cover-slip. Influenza subtypes can be determined by the haemagglutination inhibition (HI) and neuraminidase inhibition (NI) tests, or by RT-PCR with primers validated for sensitive and specific amplification of individual HA and NA genes.

- **Egg inoculation (30)**
  i) Use 10–11-day-old embryonated chicken eggs.
  ii) Inoculate 0.1–0.3 ml of inoculum into the allantoic cavity and amniotic sac; many laboratories only inoculate via the allantoic route with similar sensitivity. Generally, 3–4 eggs are inoculated per sample.
  iii) Incubate eggs at 35–37°C for 3–4 days and candle daily. Eggs with embryos that have died within 24 hours of inoculation are discarded.
  iv) Refrigerate eggs with embryos that have died later than 24 hours after inoculation. Harvest amniotic and allantoic fluids from eggs with dead embryos and from eggs with viable embryos at the end of the incubation period. All egg materials should be considered to be potentially infectious and should be treated accordingly to prevent SIV exposure to the laboratory worker.
  v) Centrifuge fluids at 1500–1900 \( g \) for 10–20 minutes at 4°C. Transfer the supernatant to another tube for testing.
  vi) Fluids are evaluated for the presence of SIV with the haemagglutination (HA) test (see below).
  vii) Repass fluids negative for haemagglutinating activity (negative for SIV) in eggs or on cell lines as described above. Isolation may be improved by making tenfold dilutions of the fluid in cell culture medium. Antibiotics may be added to the cell culture fluid.

- **Haemagglutination test**
  i) Prepare a 0.5% erythrocyte suspension from male turkey or chicken blood. Certain strains agglutinate turkey rather than chicken erythrocytes to greater or lesser degrees. Therefore, it may be necessary to choose the species of erythrocytes based on the strains circulating in a given area. Washed erythrocytes and 0.5% suspensions of erythrocytes can be stored at 4°C for up to 1 week. Discard if haemolysis is observed.
  ii) Dispense 50 µl PBS in a row of 8–12 wells on a 96-well V- or U-bottom microtitre plate for each unknown virus. U-bottom plates are generally preferred over V-bottom plates. One additional row of wells should be included for a positive control.
  iii) Add 50 µl of undiluted isolate to the first well of each corresponding row.
  iv) Serially dilute the isolate with a micropipette set to deliver 50 µl. The resulting dilutions will range from 1/2 (well 1) to 1/2048 (well 11). Well 12 contains PBS only and serves as a cell control.
  v) Add 50 µl of 0.5% erythrocyte suspension to each well and agitate the plate to mix thoroughly. Note: keep erythrocytes thoroughly suspended during the dispensing process.
  vi) Cover the plate with sealing tape and incubate at room temperature until a distinct button has formed (30–60 minutes) in the control well.
  vii) Wells with complete haemagglutination (positive HA, SIV present) will have erythrocytes spread throughout the well in a ‘mat’ type appearance. Wells with a distinct button of erythrocytes at the bottom of the well are negative for haemagglutinating activity (negative for SIV). Incomplete HA activity is demonstrated by partial buttons characterised by fuzzy margins or ‘donut-like’ appearance. When interpretation between negative and incomplete inhibition is doubtful, tilt the microtitre plate to about a 45-degree angle for 20–30 seconds and look for streaming, which produces a tear-drop appearance and translucency around the cells in wells with negative hemagglutination. Wells with partial inhibition will not produce a tear drop.

b) **Typing SIV isolates**

- **Haemagglutination inhibition test**
  i) Dilute reference HA antigens (H1, H3, etc.) to a concentration of 8 HA units (HAU) per 50 µl (4 HAU/25 µl) in 0.01 M PBS, pH 7.
ii) Standardise unknown influenza A viruses to contain 8 HAU in 50 µl.

iii) Conduct a back titration (HA test) for all unknown isolates and the H subtype antigens to assure that the correct HAUs are present. The back titration is performed as described in the HA procedures except that six well dilutions are used instead of eleven.

iv) Treat each reference serum (specific for an individual HA subtype) with RDE (receptor-destroying enzyme); add 50 µl serum to 200 µl RDE (1/10 dilution in calcium saline solution equalling 100 units per ml). Incubate overnight (12–18 hours) in a 37°C water bath. Add 150 µl 2.5% sodium citrate solution and heat inactivate at 56°C for 30 minutes. Combine 200 µl treated sample and 25 µl PBS. Note: RDE treatment is recommended as it will reduce nonspecific reactions and will enhance the identification of H1N2 and H3N2 isolates.

v) Remove natural serum agglutinins from the sera by treating diluted serum with 0.1 ml packed, washed erythrocytes per 1 ml diluted serum. Incubate for 30 minutes at room temperature with occasional mixing to keep the erythrocytes suspended. Centrifuge the treated serum at 800 g for 10 minutes and then retain the serum.

vi) Dispense 25 µl of standardised antigen (unknown isolate or positive control antigen) into three wells of a 96-well V- or U-bottom microtitre plate. Add 50 µl of PBS to several wells to serve as an erythrocyte cell control. Note: 25 µl of PBS can be used in place of the 25 µl of standardised antigen.

vii) Add 25 µl of the appropriate standardised antiserum to the first well of the H subtype being evaluated. Serially dilute the antiserum in 25 µl volumes in the antigen wells with a pipette set to deliver 25 µl. Repeat this procedure for each H subtype being evaluated. Note: If 25 µl of PBS was used in place of the 25 µl of standardised antigen in step vi, add 25 µl of standardised antigen to each well containing the standardised antiserum.

viii) Cover plate(s) and incubate at room temperature for 10–30 minutes.

ix) Add 50 µl 0.5% erythrocyte suspension to each well and shake/agitate the plate(s) to mix thoroughly. Keep the erythrocytes thoroughly suspended during the dispensing process.

x) Cover the plate(s) with sealing tape and incubate at room temperature until a distinct button has formed in the positive control wells (usually 30–60 minutes). Observe the plates after about 20 minutes' incubation for evidence of haemagglutination as some isolates may begin to elute (detach from erythrocytes) in 30 minutes.

xi) Read test results as described above for the HA test. A sample is considered positive for a specific H subtype if haemagglutination is inhibited. The test is considered valid if the positive reference antigen and its homologous antiserum demonstrate the expected HI titre and the back titration of each antigen (unknown and positive control) is 8 HAUs. If these conditions are not met, the test should be repeated.

xii) If erythrocytes in the cell control wells do not settle into a well-defined button, check the following as possible causes: incorrect formulation of PBS, excessive evaporation from the plates, erythrocytes too old, or incorrect concentration of erythrocytes.

- Neuraminidase inhibition test

Subtype identification based on the NI test is beyond the scope of many laboratories. Reference laboratories should be consulted for N typing of isolates.

c) Fluorescent antibody test

i) This technique can be used for tissue sections, cover-slips/slides, or 96-well plates of infected cell monolayers. Positive and negative controls should be included with all staining procedures.

ii) Inoculated cells are incubated for an appropriate length of time to allow 10–25% of the cells to become productively infected with virus. Rinse the cover-slip or slide once in PBS, place in 100% acetone for 5–10 minutes and air-dry. Acetone should be used in a vented hood.

iii) Prepare frozen tissue sections on glass slides. Fix the glass slides in acetone for 5–10 minutes and air-dry.

iv) Apply conjugate (fluorescein-labelled swine influenza antibody) and incubate in a humid chamber at 37°C for 30 minutes. Preferably the conjugate contains Evans blue for counter staining.

v) Rinse in PBS, pH 7.2, soak for 5–10 minutes in fresh PBS, rinse in distilled water, and air-dry.

vi) Place cover-slips on glass slides, cell side down, with mounting fluid. Remove the rubber gasket from chamber slides and add mounting fluid followed by a glass cover-slip. Mounting fluid followed by a glass cover-slip is also placed over tissue sections on the slide. If 96-well plates are used, mounting medium and cover-slips are not required.

vii) Observe stained slides in a darkened room with the use of an ultraviolet microscope. Cells infected with SIV are identified by the presence of bright apple-green fluorescence. It is recommended that the person examining the slides receive training in reading fluorescein-labelled slides as they can be
difficult to interpret. It is also important to use an antibody that recognises all possible viruses circulating in the area (e.g. a pan-anti-influenza A nucleoprotein antibody).

d) Immunohistochemistry (36)

i) Slice formalin-fixed, paraffin-embedded lung in 4-µm thick sections and place on poly-L-lysine-coated slides. Positive and negative control tissues should be included with all tests.

ii) Heat slides at 60°C for 15 minutes, deparaffinise, and rehydrate through immersions in decreasing concentrations of ethanol and then in distilled water.

iii) Treat samples with 3% hydrogen peroxide for 10 minutes and rinse twice in distilled water.

iv) Digest samples with 0.05% protease for 2 minutes and rinse twice for 2 minutes in 0.1 M Tris/PBS buffer, pH 7.2, at room temperature.

v) Apply primary mouse anti-SIV monoclonal antibody (directed against the viral nucleoprotein) to each slide and incubate at room temperature for 1 hour or overnight at 4°C. Rinse slides with Tris/PBS buffer.

vi) Apply secondary antibody (biotinylated goat anti-mouse antibody) for 10 minutes at room temperature. Rinse with Tris/PBS buffer.

vii) Apply tertiary antibody (peroxidase-conjugated streptavidin) for 10 minutes at room temperature. Rinse with Tris/PBS buffer.

viii) Apply diaminobenzidine tetrahydrochloride solution for 5 minutes at room temperature. Rinse twice in distilled water.

ix) Counterstain slides in Gill’s haematoxylin for 10–30 seconds, wash in water for 2 minutes, dehydrate, clear, and add cover-slips.

x) SIV-infected tissues are identified by the presence of brown staining in bronchiolar epithelium and pneumocytes.

e) Antigen-capture enzyme-linked immunosorbent assays

Type A antigen-capture enzyme-linked immunosorbent assays (ELISAs) are commercially available for detection of human and animal influenza viruses. These types of assays have been used for detection of SIV in lung tissue and nasal swabs (19, 33). The assays are generally available through human health care companies.

f) Polymerase chain reaction

RT-PCR tests have been developed for the diagnosis of swine influenza (8, 17). Population-wide validation data for these tests are not currently available.

2. Serological tests

The primary serological test for detection of SIV antibodies is the HI test and it is subtype specific. It should be conducted on paired sera collected 10–21 days apart. A four-fold or greater increase in titre between the first and second sample is suggestive of a recent SIV infection. Additional serological tests that have been described but not commonly used are the virus neutralisation, agar gel immunodiffusion test, and indirect fluorescent antibody test. ELISA technology for detection of SIV antibodies has been described in the literature and commercial kits have been marketed. The validation of the ELISA kits is ongoing.

- Haemagglutination inhibition test

i) Dilute reference HA antigens (H1, H3, etc.) to a concentration of 4–8 HAU/25 µl in 0.01 M PBS, pH 7.2.

ii) **H1N1 test:** Heat inactivated sera for 30 minutes at 56°C. Add 0.1 ml packed, washed erythrocytes to 1 ml of heat-inactivated, diluted serum and mix. Incubate at room temperature for 30 minutes with periodic shaking every 10–15 minutes. Centrifuge at 800 \(g\) for 10 minutes at 4°C. Note: Sera can be treated with RDE and erythrocytes as described below in step iii as an alternative to heat inactivation and treating with packed erythrocytes.

iii) **H1N2 and H3N2 test:** Add 50 µl serum to 200 µl RDE (1/10 dilution in calcium saline solution equalling 100 units per ml). Incubate overnight (12–18 hours) in a 37°C water bath. Add 150 µl 2.5% sodium citrate solution and heat inactivate at 56°C for 30 minutes. Combine 200 µl treated sample and 25 µl PBS. Add 50 µl of 50% erythrocytes. Shake and incubate for 30 minutes at room temperature or overnight at 4°C. Centrifuge at 800 \(g\) for 10 minutes at 4°C.
iv) Dispense 50 µl treated serum into two wells of a V- or U-bottom 96-well plate. Dispense 25 µl of
treated serum into two wells to be used as a serum control. Positive and negative control sera are
treated in the same way as the unknown sera.
v) Dispense 25 µl PBS in the serum control wells and all empty wells except two wells identified as the
cell control wells. Add 50 µl PBS in the cell control wells.
vi) Make serial twofold dilutions of the serum in 25 µl volumes in the plate and then add 25 µl of
appropriate antigen to all test wells except the serum control wells and the cell control wells.
vii) Incubate covered plates at room temperature for 30–60 minutes.

viii) Add 50 µl of 0.5% erythrocyte suspension to each well, shake, and incubate at room temperature for
20–30 minutes until a distinct button forms at the bottom of the cell control wells. Keep erythrocytes
thoroughly suspended during the dispensing process.
ix) Conduct a HA test using the HI test antigens prior to and simultaneously to conducting the HI test to
verify that antigen concentrations are appropriate.

x) For the test to be valid, there should be no haemagglutination in the serum control well, no inhibition of
haemagglutination with the negative serum, the positive serum should have its anticipated HI titre and
the HA back titration should indicate 4–8 HAU per 25 µl.

b) Enzyme-linked immunosorbent assay (18)
ELISA technology for detection of SIV antibodies has been described in the literature and ELISAs are
available as commercially produced kits.

C. REQUIREMENT FOR VACCINES AND DIAGNOSTIC BIOLOGICALS
Guidelines for the production of veterinary vaccines are given in Chapter 1.1.8 Principles of veterinary vaccine
production. The guidelines given here and in Chapter 1.1.8 are intended to be general in nature and may be
supplemented by national and regional requirements.

1. Seed management

a) Characteristics of the seed
Identity of the seed should be well documented, including the source and passage history of the organism.
All defining characteristics such as haemagglutinin and neuraminidase subtype should be established.
Haemagglutination inhibition and neuraminidase inhibition by subtype-specific antisera or RT-PCR and
sequencing can be used to establish the H and N subtypes. Also, aliquots of the master seed virus (MSV)
can be neutralised with specific antiserum, e.g. antiserum produced against H1N1 or H3N2 SIV, then
inoculated into the allantoic sac of 10-day old embryonated chicken eggs or on to susceptible cell lines such
as the MDCK cell line. Allantoic fluid or cell culture supernatant is harvested 72–96 hours post-inoculation
and tested for HA activity. Identity is demonstrated by the lack of HA activity in the neutralised seed, and the
presence of HA activity in the non-neutralised seed. Significant antigenic differences present in a given
strain that set it apart from other members of its subtype, and that purportedly have a beneficial impact on
its use as a vaccine, should be confirmed.

b) Method of culture
SIV seed can be grown in eggs or in cell culture. Selection of a culture method is dependent on the degree
of virus adaptation, growth in medium, rate of mutation, and viral yield in the specific culture system. SIV
vaccine products should be limited to five passages from the MSV to avoid genetic/antigenic variation.

c) Validation of culture
The purity of the seed and cells to be used for vaccine production must be demonstrated. The MSV should
be free from adventitious agents, bacteria, or Mycoplasma, using tests known to be sensitive for detection
of these microorganisms. The test aliquot should be representative of a titre adequate for vaccine
production, but not such a high titre that hyperimmune antisera are unable to neutralise seed virus during
purity testing. Seed virus is neutralised with monospecific antiserum or monoclonal antibody against SIV
and the virus/antibody mixture is cultured on several types of cell line monolayers. Cultures are
subpassaged at 7-day intervals for a total of at least 14 days, then tested for cytopathogenic and
haemadsorbing agents. Cells are also examined for adventitious viruses that may have infected the cells or
seed during previous passages. Potential contaminants include bovine viral diarrhoea virus, reovirus, rabies
virus, Aujeszky’s disease (pseudorabies) virus, transmissible gastroenteritis virus, porcine respiratory
coronavirus, porcine parvovirus, porcine adenovirus, haemagglutinating encephalomyelitis virus, porcine
rotavirus, porcine circovirus, and porcine reproductive and respiratory syndrome virus. Cell lines on which the seed is tested include: an African green monkey kidney (Vero) cell line (rabies and reoviruses), a porcine cell line, a cell line of the species of cells used to propagate the seed, if not of porcine origin, and cell lines for any other species through which the seed has been passaged. Additionally, a cell line highly permissive for bovine viral diarrhoea virus, types 1 and 2, is recommended. Bovine viral diarrhoea virus is a potential contaminant introduced through the use of fetal bovine serum in cell culture systems.

Factors that may contribute to instability during production, such as replication on an unusual cell line, should be investigated. If production is approved for five passages from the master seed, then sequencing of the genes for H and N at the maximum passage may be warranted to confirm the stability of the viral seed.

d) Validation as a vaccine

Vaccine candidates should be shown to be pure, safe, potent, and efficacious.

Strains used in vaccine production should be antigenically relevant to SIV strains circulating in the field (6, 11, 34, 35). Haemagglutination inhibition and neutralisation tests demonstrating cross-reactivity between antisera from animals vaccinated with the candidate vaccine strain and current field isolates can be used for the selection. A vaccination/challenge study in swine, using homologous and heterologous challenge strains, will indicate the degree of protection afforded by the vaccine. Swine used in vaccination/challenge studies should be free of antibodies against SIV at the start of the experiments. Vaccination/challenge studies should be conducted using virus produced by the intended production method, at the maximum viral passage permitted, and using swine of the minimum recommended age listed on the label. Initially, lots are formulated to contain varying amounts of viral antigen. The test lot containing the least amount of antigen that demonstrates protection becomes the standard against which future production lots are measured. The most valuable criterion for blind trial evaluations of treatment groups is a statistically significant reduction of virus (titres and duration of shedding) in the respiratory tract of vaccinated pigs. Differences in clinical observations and lung lesions are also among the criteria used in evaluation of a successful trial. If in-vivo or in-vitro test methods are to be used to determine the potency of each production lot of vaccine, those assays should be conducted concurrent with the minimum antigen studies in order to establish the release criteria. Combination vaccines containing more than one strain of SIV are available in some countries. The efficacy of the different components of these vaccines must each be established independently and then as a combination in case interference between different antigens exists.

2. Method of manufacture

Once the vaccine is shown to be efficacious, and the proposed conditions for production are acceptable to regulatory authorities, approval may be granted to manufacture vaccine. Generally, large-scale monolayer or suspension cell systems are operated under strict temperature-controlled, aseptic conditions and defined production methods, to assure lot-to-lot consistency. When the virus has reached its maximum titre, as determined by HA, CPE, fluorescent antibody assay, or other approved technique, the virus is clarified, filtered, and inactivated. Several inactivating agents have been used successfully, including formalin or binary ethylenimine. An inactivation kinetics study should be conducted using the approved inactivating agent on a viral lot with a titre greater than the maximum production titre and grown using the approved production method. This study should demonstrate that the inactivation method is adequate to assure complete inactivation of virus. Samples taken at regular timed intervals during inactivation, then inoculated on to a susceptible cell line or into the allantoic sac of embryonated eggs, should indicate a linear and complete loss of titre by the end of the inactivation process. This is represented as less than one infectious particle per 10^4 litres of fluids following inactivation. Typically, adjuvant is added to enhance the immune response.

3. In-process control

Cell cultures should be checked macroscopically for abnormalities or signs of contamination and discarded if unsatisfactory. A lot is ready to harvest when viral CPE has reached 80–90%. Virus concentration can be assessed using antigenic mass or infectivity assays.

4. Batch control

a) Sterility

During production, tests for bacteria, *Mycoplasma*, and fungal contamination should be conducted on both inactivated and live vaccine harvest lots and confirmed on the completed product (see Chapter 1.1.9 Tests for sterility and freedom from contamination of biological materials).

b) Safety

Mice or guinea-pigs can be used to evaluate the safety of an inactivated product. In one model, eight mice are inoculated intraperitoneally or subcutaneously with 0.5 ml and observed for 7 days. The mouse safety
test may not be applicable when certain adjuvants are used, especially saponin-based products. In the other model, two guinea-pigs are each injected with a 2-ml dose either intramuscularly or subcutaneously and observed for 7 days. Adverse clinical signs or mortality attributable to the vaccine is indicative of a lot that is not acceptable for use. The completeness of viral inactivation in a killed product can be determined by multiple passages in cell culture or eggs of the post-inactivation, pre-adjuvant production fluids, followed by HA testing for the presence of virus.

Final product may be evaluated in the host animal using two animals of the minimum age recommended for use, according to the instructions given on the label; the animals are observed for 21 days. Field safety studies conducted on vaccines, in at least three divergent geographical areas, with at least 300 animals per area, are also recommended. If the vaccine is to be used in swine destined for market and intended for human consumption, a withdrawal time consistent with the adjuvant used (generally 21–60 days) should be established by such means as histopathological examination submitted to the appropriate food safety regulatory authorities.

c) Potency

During production, antigen content is measured to establish that minimum bulk titres have been achieved. Antigen content is generally measured before inactivation and prior to further processing. Relative potency ELISA, HA, and HI are among the assays that can be used to determine antigen content in final product. It is necessary to confirm the sensitivity, specificity, reproducibility, and ruggedness of such assays.

d) Duration of immunity

The duration of immunity and recommended frequency of vaccination of a vaccine should be determined before a product is approved. Initially, such information is acquired directly using host animal vaccination/challenge studies. The period of demonstrated protection, as measured by the ability of vaccinates to withstand challenge in a valid test, can be incorporated into claims found on the vaccine label. Once a suitable potency assay has been identified, should antigenic drift require replacement of strains within the vaccine, strains of the same subtype can be evaluated in either the host animal or a correlated laboratory animal model. However, circulating strains may show significant antigenic differences from the vaccine strain, but the vaccine strain may still provide protection (34). Also, the vaccine may not protect against a new strain that appears to be antigenically similar to the vaccine. Other factors that play a role include the adjuvant and the antigenic dose. Consequently, it would appear that the efficacy of a vaccine will always have to be evaluated in swine.

e) Stability

Vaccines should be stored with minimal exposure to light at 4°C ± 2°C, or as approved by the designated regulatory authorities. The shelf life should be determined by use of the approved potency test (Section C.5.b) over the proposed period of viability.

f) Preservatives

The most common preservative is thimerosol, at a final concentration not to exceed 0.01% (1/10,000). Addition of thimerosol or other mercury-based compounds should be avoided if possible. Antibiotics may be used as preservatives in SIV vaccines but are limited as to kinds and amounts. Also restricted are residual antibiotics from cell culture media that may be present in the final product. For example, the total amount of preservative and residual gentamicin is not to exceed 30 mcg per ml of vaccine.

g) Precautions

Inactivated SIV vaccines present no special danger to the user, although accidental inoculation may result in an adverse reaction due to the adjuvant and secondary components of the vaccine. Generally, healthy pigs of weaning age or older and pregnant sows at any stage of gestation may be safely vaccinated with inactivated SIV vaccines.

5. Tests on the final product

a) Safety

Final container samples of completed product from inactivated vaccines should be tested in young mice as described above in Section C.4.b.

b) Potency

The potency assay established at the time of the minimum antigen protection study should be used to evaluate new lots for release. The assay needs to be specific and reproducible. It must reliably detect vaccines that are not sufficiently potent. If laboratory animal serology is used instead of swine serology, it should first be
demonstrated that vaccination of the laboratory animal induces a specific, sensitive, dose-dependent response as measured in the potency assay and is correlated to protection in swine (Section C.1.d).

REFERENCES


CHAPTER 2.8.9.

SWINE VESICULAR DISEASE

SUMMARY

Swine vesicular disease (SVD) is a contagious disease of pigs, caused by an enterovirus and characterised by vesicles on the coronary bands, heels of the feet and occasionally on the lips, tongue, snout and teats. Strains of SVD virus may vary in virulence, and the disease may be subclinical, mild or severe, the latter usually only being seen when pigs are housed on abrasive floors in damp conditions. The main importance of SVD is that it is clinically indistinguishable from foot and mouth disease (FMD), and any outbreaks of vesicular disease in pigs must be assumed to be FMD until investigated by laboratory tests and proven otherwise. However, subclinical infection has been the most frequent condition observed during recent years.

Identification of the agent: Where a vesicular condition is seen in pigs, the demonstration by enzyme-linked immunosorbent assay (ELISA) of SVD viral antigen in a sample of lesion material or vesicular fluid is sufficient for a positive diagnosis. If the quantity of lesion material submitted is not sufficient (less than 0.5 g), or if the test results are negative or inconclusive, a more sensitive test, such as the reverse transcription polymerase chain reaction (RT-PCR) or isolation of virus (VI) in porcine cell cultures, may be used. If any inoculated cultures subsequently develop a cytopathic effect, the demonstration of SVD viral antigen by ELISA or by RT-PCR will suffice to make a positive diagnosis. Subclinical infection may be detected by random sampling of pen-floor faeces followed by identification of SVD viral genome using RT-PCR or VI tests.

Serological tests: Serological tests can be used to help confirm clinical cases as well as to identify subclinical infections. Specific antibody to SVD virus can be identified using the microneutralisation test or ELISA. Although the microneutralisation test requires 2–3 days to complete, it remains the definitive test for antibody to SVD virus. A small proportion (up to 0.1%) of normal, uninfected pigs will react positively in serological tests for SVD. The reactivity of these singleton reactors is transient, so that they can be differentiated from infected pigs by resampling of the positive animal and its cohorts.

Requirements for vaccines and diagnostic biologicals: There are currently no commercial vaccines available against SVD. Diagnostic and standard reagents are available from regional reference laboratories.

A. INTRODUCTION

Swine vesicular disease (SVD) can be a subclinical, mild or severe vesicular condition depending on the strain of virus involved, the route and dose of infection, and the husbandry conditions under which the pigs are kept. Clinically, SVD is indistinguishable from foot and mouth disease (FMD) and this is its main importance. It is therefore urgent that cases of SVD be distinguished from FMD by laboratory investigation. Recent outbreaks of SVD have been characterised by less severe, or no clinical signs; infection has been detected when samples are tested for a serosurveillance programme or for export certification.

The incubation period for SVD is between 2 and 7 days, after which a transient fever of up to 41°C may occur. Vesicles then develop on the coronary band, typically at the junction with the heel. These may affect the whole coronary band resulting in loss of the hoof. More rarely, vesicles may also appear on the snout, particularly on the dorsal surface, on the lips, tongue and teats, and shallow erosions may be seen on the knees. Affected pigs may be lame and off their feed for a few days. Abortion is not a typical feature of SVD. Recovery is usually complete in 2–3 weeks, with the only evidence of infection being a dark, horizontal line on the hoof where growth has been temporarily interrupted. The clinical signs vary according to the age of pigs affected, the conditions under which they are kept and the strain of SVD virus involved (13). Disease caused by mild strains may remain
unobserved, particularly in pigs kept on grass or housed on deep straw. Younger animals are more severely affected, although mortality due to SVD is very rare, in contrast with FMD in young stock. Nervous signs have been reported, but are unusual. Affected pigs may excrete virus from the nose and mouth and in the faeces up to 48 hours before the onset of clinical signs. Most virus is produced in the first 7 days after infection, and virus excretion from the nose and mouth normally stops within 2 weeks. Virus may continue to be shed for up to 3 months in the faeces. The SVD virus is extremely resistant to inactivation in the environment, and is stable within the pH range 2.5–12.0 (14). This is in contrast to the FMD virus, which is very labile outside the pH range 6.0–8.0.

Because SVD may be mild or subclinical, it is essential when submitting samples from suspect clinical cases that serum samples from both the suspect pigs and other apparently unaffected animals in the group be included. It is possible for SVD to circulate unnoticed until it affects a particularly susceptible group, and therefore, in order to ascertain how long infection has been present, it is necessary to look for seroconversion to SVD virus in apparently healthy animals. Also the identification of the isotype of the immunoglobulins (M or G) to SVD virus may help to ascertain the time of exposure to infection.

SVD is clinically very similar to FMD. Samples for virus isolation or antigen detection must be handled and submitted as though they contained FMD virus and must be transported in phosphate buffered saline (PBS) mixed with glycerol (1/1), pH 7.2–7.6, with antibiotics such as (final concentration per ml) penicillin (1000 International Units [IU]), neomycin sulphate (100 IU), polymyxin B sulphate (50 IU), and mycostatin (100 IU) (10).

SVD virus has been classified as a pig enterovirus, in the family Picornaviridae. All isolates are classified in a single serotype, with four distinguishable antigenic/genomic variants (2). Antigenically, SVD virus is related to the human virus coxsackievirus B5. There are reports of seroconversion to SVD virus in laboratory workers handling the agent. Clinical disease was reported to be mild with the exception of a single case of meningitis associated with SVD virus infection. However, there have been no reported cases of seroconversion or disease in farmers or veterinarians working with infected pigs. Under experimental conditions, it has not been possible to show transmission of coxsackievirus B5 between pigs.

**B. DIAGNOSTIC TECHNIQUES**

1. **Identification of the agent**

Any vesicular condition in pigs may be FMD. Once suspicion of FMD has been eliminated, the diagnosis of SVD requires the facilities of a specialised laboratory. Countries that lack such a facility should send samples for investigation to an OIE Reference Laboratory for SVD¹. In the Americas, parallel testing for vesicular stomatitis viral antigen should also be conducted.

The detection of antigens or genome of SVD virus by means of enzyme-linked immunosorbent assay (ELISA) and reverse transcription polymerase chain reaction (RT-PCR) has the same diagnostic value as virus isolation. Due to their speed, ELISA and RT-PCR make suitable screening tests. However, virus isolation is the reference method and should be used if a positive ELISA or RT-PCR result is not associated with the detection of clinical signs of disease, the detection of seropositive pigs, or a direct epidemiological connection with a confirmed outbreak.

If there are clinical signs, investigation should start with the examination of a 10% suspension of lesion material in PBS. Faecal samples are the specimen of choice for the detection of virus where subclinical SVD is suspected. Faecal samples can be collected from individual pigs or from the floor of premises suspected to contain, or to have contained, pigs infected with SVD. The level of virus in faeces is usually insufficient for detection by ELISA and the use of RT-PCR and/or virus isolation is required. A significant proportion of faecal samples inoculated into cell cultures will give rise to the growth of other enteroviruses. These can be differentiated from SVD virus by ELISA or RT-PCR, but they may also outgrow SVD virus that is present, and give rise to false negative results.

- **Preparation of samples**

  *Lesion material:* a suspension is prepared by grinding the sample in sterile sand in a sterile pestle and mortar with a small volume of tissue culture medium and antibiotics. Further medium should be added to

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¹ FAO (Food and Agriculture Organization of the United Nations) World Reference Laboratory for FMD, Institute for Animal Health, Pirbright, Woking, Surrey GU24 0NF, United Kingdom (also an OIE Reference Laboratory for FMD and SVD). Istituto Zooprofilattico Sperimentale della Lombardia e dell’Emilia Romagna (IZSLER), Brescia, Via Bianchi 9, Italy.
obtain approximately a 10% suspension. This is clarified by centrifugation at 10000 rpm for 20–30 minutes in a high speed centrifuge and the supernatant is harvested.

*Faecal samples:* faecal material (approximately 20 g) is resuspended in a minimal amount of tissue culture medium or phosphate buffer (0.04 M phosphate buffer or PBS). The suspension is homogenised by vortexing and clarified by centrifugation at 10000 rpm for 20–30 minutes in a high speed centrifuge; the supernatant is harvested and filtered through a 0.45 µm filter.

### a) Virus isolation

A portion of the clarified epithelial or faecal suspension is inoculated on to monolayers of IB-RS-2 cells or other susceptible porcine cells, grown in appropriate containers (25 cm² flasks, rolling tubes, 24-, 12-, 6-well plates). For differential diagnosis (e.g. FMD) in case of clinical lesion bovine cell culture systems should also be employed. Generally SVD virus will grow in cells of porcine origin only, however there is a report that the virus can be isolated in secondary lamb kidney cells. Tissue culture medium is supplemented with, 10% bovine serum for cell growth, with 3% bovine serum for maintenance, and with antibiotics.

Cultures are examined daily. If a cytopathic effect (CPE) is observed, the supernatant fluid is harvested and virus identification is performed by ELISA (or other appropriate test, e.g. RT-PCR). Negative cultures are blind-passaged after 48 or 72 hours, and observed for a further 2–3 days. If no CPE is evident after the second passage, the sample is recorded “NVD” (no virus detected). When isolating virus from faeces in which the amount of virus present may be low, a third tissue culture passage may be required.

### b) Immunological methods

- **Enzyme-linked immunosorbent assay**

  The detection of SVD viral antigen by an indirect sandwich ELISA has replaced the complement fixation test as the method of choice. The test is the same as that used for FMD diagnosis. Wells of ELISA plates are coated with rabbit antisera to SVD virus. This is the capture serum. Test sample suspensions are added and incubated. Appropriate controls are also included. Guinea-pig detection serum is added at the next stage followed by rabbit anti-guinea-pig serum conjugated to horseradish peroxidase. Extensive washing is carried out between each stage to remove unbound reagents. A positive reaction is indicated if there is a colour reaction on the addition of chromogen (for example orthophenylenediamine) and substrate (H₂O₂). With strong positive reactions this will be evident to the naked eye, but results can also be read spectrophotometrically at the appropriate wavelength, in which case an absorbance reading ≥0.1 above background indicates a positive reaction. As an alternative to guinea-pig and rabbit antisera, suitable monoclonal antibodies (MAbs) can be used, coated to the ELISA plate as the capture antibody, or peroxidase conjugated as tracing antibody.

  An MAb-based ELISA can also be used to study antigenic variation among strains of SVD virus. Tissue culture grown viral strains are trapped by a rabbit hyperimmune antiserum to SVD virus adsorbed to the solid phase. Appropriate panels of MAbs are then reacted and the binding of MAbs to field strains is compared with the binding of MAbs to the parental strains. Strong binding indicates the presence of epitopes shared between the parental and the field strains (2).

- **Nucleic acid recognition methods**

  Reverse transcription followed by the PCR (RT-PCR) is a useful method to detect SVD viral genome in a variety of samples from clinical and subclinical cases. Several methods have been described (3, 7, 12, 15–18), employing different techniques for RNA extraction, targeting different parts of the SVD virus genome and using different approaches to detect the DNA products of amplification. In the case of faecal samples, an immunocapture technique using a SVD virus-specific MAb has been shown to be effective (7). This method (described below) is suitable for laboratories without sophisticated equipment for real-time detection of DNA amplification products, but where such facilities are available an approach such as that described by Reid *et al.* (16, 17) offers advantages in terms of ease of use and reduced risk of laboratory contamination by PCR products.

  i) RNA Immune-extraction: Coat wells of an ELISA plate with a saturating solution of MAb 5B7 (200 µl/well, diluted in carbonate-bicarbonate buffer) by overnight incubation at 4°C. Wash plates three times with PBS. Use plates immediately or store at –20°C for up to 2–3 weeks, or more if stabilised. **NOTE:** As an alternative to immunocapture, RNA extraction can be performed using a suitable commercially available kit (such as RNeasy kit from Qiagen).

  ii) Distribute each sample (faeces suspension) into three wells of the 5B7-coated plate (200 µl/well, 600 µl of sample in total).

  iii) After incubation for 1 hour at 37°C with very slow shaking, wash wells three times with PBS. Washing is performed manually, in order to avoid cross-contamination between wells.
iv) RNA is extracted from each sample by adding approximately 100 µl/well of lysis buffer (4 M guanidine thiocyanate, 25 mM sodium citrate, pH 7, 0.5% Sarkosyl). Incubate wells for 3–5 minutes and recover the sample from the three wells (300–350 µl total), and transfer into a single tube.

v) RNA is then precipitated by adding a mixture of 750 µl of absolute ethanol and 35 µl of 3 M sodium acetate (pH 5.2); vials are vortexed and incubated at –20°C for a minimum of 1 hour (prolonged overnight precipitation at –20°C may also be suitable).

vi) Centrifuge the sample at 13,000 rpm for 30 minutes at 4°C, after which a pellet should be visible which should be washed with 500 µl of 70% cold ethanol (centrifuged at 13000 rpm for 10 minutes at 4°C) and dried.

vii) Assemble the reaction mixes for the reverse transcription of SVDV RNA. Mixes (20 µl total volume) contain 4 µl of 5X AMV reaction buffer (final reaction concentration is 8 mM MgCl₂, 30 mM KCl, 1 mM dithiothreitol, 50 mM Tris/HC1, pH 8.5), 2 µl 10 mM dNTP mix, 1 µl Random Primer pd(N)₆ (100 pmol), 0.3 µl RNase inhibitor (12 units), 0.2 µl AMV reverse transcriptase (5 units) 0.5 µl BSA (0.5 µg) and 12 µl of nuclease-free water.

viii) Resuspend the RNA pellet in 20 µl of reverse transcription mix (see above).

ix) Incubate reactions at 42°C for 60 minutes followed by 95°C for 3 minutes.

x) PCR for SVDV Genome: Add 5 µl of prepared cDNA to 20 µl PCR amplification mix containing 0.55 µl 2M KCl (final reaction concentration is 44 mM), 2 µl 10 mM dNTP mix, 1 µl pSVDV-SA2 rev primer (10 pmol: 5'-TCA-CGT-TTG-TCC-AGG-TTA-CC-3'), 1 µl pSVDV-SS4 dir primer (10 pmol: 5-TTC-AGA-ATG-ATT-GCA-TAT-GGG-G-3'), 0.25 µl Taq polymerase (1.24 units) and 15.2 µl of nuclease-free water.

xi) Mix a 20 µl aliquot of each sample with 4 µl of staining solution and load onto a 2% agarose gel. After electrophoresis, a positive result is indicated by the presence of a 154 bp fragment of SVDV RNA polymerase (3D) gene in the gel.

xx) Place the plate in a PCR thermocycler and run the following programme:

One cycle at 94°C for 3 minutes,

40 Cycles of 94°C for 20 seconds, 60°C for 20 seconds, 72°C for 30 seconds,

One cycle at 72°C for 5 minutes.

xii) Mix a 20 µl aliquot of each sample with 4 µl of staining solution and load onto a 2% agarose gel. After electrophoresis, a positive result is indicated by the presence of a 154 bp fragment of SVDV RNA polymerase (3D) gene in the gel.

Comparative analysis of sequences of the viral genome is useful to establish relationships between isolates of SVD virus. By sequencing the 3D gene, which codes for the major structural protein VP1, it has been possible to group strains of SVD virus according to their sequence homology, and to relate epidemiologically strains causing disease in different regions or at different times (2). A database of 3D gene sequences of SVD viruses is held at the OIE Reference Laboratory, Pirbright, UK.

2. Serological tests

These are used in the laboratory confirmation of outbreaks, for serological surveillance and for export certification of pigs. SVD is often diagnosed solely on the evidence of serological tests. Because of the subclinical or mild nature of the disease, it is often first suspected following routine serology for disease surveillance or export certification. The virus neutralisation (VN) test, the double immunodiffusion test, the radial immunodiffusion test, the counter immunoelectrophoresis test and the ELISA have all been described for the detection of antibodies to SVD virus (1, 5, 8). However, the VN test and the ELISA are the only techniques commonly used. The VN test is the accepted standard test, but has the disadvantage that it takes 2–3 days to complete and requires tissue culture facilities. The ELISA is more rapid and can be more easily standardised. A small proportion of sera from animals with no previous exposure to SVD virus will react positively in serological tests for antibody to SVD virus. The SB7 MAb competitive ELISA (MAC-ELISA) is a reliable technique for detecting SVD antibody (1, 9) and similar results have been obtained with other ELISAs (4, 11). Results from a small proportion, 0.25%–0.45%, of sera from normal pigs are borderline or positive by the MAC-ELISA and should be retested by the VN test. Up to 0.25%–0.45% of sera from infected pigs usually contain specific IgG alone or both IgG and IgM, whereas sera from ‘singleton’ reactors usually contain exclusively IgM and do not convert to IgG (6). IgM/IgG isotype-specific ELISAs are also helpful in assessing the time of infection in the pig or on the infected premises. The presence of IgM, alone or together with IgG, is evidence of recent infection and indicative of virus shedding, while detection of IgG alone suggests an older exposure to infection (1).
a) **Virus neutralisation (the prescribed test for international trade)**

The quantitative VN microtest for antibody to SVD virus is performed using IB-RS-2 cells (or suitable susceptible porcine cells) in flat-bottomed tissue-culture grade microtitre plates.

Virus is grown on IB-RS-2 cell monolayers and stored at –20°C after the addition of an equal volume of glycerol. SVD virus has been found to be stable under these conditions for at least 1 year. The sera are inactivated at 56°C for 30 minutes before testing. A suitable medium is Eagle’s complete medium/LYH with antibiotics.

The test is an equal volume test in 50 µl volumes:

i) Starting from a 1/4 dilution, sera are diluted in a twofold dilution series across the plate, two rows of wells per serum.

ii) Previously titrated virus is added; each 50 µl unit volume of virus suspension contains about 100 TCID$_{50}$ (50% tissue culture infective dose).

iii) Controls include at least a weak positive serum and a negative serum, a cell control, a medium control and a virus titration used to calculate the actual virus titre used in the test.

iv) Incubate at 37°C for 1 hour with the plates covered.

v) A cell suspension at 10$^6$ cells/ml is prepared in medium containing 10% bovine serum for cell growth. 50 µl of cell suspension is added to each well.

vi) Plates are sealed with pressure-sensitive tape and incubated at 37°C for 2–3 days. Alternatively, the plates may be covered with loosely fitting lids and incubated in an atmosphere of 5% carbon dioxide at 37°C for 2–3 days.

vii) Microscopic readings are feasible after 48–72 hours; the plates may be finally fixed and stained routinely on the third day. Fixation is effected with 10% formalin/saline for 30 minutes; staining is done by immersion in 0.05% methylene blue in 10% formalin for 30 minutes. The plates are rinsed in tapwater.

viii) Positive results are blue-stained cell sheets; the negative wells are empty. Titres are expressed as the final dilution of serum present in the serum/virus mixture at the 50% end-point. The test is considered to be valid when the amount of virus actually used per well is between 10$^{1.5}$ and 10$^{2.5}$ TCID$_{50}$, and the positive standard sera are within twofold of their expected titre.

ix) **Interpretation of the results:** At the Pirbright, OIE Reference Laboratory for SVD (see footnote 1), VN titres less than or equal to 1/11 are considered to be negative. Titres of 1/16 to 1/32 are doubtful and VN titres of 1/45 or more are regarded as positive. However, as titres depend on the cell system used, laboratories should establish their own criteria by reference to standard reagents available from the OIE Reference Laboratory.

b) Enzyme-linked immunosorbent assay

In the ELISA developed by Brocchi et al. (1), the SVD viral antigen is trapped to the solid phase using the MAb 5B7. The ability of test sera to inhibit the binding of peroxidase-conjugated MAb 5B7 to the trapped antigen is then evaluated. Finally, the amount of conjugated MAb bound is detected by the addition of substrate and chromogen.

i) ELISA plates are coated with 50 µl/well of MAb 5B7 at a saturating dilution in carbonate/bicarbonate buffer, pH 9.6, by overnight incubation at 4°C.

ii) The plates are washed three times with PBS containing 0.05% Tween 20, and 50 µl of SVD antigen (SVD virus grown in IB-RS-2 cells, clarified, filtered and inactivated) at a predetermined optimal dilution, is added to each well. The optimal dilution of antigen is determined by checkerboard titrations of antigen and conjugated MAb that define the working dilution giving an absorbance on the upper part of the linear region of the antigen titration curve (between 1.5 and 2.0 optical density units). Plates are then incubated for 1 hour at 37°C.

iii) After three additional washes, 50 µl of diluted test sera (not inactivated) and control sera are incubated with the trapped antigen for 1 hour at 37°C. Three-fold dilution series of sera are obtained directly in ELISA wells by adding 10 µl of serum to 65 µl of buffer (1/7.5 dilution) then transferring 25 µl to sequential wells containing 50 µl of buffer, mixing, and finally discarding 25 µl.

iv) After incubation for 1 hour, 25 µl of an optimal dilution of peroxidase-conjugated MAb 5B7 (see step ii above) is added to each well and the plates are incubated at 37°C for a further 1 hour.
v) After a final series of washes, the colorimetric reaction is developed by distributing 50 µl per well of the substrate solution (for example 0.5 mg/ml orthophenyldiamine in phosphate/citrate buffer, pH 5, containing 0.02% H₂O₂).

vi) The reaction is stopped after 10 minutes by adding 50 µl of 2 N H₂SO₄. The absorbance is read at the appropriate wavelength using a microplate reader.

Antigen, sera and conjugate are diluted in PBS, pH 7.4, containing 0.05% Tween 20 and 1% yeast extract; the dilution buffer for sera contains, in addition, 1.0% mouse serum to prevent nonspecific binding of pig serum to MAb 5B7 either coated to the plate or conjugated to peroxidase.

vii) Controls: Four wells on each plate containing all reactants except test serum confirm the maximum absorbance reading for the antigen; convalescent pig serum at four selected dilutions; negative pig serum; a low positive standard pig serum.

viii) Interpretation of the results: Reactions are expressed as the percentage inhibition by each test serum of the MAb reaction with the SVD antigen. Sera are considered to be positive when producing an inhibition ≥80% at the 1/7.5 dilution; negative when producing an inhibition <70% at the 1/7.5 dilution; doubtful when producing an inhibition ≥70% and <80% at the 1/7.5 dilution. The second dilution (1/22.5) provides an indication of the level of antibodies: strongly positive sera show >80% inhibition at both 1/7.5 and 1/22.5 dilutions, while sera registering >80% inhibition at the 1/7.5 dilution but <50% inhibition at the 1/22.5 dilution are considered to be low positive or borderline. All positive, borderline and doubtful sera should be confirmed using the VN test.

STANDARD REFERENCE SERA FOR SVD SEROLOGY

The OIE Reference Laboratory, Pirbright, UK maintains a panel of reference sera that have been extensively validated by the National SVD Reference Laboratories of the Member States of the European Union.

C. REQUIREMENTS FOR VACCINES AND DIAGNOSTIC BIOLOGICALS

No commercial SVD vaccines are currently available. Standard sera can be obtained from the OIE/FAO WRL for FMD (see footnote 1). MAb 5B7 is available from the OIE Reference Laboratory for swine vesicular disease in Italy (see Table given in Part 3 of this Terrestrial Manual).

REFERENCES


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**NB:** There are OIE Reference Laboratories for Swine vesicular disease (see Table in Part 3 of this Terrestrial Manual or consult the OIE Web site for the most up-to-date list: www.oie.int).
CHAPTER 2.8.10.
TESCHOVIRUS ENCEPHALOMYELITIS
(previously enterovirus encephalomyelitis or Teschen/Talfan disease)

SUMMARY

Teschovirus encephalomyelitis was first described as a particularly virulent, highly fatal encephalomyelitis of pigs and was previously known as Teschen disease (or enterovirus encephalomyelitis). It is caused by strains of porcine teschovirus serotype 1 (PTV-1) of the genus Teschovirus, family Picornaviridae. Less severe forms of the disease were first recognised in the United Kingdom, where it was called Talfan disease, and in mainland Europe, where it was called poliomyelitis suum or benign enzootic paralytic. In addition to PTV-1 strains, the milder form of the disease can be caused by other PTV serotypes, including PTV-2, -3, -4, -5, -6, -9 and -10.

The disease was first described in Teschen, Czechoslovakia in 1929. During the 1940s and 1950s it caused serious losses in European countries and was spread to other continents. The clinical disease is now rare and has not been reported in Western Europe since 1980. However, there has been serological evidence that virus variants, that are not pathogenic or of low pathogenicity, circulate in pig populations.

Identification of the agent: The virus has affinity for the central nervous system and therefore suspensions of brain and spinal cord from affected pigs are used as inocula for virus isolation. The virus propagates successfully on monolayers derived from swine tissue, in particular from kidney. If PTV is present, it gives rise to specific cytopathic effects characterised by rounded refractile cells. For PTV identification and serotyping, suitable tests are employed using specific antisera or monoclonal antibodies against standard strains of PTV. Virus neutralisation tests and indirect fluorescent antibody tests are preferred. Reverse-transcription polymerase chain reaction amplification of parts of the viral genome is possible, but as yet no specific tests have formally been accepted for diagnosis.

Serological tests: Because the seroprevalence of PTV-1 may exceed 60% in healthy pig populations in Central Europe, and identical clinical signs may be caused by other viruses, including other serotypes of PTV, a single serological test for PTV-1 giving positive results does not indicate that the neurological signs observed are actually caused by a PTV-1 infection. A four-fold rise in titre together with typical signs should be considered to be an indication that PTV-1 infection caused clinical disease. For screening for specific antibodies in pig populations, it is recommended to use the virus neutralisation test in microtitre plates or the enzyme-linked immunosorbent assay.

Requirements for vaccines and diagnostic biologicals: When clinical disease was common, vaccines were available and used; however, as the disease is now rare, vaccines are no longer available.

A. INTRODUCTION

Teschovirus encephalomyelitis (previously Teschen/Talfan diseases, and later enterovirus encephalomyelitis) is an acute condition of pigs characterised by central nervous system (CNS) disorders. Teschen is the name of the town in the Czech Republic where the disease was first recognised in 1929 (4, 5). In the 1950s, the disease spread throughout Europe and caused huge losses to the pig breeding industry. Less severe forms of the disease were first recognised in the United Kingdom, where it was called Talfan disease, and in Denmark, where it was called poliomyelitis suum; these were benign enzootics of swine. Teschovirus encephalomyelitis has not
been reported in Western Europe since 1980 (Austria) and the disease is now considered rare. In the last 12 years (since 1996) disease was reported to the OIE by the following countries: Belarus (1996, 1999 and 2005), Japan (2002), Latvia (1997 and 2000–2002), Madagascar (1996–2000, 2002 and 2004–2005), Moldavia (2002–2004), Romania (2002), Russia (2004), Uganda (2001) and Ukraine (1996–2005). In most of these cases it is not known if diagnosis was made purely on clinical grounds or in conjunction with laboratory tests; the exception being in Japan in 2002 (17).

The causal agent of teschovirus encephalomyelitis is porcine teschovirus serotype 1 (PTV-1), which belongs to the species *Porcine teschovirus*, genus *Teschovirus*, family *Picornaviridae* (2, 5). Originally the PTVs were classified within the genus *Enterovirus* and the original 11 porcine enterovirus (PEV) serotypes, PEV-1 to PEV-11, were placed in three groups – I, II and III – on the basis of cytopathic effect (CPE) produced, serological assays and replication in different cell cultures (7). PEV-1 to PEV-7 and PEV-11 to PEV-13 were identified as group I. Based on nucleotide sequencing and phylogenetic analysis, the PEV group I viruses have now been placed in the genus *Teschovirus*. PEV-1 to -7 have been renamed PTV-1 to -7 and PEV-11 to -13 were renamed PTV-8 to 10; an additional serotype, PTV-11, has also recently been described (8, 14). PEV group II contains PEV-8 (species *Porcine enterovirus A*) and group III consists of PEV-9 and PEV-10 (species *Porcine enterovirus B*). These two groups currently belong to the genus *Enterovirus* (14, 18), although it has been suggested that PEV-8 may be reclassified in another new picornavirus genus (8).

PTV-2, -3, -4, -5, -6, -9 and -10 have been isolated from pigs with milder forms of the disease (16). PTV infections often do not produce clinical signs. Serotypes may be differentiated using a virus neutralisation (VN) test (2, 7), complement fixation test (6) or indirect fluorescent antibody (IFA) test (1, 13).

PTV infections only occur in swine; other animal species are not known to be susceptible.

Differential diagnoses include pseudorabies (Aujeszky's disease) and classical swine fever (acute form). In addition, Japanese encephalitis, *Streptococcus suis* and haemagglutinating encephalomyelitis may occasionally produce similar clinical signs. Non-infectious aetiologies, in particular toxicities, must also be considered.

PTV may be identified serologically using standard antisera that have been prepared by hyperimmunisation of guinea-pigs, rabbits, or colostrum-deprived piglets with standard strains of PTV serotypes 1–11.

The virus enters the animal via the oral or nasal cavity. The incubation period is about 14 days. The main signs of the prodromal stage are fever up to 41.5°C, lassitude, anorexia and locomotor disturbances. This stage is followed by hypersensitivity, tremors, clonic spasms of the legs, flaccid paralysis, opisthotonos and nystagmus; convulsions may be observed in young pigs. In the final clinical stage, paralysis proceeding from the hind part through the loins to the fore part of the body is observed. Paralysis of the thermoregulatory centre results in hypothermy. When respiratory muscles are paralysed, the animal dies of suffocation.

Laboratory diagnosis of the disease is based on typical clinical signs plus histological lesions of the brain and spinal cord, identification of the virus in the CNS of affected pigs, and on the detection of specific antibodies in the blood of convalescent animals.

### B. DIAGNOSTIC TECHNIQUES

#### 1. Histological examination and immunohistochemistry

For histological diagnosis, samples of cerebrum, cerebellum, diencephalon, medulla oblongata and cervical and lumbar spinal cord are collected. The samples are fixed in formaldehyde and sections are stained using conventional histological methods. The virus multiplies in the CNS causing a nonsuppurative poli-encephalomyelitis with lymphocytic perivascular cuffs, especially in the spinal cord (4). Pathological changes are observed in the grey matter of the diencephalon, cerebellum, medulla oblongata and in the ventral horns of the spinal cord, consistently including dorsal root ganglia and trigeminal ganglia (ganglioneurites) and to a lesser extent in the cerebral hemispheres. Lesions may involve the dorsal horns of the spinal cord in very young animals. Degeneration of neurons (swelling, chromatolysis, necrosis, neuronophagia, axonal degeneration) and their replacement by microgliosis (astrobytosis, astrogliosis) develops in the late stage of the disease.

Detection of teschovirus antigens by immunohistochemistry on fixed, paraffin-embedded CNS sections is very difficult and not consistently possible. If suitable specific antisera or monoclonal antibodies are available, as well as specific detection techniques, correlation of pathological changes with the location of the agent may be possible on fixed, paraffin-embedded sections of the CNS.
2. **Identification of the agent**

a) **Isolation of the virus**

Progress in diagnosis of teschovirus encephalomyelitis and vaccine production has been made possible by the propagation of virus in cell culture (9, 11).

Samples of brain and spinal cord are collected from pigs slaughtered at an early clinical stage of the disease. When not processed immediately, the samples should be placed in a solution prepared from equal parts of phosphate buffered isotonic saline solution (PBS), pH 7.4, and glycerol. Pieces of tissue are minced to prepare a 10% (w/v) suspension in PBS. The suspension is centrifuged at 800 g for 10 minutes and the supernatant fluid is used for inoculation of cell cultures. Monolayer cultures of primary porcine kidney or established cell lines derived from porcine tissue are suitable for isolation of PTV.

- **Test procedure**
  
i) Test tubes or tissue culture vessels with monolayer cell cultures are used. Growth medium is discarded and tubes or vessels are inoculated with 0.1 ml of suspect tissue homogenate.
  
ii) Inoculated test tubes are placed on a roller drum or tissue culture vessels are placed on a tray and incubated for 1 hour at 37°C.
  
iii) The inoculum is discarded; the tubes or tissue culture vessels are washed with PBS and replenished with 1–20 ml (depending on the type of tissue culture vessel used) of maintenance medium without calf serum.
  
iv) The tubes are examined microscopically each day. If the sample contains PTV, characteristic CPE will be seen after 3–4 days. The CPE is characterised by small foci of rounded refractile cells. After several passages the virus grows better and produces complete CPE. The identity of PTV can be confirmed by the use of specific antiserum or monoclonal antibodies. The VN or the IFA test is best suited to this purpose. Once an isolate has been identified serologically as PTV, piglet inoculation is the only certain means of determining that the given isolate is pathogenic.

b) **Virus neutralisation test for porcine teschovirus identification**

The virus harvested from cell cultures is diluted in cell culture maintenance medium over the range 10^{-1} to 10^{-5} in tenfold steps. For teschovirus serotyping, 12 rows of each dilution are prepared; 50 µl of standard antiserum to PTV-1–11 diluted 1/10 is added to rows 1–11 and 50 µl of negative serum is added to the last row. Mixtures are incubated overnight at 4°C or for 1 hour at 37°C and thereafter inoculated into roller tube cultures or into wells of microtitration plates with confluent monolayer cell cultures. The inoculated cell cultures are incubated at 37°C. Assessment is carried out 72 hours later and every following day up to day 10, depending on when the CPE is seen. The identification of a PTV serotype is confirmed if the titre of the isolated virus in the presence of that antiserum is at least 10^3 lower than that virus incubated with negative serum.

c) **Indirect fluorescent antibody test for the confirmation of porcine teschovirus antigen in cells**

The IFA test is based on the reaction of the antigens in infected cells with specific antibodies in positive serum (13). The reaction is visualised by a fluorescein isothiocyanate (FITC)-conjugated antiglobulin, using a microscope with a UV or a blue light source. The antigen is detectable in cells 12 hours after the infection with PTV, i.e. before the development of CPE. Polyclonal antisera often show cross-reactivity with different PTV types, which can confuse the interpretation of results.

- **Test procedure**
  
i) Monolayers of porcine kidney cells on cover-slips are inoculated with the suspected material. Positive and negative controls should be processed in parallel with the test specimens.
  
ii) After incubation for 12–16 hours, the cover-slips are removed, washed twice in PBS, air-dried and fixed in cold acetone for 5–15 minutes.
  
iii) The cover-slips are placed into a wet box and flooded with rabbit or pig hyperimmune anti-PTV serum optimally diluted 1/10 with PBS or with PTV-specific monoclonal antibody at working dilution.
  
iv) The wet box is closed and incubated at 37°C for 60 minutes.
  
v) The cover-slips are removed and washed three times in PBS, then flooded with FITC-conjugated anti-rabbit or anti-pig goat serum, at a previously assessed working dilution, and incubated at 37°C for 30 minutes.
  
vi) The cover-slips are then washed three times with PBS, air-dried and mounted in 0.1 M Tris-buffered glycerol, pH 8.6.
After processing, the cover-slips are examined microscopically. The control slides are examined first to confirm that the fluorescence observed is specific. The fluorescence is apple green in colour and occurs in the cell cytoplasm and at the periphery of the nucleus. Instead of cover-slips, multipot slides or multiwell plates can also be used.

d) Reverse-transcription polymerase chain reaction

The reverse-transcription polymerase chain reaction (RT-PCR) provides a method for detection and differentiation of specific gene regions of porcine teschoviruses (12, 19). The nested RT-PCR with specific primer sets has been used to differentiate between PTVs and PEVs (19). PCR is more rapid and less laborious than virus isolation by tissue culture technique and serotyping. However, the PCR technique is currently restricted to specialised laboratories.

3. Serological tests

Because the seroprevalence of PTV-1 may exceed 60% in healthy pig populations in some countries of Central Europe, and identical clinical signs may be caused by other viruses – including other serotypes of PTV – a single serological test for PTV-1 giving positive results does not indicate that neurological signs observed are actually caused by PTV-1. A four-fold rise in titre together with typical signs should be considered to be an indication that PTV-1 infection caused clinical disease. Another reason that paired serum samples are needed for confirmation of the significance of titres is that cross-reactions have been reported with orphan teschoviruses.

Pigs that have recovered from disease, or those with inapparent disease, produce specific antibodies. Several serological methods are available for their detection, of which the microtitre VN test using pig kidney cell cultures is the most useful (10). An ELISA has been developed that is more sensitive and rapid (3).

For serological diagnosis it is necessary to have standard strains of PTV serotypes propagated in cell cultures and hyperimmune serum monospecific for PTV types.

- Standard strains of porcine teschoviruses

**Characteristics:** Following long experience, the strain ‘Zabreh’, isolated in Czechoslovakia during the period of peak incidence of the disease, was selected as the standard strain to generate the severe form of teschovirus encephalomyelitis. The pathogenicity of the strain is maintained by intracerebral passages in healthy, colostrum-deprived piglets. The virus produces typical signs of teschovirus encephalomyelitis after an incubation period of 5–7 days. For serological diagnosis, the following strains of PTV serotypes should be used as standard strains:

- type 1: Talfan
- type 2: T80
- type 3: O2b
- type 4: PS36
- type 5: F26
- type 6: PS37
- type 7: F43
- type 8: UKG/173/74
- type 9: Ger-2899/84
- type 10: Ger-460/88
- type 11: Dresden

**Stock virus:** Standard strains are propagated on monolayers of cell culture either from primary porcine kidney or testes or on an established cell line, for example PK-15. A 10% suspension in PBS, pH 7.4, is prepared from the brain and spinal cord of piglets infected experimentally with PTV. Some types are isolated from faeces. The suspension is centrifuged and the supernatant is used for the inoculation of cell cultures. The procedure for the cultivation of PTV in cell cultures is as follows:

The growth medium is removed from the cell culture and after rinsing with buffered saline, cells are inoculated with the virus suspension at 37°C. The size of the inoculum should be equal to 10% of the growth medium. After 1 hour of incubation at 37°C, the inoculum is decanted, the culture vessel is rinsed with buffered saline, and the cells are overlayed with the appropriate volume of serum-free medium supplemented with antibiotics. CPE is apparent within 48 hours, and the monolayer disintegrates more or less completely during the next 48–72 hours. In the subsequent three to five passages in cell culture, the development of the CPE accelerates and the concentration of virions increases. Titration of the virus is performed in tube cultures or on microtitre plates. A cell-adapted strain usually reaches TCID$_{50}$ (50% tissue culture infective dose) titres of $10^6$–$10^7$ per ml.

The fluid harvest is checked for specificity using known specific hyperimmune antiserum. Treatment with 5% chloroform and cultivation in human and bovine cell cultures and chicken embryos is used to exclude contamination with other viruses. PTV is chloroform resistant and multiplies only in cultures of swine origin. Immunofluorescent antibody staining is useful to detect possible contaminants that are also chloroform resistant and propagate on cells of swine origin (e.g. parvovirus), or that are non-cytopathic. The stock virus should be dispensed into small aliquots and preserved at –60°C. Frozen virus retains its properties for several years. For stock virus that is to be used in the neutralisation test, a constant dose of 100 TCID$_{50}$ is recommended.

- Specific hyperimmune serum

Specific hyperimmune serum is obtained by repeated immunisation of guinea-pigs, rabbits or colostrum-deprived piglets with PTV. Although the animals are selected from specific pathogen free breeds, they are nonetheless
tested before immunisation for absence of antibodies against PTV. The standard strains should be used. Rabbits are immunised either intravenously, using virus suspension alone, or subcutaneously or intraperitoneally, using the virus suspension with 10% oil adjuvant. Good results may be obtained by administering three doses of 2 ml of virus suspension plus 0.2 ml oil adjuvant, at intervals of 2 weeks. The rabbits are bled 10 days after the last immunisation. Piglets are immunised in the same way. The harvested sera are clarified by centrifugation and stored in small aliquots at –20°C. The sera are tittered using a neutralisation test and constant antigen. Only sera with an antibody titre of at least 1/256 can be used for the identification of the virus.

a) Virus neutralisation test in microtitre plates

The test is performed in flat-bottomed cell culture microtitre plates, using low passage porcine kidney or testes cells or cell lines derived from porcine cells. Stock virus is grown in cell monolayers. The virus harvested from cell cultures is clarified by centrifugation and stored in aliquots at –20°C. Culture medium, such as Eagle’s complete medium or LYH (Hanks balanced salt solution with yeast extract, lactalbumin and antibiotics), is used as diluent. The virus harvested from cell cultures is clarified by centrifugation and stored in aliquots at –70°C or as 50/50 mixture with glycerol and can be stored at –20°C.

- **Test procedure**
  1. Inactivate swine sera for 30 minutes at 56°C.
  2. The sera to be tested are diluted in cell culture medium in twofold steps from 1/2 to 1/64, four wells per dilution and 50 µl volumes per well.
  3. Controls include positive and negative sera, cells and medium control.
  4. Add to each well 50 µl of virus stock previously diluted in culture medium to provide 100 TCID₅₀.
  5. Incubate for 1 hour at 37°C with the plates covered. The residual virus stock is also incubated.
  6. Make back titrations of the residual virus stock in four tenfold dilution steps using 50 µl per well and four wells per dilution.
  7. Add 50 µl of porcine kidney cell suspension at 5 × 10⁵ cells per ml.
  8. After further shaking, lids are put on and the plates are incubated at 37°C in a 5% CO₂ atmosphere for 2–3 days or longer, to a maximum of 8 days.
  9. Examine the plates microscopically for CPE. The test should be validated by checking the back titration of virus and titration of positive control serum. Virus should give a value of 100 TCID₅₀ with a permissible range of 30–300. The standard positive serum should give a titre within 0.3 log₁₀ units from its predetermined mean. A negative serum should give no neutralisation at the lowest dilution tested, i.e. 1/2.
  10. The VN results are determined by the Spearman–Kärber method as the dilution of serum that neutralised the virus in 50% of the wells.
  11. Virus neutralisation titres are regarded as positive if the corresponding serum neutralises the virus at an initial serum dilution of 1/8 or higher.

b) Enzyme-linked immunosorbent assay

An alternative method for the detection and titration of specific antibodies against PTV is the ELISA technique (3). The test is performed in microtitre plates using PTV grown on cell cultures as antigen. The technique can be carried out using the following steps.

- **Antigen preparation**
  1. Virus is propagated on monolayers of cell culture either from primary porcine kidney or testes or on an established cell line, for example PK-15. The growth medium is removed from the cell culture and after rinsing with buffered saline, cells are inoculated with virus suspension at a low multiplicity of infection. After 30 min of incubation at 37°C, cells are overlayed with the appropriate volume of serum-free medium supplemented with antibiotics. Incubation at 37°C is continued with daily microscopic observations. CPE should be apparent within 48 hours, and the monolayer disintegrates more or less completely during the next 48–72 hours. A cell-adapted strain usually reaches TCID₅₀ (50% tissue culture infective dose) titres of 10⁵–10⁶ per ml.
  2. The harvested virus is clarified by centrifugation at 200 g for 15 minutes, and then precipitated with a final 50% saturated (NH₄)₂SO₄ for 120 minutes at 4°C.
  3. After centrifugation at 2000 g, the resulting precipitate is suspended in TEN buffer (Tris-hydroxymethyl-methylamine [0.01 M], ethylene diamine tetra-acetic acid [1 mM] and NaCl [0.15 M]), pH 7.4, to 1/100 of the initial volume.
  4. The concentrated viral suspension is extracted by shaking with freon 3/1 for 10 minutes at 4°C.
v) Following further centrifugation, the supernatant is divided into two separate phases. The upper aqueous phase, containing the viral antigen, is desalinated by passage through a 2.5 x 40 cm cylinder packed with sephadex G 25.

vi) The viral solution is finally concentrated by ultracentrifugation at 160,000 g for 3 hours.

vii) The pellet is suspended in TEN buffer, pH 7.4, in approximately 1000th the initial volume of virus.

viii) Insoluble proteins are separated by light centrifugation, and the supernatant is used as the positive antigen in the ELISA.

- Test procedure

i) Plates are sensitised with pre-diluted antigen in phosphate buffered saline (PBS), pH 7.2, by adding 100 µl to each well. The adsorption of antigen to the surface of the plate takes place overnight at 4°C. Parallel rows of the plate should be treated with negative antigen.

ii) The plate is washed five times in PBS to remove excess antigen.

iii) Test sera are diluted 1/20 with PBST (PBS solution containing 0.05% Tween 20). 50 µl of the diluted sera is placed into each of two wells with positive antigen and into two wells with negative antigen. (Negative antigen is prepared as described above except that the tissue culture is not inoculated with virus and cells are disrupted by freezing.) The plate is incubated for 1 hour at 37°C.

iv) The plates are washed five times with PBST.

v) A predetermined dilution of horseradish peroxidase conjugated with anti-swine immunoglobulin prepared in rabbits is added in 50 µl quantities to each well. The plates are further incubated for a further 1 hour at room temperature.

vi) The plates are washed five times in PBS.

vii) Substrate solution (0.1% ortho-phenylendiamine with 0.03% hydrogen peroxide in PBS, pH 6.0) is added in 100 µl quantities to each well.

viii) After the addition of substrate, positive samples change colour to dark brown. When a sufficient degree of colour reaction is seen in the wells of known positive sera, the reaction is stopped by addition of 50 µl of 2 M sulphuric acid to each well. The absorbance of the wells is measured at a wavelength of 492 nm, preferably using an automatic multi-channel spectrophotometer with print-out mechanism. Positive and negative sera and non-infected cells should be processed as controls in parallel with the test specimens.

ix) The absorbance of a serum is the mean reading of two wells with positive antigen minus the mean reading of two wells with negative antigen. Absorbance readings of test sera that exceed by more than twofold the mean reading of standard negative sera are regarded as positive.

C. REQUIREMENTS FOR VACCINES AND DIAGNOSTIC BIOLOGICALS

- Vaccines against teschovirus encephalomyelitis

During the period of highest incidence of the disease in central Europe and Madagascar, active immunoprophylaxis was an important means for the control of this infection (15). As severe clinical disease has disappeared, vaccination has been discontinued and the vaccine is no longer being produced or used anywhere in the world.

REFERENCES


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CHAPTER 2.8.11.

TRANSMISSIBLE GASTROENTERITIS

SUMMARY

Transmissible gastroenteritis (TGE) is an enteric disease of pigs caused by TGE virus (TGEV), a member of the Coronaviridae. Since 1984, a distinct respiratory variant (porcine respiratory coronavirus or PRCV) has spread throughout many parts of the world. This virus is probably a deletion mutant of TGEV. PRCV does not appear to be an important primary pathogen, but it contributes to the porcine respiratory disease complex and it has greatly complicated the diagnosis of TGE, particularly by serological means.

Laboratory diagnosis is made by demonstrating the presence of virus, viral antigens or viral nucleic acid in material from suspected cases, or by demonstrating virus-specific humoral antibodies.

Identification of the agent: Virus may be identified by virus isolation in tissue culture, electron microscopy, various immunodiagnostic assays, and more recently by specific detection of viral RNA. The most commonly employed rapid assays are probably the immunodiagnostic ones, particularly enzyme-linked immunosorbent assays (ELISAs) on faeces and fluorescent antibody tests on cryostat sections of intestine. Another enteric disease, porcine epidemic diarrhoea, is caused by a serologically distinct coronavirus that nevertheless has an identical appearance under the electron microscope. Diagnostically, immune electron microscopy circumvents this problem.

Serological tests: The most widely used methods are virus neutralisation tests and ELISAs. Only in the latter case is differentiation from PRCV possible, as TGEV and PRCV antibodies show complete cross-neutralisation.

Requirements for vaccines and diagnostic biologicals: There are no commercial biological products available internationally. However, several countries practise vaccination, and in the United States of America, licences have been issued authorising the production and distribution of monovalent and combined vaccines.

A. INTRODUCTION

Transmissible gastroenteritis (TGE) is an enteric disease of pigs caused by TGE virus (TGEV), a member of the Coronaviridae. Since 1984, a distinct respiratory variant (porcine respiratory coronavirus or PRCV) has spread throughout many parts of the world and is now found in most countries where surveys for it have been conducted, one exception being Oceania. Occurrences of TGE have become more sporadic. The disease is still reported on an occasional basis from parts of Europe, North America and Asia (31). TGEV multiplies in and damages the enterocytes lining the small intestine, producing villous atrophy and enteritis. Diarrhoea and vomiting occur in pigs of all ages; mortality is highest in neonates. Extra-intestinal sites of virus multiplication include the respiratory tract and mammary tissues (20), but the virus is most readily isolated from the intestinal tract and from faeces. By contrast, PRCV is most readily isolated from the upper respiratory tract, the trachea, tonsils or the lungs, and little enteric multiplication of virus occurs (10, 32, 39) although PRCV can be detected by nested reverse-transcription polymerase chain reaction (RT-PCR) in nasal swabs and faeces of PRCV-infected swine (9). PRCV is probably a deletion mutant of TGEV (41) as confirmed by recent data comparing the complete 30 Kb genome sequences of TGEV and PRCV strains (66).

As TGE is a contagious disease that can occur as explosive epizootics, rapid diagnostic methods for its confirmation are particularly important. The disease can also take the form of a low-level endemic problem of post-weaning diarrhoea, which is more difficult to diagnose. The occurrence of TGEV in PRCV-immune herds also leads to milder and sporadic clinical cases of TGEV, further complicating TGEV diagnosis in such scenarios (22).

Possible wild and domestic animal reservoirs for TGEV have been suggested. Wild and domestic carnivores (foxes, dogs, possibly mink) and cats seroconvert to TGEV and are suggested as potential subclinical carriers of
TGEV, serving as reservoirs between seasonal (winter) epidemics. However only virus excreted by serially TGEV-infected dogs has been confirmed as infectious for pigs (46). Based on genetic and antigenic similarities, it has been proposed that TGEV, PRCV, feline and canine coronaviruses represent host-range mutants of an ancestral coronavirus. Wild birds (Sturnus vulgaris) and flies (Musca domestica) have been proposed as mechanical vectors for TGEV, excreting virus for 32–72 hours, respectively (46).

**B. DIAGNOSTIC TECHNIQUES**

1. **Identification of the agent**

Virus may be identified by virus isolation in tissue culture (12), immunofluorescence, reversed passive hemagglutination, enzyme-linked immunosorbent assays (ELISAs), radioimmunoassay (RIA), hybridisation with DNA probes, electron microscopy, and, more recently, by specific detection of viral RNA (14, 21, 37, 46, 55, 64). Molecular techniques such as RT-PCR and nested RT-PCR developed in the past few years have increased the sensitivity and specificity of detection and differentiation of TGEV and PRCV directly from field samples (9, 21, 22, 37). An alternative diagnostic method that has been recommended for laboratories lacking facilities for specialised tests is the oral dosing of susceptible TGEV/PRCV seronegative piglets with suspect intestinal contents. However, laboratory tests are still required to confirm susceptibility of the pigs prior to inoculation and to show that any illness induced in these animals is due to TGE. The most commonly employed rapid assays are probably the immunodiagnostic ones, particularly enzyme-linked immunosorbent assays (ELISAs) on faeces (4, 25, 62), fluorescent antibody tests (FAT) on cryostat sections of intestine (40) and immunohistochemistry (IHC) on formalin-fixed, paraffin sections (53). Detection of virus by reversed passive hemagglutination has also been described (1). Another enteric disease, porcine epidemic diarrhoea (PED), is caused by a serologically distinct coronavirus that nevertheless has an identical appearance under the electron microscope. Diagnostically, immune electron microscopy circumvents this problem (44, 61) as does the application of PED virus-specific detection assays (23).

a) **Virus isolation in tissue culture**

Apart from the inoculation of live piglets (12), this is the most definitive method of diagnosis. However, for routine use it is slow and laborious. TGEV does not grow well in cell culture, making this technique impractical as a routine diagnostic procedure. Moreover isolation of TGEV from pigs in PRCV seropositive herds is also problematic and often requires placement of TGEV/PRCV seronegative pigs in the suspect herd to serve as sentinels, followed by collection of samples from the sentinel pigs for TGEV isolation or detection (9, 22). PRCV can be isolated in tissue culture using similar cell types and techniques as for TGEV, but using nasal cells or fluids and tracheal, tonsil or lung tissues or homogenates as the optimal specimens (9, 39).

For TGEV, isolation is usually attempted ante-mortem from faeces or post-mortem from the small intestine. Loops of affected small intestine, ligated at each end to retain the contents, or mucosal impression smears of the the small intestinal luminal surface are the preferred specimens. As the virus is heat labile, all samples should be fresh or chilled.

Sample material is homogenised in cell culture medium or phosphate buffered saline (PBS), pH 7.2, containing antibiotics, e.g. penicillin (1000 U/ml), dihydrostreptomycin (1000 µg/ml), and mycostatin (20 U/ml), to produce a 10% suspension. This is allowed to stand out of direct sunlight for 30 minutes at room temperature. The suspension is then sonicated and clarified by low-speed centrifugation. The supernatant fluid may be mixed with an equal volume of heat-inactivated bovine serum in order to reduce the cytotoxic effect of the material and it is then used to inoculate susceptible tissue cultures, such as 3–4-day-old primary or secondary pig kidney monolayers. Other low passage porcine cultures (such as thyroid or testis) and some cell lines (17, 27) may also be used for primary virus isolation. After incubation at 37°C for 1 hour, the cell sheets are overlayed with a medium, such as Earle’s yeast lactalbumin (EYL) balanced salt solution, containing sodium bicarbonate and antibiotics, e.g. penicillin (100 U/ml), dihydrostreptomycin (100 µg/ml), mycostatin (20 U/ml), and 1% fetal calf serum. Incorporation of trypsin into the culture medium may enhance primary viral recovery (5, 17). Uninoculated control cultures are established concurrently and all cultures are incubated at 37°C.

Viral cytopathic effect (CPE) may be observed after 3–7 days, characterised by cells rounding, enlarging, forming syncytia and detaching into the medium. Plaque formation is sometimes more reliable and easier to recognise. A suitable plaquing overlay is 1.6% noble agar in 2 × minimal essential medium with 1% NaCO₃, antibiotics (as above), 0.7% neutral red and 1% DEAE (diethylaminoethyl) (100 µg/ml). Wild-type TGEV does not grow readily in tissue culture, so several subpassages may be necessary before these distinctive changes become apparent. Cytopathic isolates must be confirmed as TGEV by immunostaining or by in-vitro neutralisation tests using appropriate TGEV-specific antisera (5). If suitable monoclonal antibodies (MAbs) are available they can be used to distinguish between TGEV and PRCV by immunostaining.
methods (15, 54). Differentiation of TGEV from PRCV can also be accomplished by TGEV-specific cDNA probes (2) or by discriminatory RT-PCR or nested RT-PCR (9, 21, 22, 37).

b) Fluorescent antibody test for viral antigens

The fluorescent antibody test is a rapid, sensitive and specific means of identifying TGE viral antigens in cryostat sections of intestine. A freshly dead pig is required, and the ideal animal should be under 4 weeks of age (preferably less than 1 week) and just starting to show clinical signs of the disease (that is, within 24–28 hours of infection). Within 30 minutes of death, 2 cm lengths from four different regions of the posterior part of the small intestine should be removed. Lengths of 5–10 mm are cut from these for snap freezing with solid CO₂. Correct orientation of the material is important to ensure that subsequent cutting by cryostat yields true transverse sections. Sections are cut 6 µm thick, mounted on cover-slips, air-dried and fixed in acetone. An alternative and faster procedure is to excise and longitudinally cut open a piece of the distal small intestine, gently wash the mucosal surface with PBS and prepare impression smears of the luminal intestinal surface on ethanol-cleaned microscope slides followed by air drying and acetone fixation (5). The slides are then processed and stained like the cryostat sections as follows. Fixed positive and negative control sections or smears are stored at −20°C for staining in parallel. After washing with Tris buffer, pH 8.7, or PBS, the sections are stained with a diluted solution of fluorescein isothiocyanate (FITC)-conjugated TGEV antibody, and placed in a humid incubator at 37°C for 30 minutes. Any unbound stain is removed by washing in Tris buffer. If desired, the sections are counterstained with a 10⁻⁵ dilution of Evans blue in Tris buffer and mounted in glycerol.

Stained sections or smears should be examined by ultraviolet light microscopy as soon as possible. The quality of the staining is assessed by reference to the controls. An accurate interpretation depends on the preservation of the villous architecture, the epithelial cells of which are examined for intracytoplasmic fluorescence.

A peroxidase–antiperoxidase IHC method for the demonstration of TGEV has been developed for detection of TGEV and PRCV in both frozen and formalin-fixed, paraffin-embedded tissues (19, 53). The IHC applied to formalin-fixed tissues is advantageous because it can be done prospectively or retrospectively on the same formalin-fixed tissues used for histopathology and the fixed tissues or slides can be more readily shipped as they are stable and they do not contain live virus (53).

c) Enzyme-linked immunosorbent assay detection of faecal virus antigens

A double antibody-sandwich system may be used, for instance with a capture MAb and a polyclonal enzyme-linked detector antibody (25, 48). This test is based on capture of the viral antigen from the faecal sample by three MAb, two specific for the S protein (site A and D) and one for the nucleoprotein N (25, 48). A negative coating is used as control for the specificity of the test, consisting of antibodies purified from the ascitic fluid of mice inoculated with SP2/0 myeloma cells that do not recognise TGEV. MAbs are applied to 96-well microplates in a bicarbonate buffer, pH 9.6, and incubated overnight at 37°C. All samples are tested in duplicate wells, one containing positive coating (TGEV MAbs) and one containing the negative coating. Faecal samples are diluted in cell culture medium (1/10), vortexed and centrifuged at low speed (2000 g) for 15 minutes. Then the supernatant is decanted into sterile tubes and tested or stored frozen. Plates are washed twice with washing buffer (PBS containing 0.05% Tween 20) before adding the prepared faecal samples. The plates are incubated overnight at 37°C. After washing four times, a biotinylated polyclonal anti-TGEV serum is added in PBS buffer containing 0.05% Tween 20. The plates are incubated at 37°C for 1 hour. The plates are washed four times before adding a horseradish peroxidase-labelled streptavidin conjugate and incubated at 37°C for 1 hour. The plates are washed six times before adding the enzyme substrate, which is ABTS (2,2’-azino-di-[3-ethyl-benzthiazoline]-6-sulphonic acid) with 0.03% H₂O₂ in 0.1 M citrate buffer, pH 4.2. The reaction is stopped after 30 minutes at room temperature by the addition of 5% sodium dodecyl sulphate and the absorbance determined in an ELISA reader at 405 nm. TGEV negative and positive faecal samples are included on each plate.

d) Nucleic acid recognition methods

In-situ hybridisation (ISH) and RT-PCR methods have been described for the direct detection of TGEV in clinical samples, with differentiation from PRCV (21, 37, 55). A second round of nested PCR may significantly enhance the sensitivity (9, 21, 22, 36). Differentiation between TGE viruses may be achieved by analysing PCR products with restriction endonuclease enzymes (64) or by sequencing (9, 22, 28, 38, 66). Duplex RT-PCR for the combined detection of TGEV and porcine epidemic diarrhoea virus has been described (23).

2. Serological tests

Serology may be diagnostic if a rising titre of antibody can be demonstrated. In addition, a single seropositive result has diagnostic value if collected from a population previously known to be seronegative. As the possibility of acquiring carrier virus status among pigs can be reduced by accepting only seronegative animals, serological testing is also a common precondition for importation.
Following infection with TGEV or PRCV, viral antibodies can be detected in serum from 6 or 7 days post-infection, and such antibodies persist at least for many months. Although PRCV and TGEV antibodies show complete neutralisation of either virus, there are differences in the specificities of some of the non-neutralising antibodies (7, 14, 15, 46, 54), as PRCV lacks certain epitopes present on the TGEV. However, virus neutralisation (VN) is not a practical method to differentiate PRCV from TGEV infection. MAbs to such regions can be incorporated into competitive ELISAs to detect serum antibody that is entirely TGEV specific. While such tests are reliable in that they do not produce false-positive results with PRCV antisera, false negatives may occur because of a reduced sensitivity compared with neutralisation tests, and because of strain variation among TGE viruses, such that a single TGEV-specific MAb may not recognise all strains (6, 54). The problem of insensitivity can be reduced by using the tests on a group or herd basis. These MAb-based ELISAs are the method of choice for differentiating PRCV from TGEV to qualify animals for export.

In addition, using such tests for differential diagnosis less than 3 weeks after exposure to PRCV produced inconsistent and unreliable results (50). More accurate results were also achieved by testing paired serum samples (acute and convalescent) in the assays and by using the recombinant spike (S) protein of TGEV as the coating antigen in place of TGEV-infected, fixed swine testicular cells (50).

a) Transmissible gastroenteritis virus/porcine respiratory coronavirus tests

These tests detect antibody to both TGEV and PRCV, and include VN tests, indirect ELISAs (16, 18, 26, 29, 42) and competitive ELISAs based on TGEV/PRCV group-specific MAbs (35).

VN tests can be performed with a variety of cell types and viral strains. Commonly used cell lines include swine testes (27) or primary or continuous porcine kidney cells. Such tests have been very widely used for many years and are commonly regarded as standards against which to assess new assays. A plaque reduction VN assay using swine testes cell monolayers in 6-well plastic plates and the attenuated Purdue strain of TGEV is commonly used (5). A modification of the method of Witte (63) described below, uses flat-bottomed tissue-culture grade microtitre plates, a cell line of A72 cells derived from a dog rectal tumour, and a field strain of virus adapted to grow in such cells: 100 TCID<sub>50</sub> (50% tissue culture infective dose) of virus is incubated with heat-inactivated test sera, and neutralisation is indicated by absence of CPE after further incubation with A72 cells in Leibovitz 15 medium (Sigma, United Kingdom) with added antibiotics, 10% fetal calf serum and 1% L-glutamine. The total volume of reagents in all wells should be 150 µl.

- Virus neutralisation: test procedure
  i) Sera are inactivated for 30 minutes in a water bath at 56°C.
  ii) Doubling dilutions of test sera are made in cell culture medium beginning with undiluted serum (this gives a neutralisation stage dilution of 1/2 when mixed with an equal volume of virus). The dilutions are prepared in a 96-well flat-bottomed cell-culture grade microtitre plate using, optimally, three wells per dilution and 25 µl volumes per well. Positive and negative control sera are also included in the test. No standard sera are available, but internal positive standards should be prepared and titrated in an appropriate range.
  iii) 25 µl TGEV stock is added to each well at a dilution in culture medium calculated to provide 100 TCID<sub>50</sub> per well. Virus should be added to two out of the three wells containing serum at each dilution. The third well serves as a serum-only control and should receive 25 µl of culture medium instead of virus.
  iv) The residual virus is back titrated in four tenfold steps using 25 µl per well and at least four wells per dilution; 25 µl of culture medium is added to each of the back-titration wells to compensate for the absence of a test serum.
  v) The plates are agitated briefly and then incubated for 1 hour in a 5% CO<sub>2</sub> atmosphere at 37°C.
  vi) 100 µl of, for example, A72 cell suspension at 2 × 10<sup>5</sup> cells per ml is added to each well.
  vii) The plates are incubated for 3–7 days in a 5% CO<sub>2</sub> atmosphere at 37°C; the test can be performed successfully, if the plates are incubated without CO<sub>2</sub>.
  viii) The plates are read microscopically for CPE. The test is validated by checking the back titration of virus (which should give a value of 100 TCID<sub>50</sub> with a permissible range of 50–200 TCID<sub>50</sub>) and the control sera. The standard positive serum should give a value within 0.3 log<sub>10</sub> units either side of its predetermined mean. Readings of each test serum dilution should be made with reference to the appropriate serum-only control to distinguish viral CPE from serum-induced cytotoxicity or contamination.
  ix) The test serum results are determined by the Spearman–Kärber method as the dilution of serum that neutralised the virus in 50% of wells.
  x) A negative serum should give no neutralisation at the lowest dilution tested (i.e. undiluted serum, equivalent to a dilution of 1/2 at the neutralisation stage).
b) Transmissible gastroenteritis virus-specific tests to differentiate TGEV from PRCV-infected pigs

TGEV-specific tests are blocking or competition ELISAs that use an MAb that recognises TGEV but not PRCV (6, 8, 50, 54, 60) and are the tests of choice for qualifying animals for export. Test sera from pigs previously infected with a strain of TGEV recognised by the MAb will contain antibodies of the same specificity that can compete with it for binding to TGEV antigen-coated ELISA plates. Pigs infected with PRCV that does not contain the TGEV unique epitope will not produce antibodies to this epitope; hence, PRCV antibodies will not compete with or block binding of the TGEV-specific MAb (6, 8, 50, 54, 60). ELISA antigens may be prepared from cell lysates of kidney cell lines that were either inoculated with tissue-culture-adapted strains of TGEV, or uninfected. Alternatively TGEV-infected or uninfected swine testes cells fixed in 80% acetone have been used as an antigen source, or antigens may be prepared from recombinant S (rec-S) protein harvested in soluble form from an insect (Sf9) cell line infected with a recombinant baculovirus expressing a TGEV S protein containing the four major antigenic sites (50, 54). Positive and negative antigens are coated to alternate rows of microtitre plates using bicarbonate buffer, pH 9.6. Diluted test sera, including known TGEV positive and known TGEV/PRCV negative controls, as well as known PRCV positive (negative in this test, positive in VN test) are added to appropriate wells and incubated overnight before further addition of diluted MAb to all wells. Bound MAb is detected by a peroxidase-conjugated anti-mouse antibody that induces a colour reaction in the presence of an appropriate substrate. The colour changes are measured using spectrophotometer, and for each test sample the net result is the difference in absorbance between the positive and negative antigen wells, expressed as a percentage of the result obtained with the negative control serum. The negative–positive cut-off value for the test must be determined by previous testing of known negative and positive populations. There are several commercial kits available that are TGEV specific.

Haemagglutination-based tests described to date (24, 30, 51) were validated before the appearance of PRCV. However, they may be TGEV specific as TGEV, but not PRCV, is haemagglutinating (47).

C. REQUIREMENTS FOR VACCINES AND DIAGNOSTIC BIOLOGICALS

Vaccination against TGE is carried out in several countries.

Guidelines for the production of veterinary vaccines are given in Chapter 1.1.8 Principles of veterinary vaccine production. The guidelines given here and in Chapter 1.1.8 are intended to be general in nature and may be supplemented by national and regional requirements.

Information on experimental work or field trials of TGEV vaccines licensed for use in the United States of America (USA) has been reviewed, including possible limitations in their field efficacy and concepts related to the design of optimal TGEV vaccines (43, 45, 46). Several manufacturers are licensed to produce TGEV vaccines in the USA: the vaccines include modified live and inactivated vaccines. The modified live vaccines are used for oral administration to pregnant sows (to induce passive immunity) or have also been licensed for oral administration to nursing or weaned pigs (to induce active immunity). Inactivated TGEV vaccines are licensed for parenteral inoculation of pregnant sows by the intramuscular route or for intraperitoneal administration to nursing or weaned pigs. In general, these vaccines induced marginal passive protection against TGEV challenge of nursing piglets when evaluated under controlled experimental conditions or in the field in TGEV/PRCV herds. Although they fail to adequately protect against epizootic TGE, data suggest that these vaccines may provide some efficacy against enzootic TGEV by stimulating an anamnestic antibody response to TGEV in serum and milk (45, 46).

The main reason proposed for TGEV vaccine failures was their inability to stimulate high levels of secretory IgA (SIgA) antibodies in milk analogous to the SIgA antibody responses found in the milk of sows naturally infected with TGEV (45, 46). Furthermore, these vaccines did not adequately protect the seronegative sow against TGE, such that illness in the sow often resulted in anorexia, agalactia and failure to passively protect her piglets. Thus the modified live vaccines may fail to replicate to the extent required to induce protective immunity in the intestine, or if given to seronegative neonatal animals, concerns exist regarding their possible reversion to virulence. Killed vaccines given parenterally do not induce SIgA antibodies; cell-mediated immune responses are often poor and the duration of immunity may be short-lived. Although use of PRCV strains as vaccine candidates for TGE has been proposed, experimental studies regarding their efficacy against TGEV have shown a lack of efficacy (34) or only partial cross-protection (3, 11, 59). However, the widespread prevalence of PRCV infections in the swine population in Europe appears to have dramatically reduced the incidence of epizootic TGE in Europe (39). Newer recombinant DNA strategies for the development of TGEV vaccines include the possible use of an S protein subunit vaccine (contingent upon the development of mucosal delivery systems and adjuvants) (33, 49, 52) or the use of live recombinant viral or bacterial vectors that express TGEV genes important for the induction of immunity (13, 43, 46, 56, 57, 65).

There are a number of general requirements (e.g. produced in a licensed facility, label rules, tracking capability, etc.) that apply to all biological products including vaccines. A set of regulations exist (called standard requirements, or SRs) that describe testing to be done on the vaccine and parent materials. Detailed information
on SRs for vaccines in the USA are contained in the Code of Federal Regulations (CFR) Title 9, Volume 1, Part 113 (abbreviated below as 9 CFR, 113) (58). The general European Pharmacopoeia monograph and EMEA (European Medicines Agency) guidelines are applicable to TGEV vaccines, even though no vaccines are currently used in the European Union.

1. Seed management

a) Characteristics of the seed

The seed virus must be tested for purity and identity. The purity includes freedom from bacteria and fungi (9 CFR 113:27), mycoplasmas (9 CFR 113:28), and extraneous viruses (9 CFR 113:55) (58). The demonstration of identity is usually accomplished by VN or FAT. Genetically engineered vaccines or naturally selected vaccines with claims of antigen-coding gene deletion/inactivation are required to provide evidence (genotypic and/or phenotypic) of that identity.

b) Method of culture

Culture must be carried out on proven uncontaminated (approved) cells, and the number of cell culture passages is limited (usually to five). It is not required that the species of origin of the cell line be that of the target species.

c) Validation as a vaccine

Vaccine validation takes two forms. The master seed is considered to be immunogenic if a vaccine made at the highest passage, and according to the outline of production, is shown to be protective. The lowest antigenic level (modified live virus titre or inactivated antigen mass) shown to be protective becomes the baseline for all future serials (lots) of the product. In the case of live products, factors for titration variation and the death curve over time would be added. These trial vaccines should be tested for purity, safety, and efficacy by the manufacturer. Protection must be shown against the natural disease with the virulent challenge virus. Virulent challenge virus is defined as the dose that causes disease in $\geq 95\%$ of the susceptible controls. Three prelicense serials must subsequently be made and tested by the manufacturer and by the licensing authority, for potency, sterility and safety.

2. Method of manufacture

This is proprietary information for each manufacturer and hence not available.

3. In-process control

This is largely proprietary. Some in-process controls refer directly to production (e.g. $O_2$ concentration in the fermenter). Another category, however, includes tests similar to the final container potency test. For all vaccines, the simpler the final batch or container potency test, the more likely it is that it may be used as a monitoring/blending test: for example, virus titration on sub-batches may be used to predict final blended batch titre. Ingredients of animal origin must be sterilised or shown to be free from contamination.

4. Batch control

Batches must be blended to the final specifications and bottling specifications (e.g. fermentation runs may be pooled, or one run may be split and pooled with each of three others, etc.). In some countries, bulk and process control define the product and are the subject of intense regulation and scrutiny. The emphasis in the USA is on the final product. Batch control techniques must be detailed in the outline of production and must be meaningful, trackable, and the manufacturer must discard product that fails to meet specifications. If a batch is to be exported to another country for bottling or blending, then it is subjected to all the testing as though it were final product.

a) Sterility

All products must be tested for sterility. The manufacturer may also run sterility tests on batches for monitoring. Tests are similar to those described in Section C.1.a.

b) Safety

Safety tests are done before the licence is granted, and then on the final container (Sections C.5.a and C.5.b).
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c) Potency

Potency would normally only be done if the potency test were a simple test (e.g. ELISA) to confirm the blending calculations before bottling.

d) Duration of immunity

Duration of immunity is tested in the prelicensure (efficacy) serial test, not the batch control. New products are required to support label claims for revaccination schedules with efficacy trials (challenge) at the specified time after vaccination.

e) Stability

Stability is established before the licence is granted. Usually accelerated ageing (37°C) is used to estimate the lifetime so that the products do not have to be kept at storage temperature (4°C) for the real-time period. This will be confirmed with real-time data later. The manufacturer is not required to do stability testing. Manufacturers are required to state the amount of antigenic material that will be in their product throughout the shelf life. Samples of product are selected (usually live) and tested within 30 days of expiration to see if, for example, the titre is at the level stated in the manufacturer’s outline. Stability is also affected by moisture. Moisture left in a desiccated product can shorten its life, so this has to be tested in the final product or in-process.

f) Preservatives

There are restrictions on the maximum amounts of antibiotics that can be in a product. Restrictions on some vaccine components are related to their safety and to whether the stated withdrawal period is long enough for the component to have cleared before the animal is slaughtered. Preservatives used are proprietary.

g) Precautions (hazards)

Any risks to vaccinates need to be clearly stated on the label. This usually applies to pregnancy warnings for abortogenic live viruses, and the general anaphylaxis warning, but may also attempt to warn the user about soreness or swelling at the injection site, or transient fever or inappetence in some cases. No unusual label precautions apply to the TGE vaccines currently licensed.

5. Tests on the final product

a) Safety

Usually this will be a mouse and/or a guinea-pig or swine safety test (9 CFR 113:33, and ref. 63). Sterility tests are also carried out on the final product.

b) Potency

There is no single test for release potency. Whatever test is used must be correlated to protection in the host animal (the efficacy tests). The potency of live TGEV vaccines can be evaluated by in-vitro titration of the viral infectious dose in cell culture (43). This titre must be correlated with the minimum viral titre required to induce protective immunity against experimental challenge, and also against natural challenge under field conditions. The potency of killed vaccines is evaluated by vaccination and challenge tests using different doses of the vaccine. Titres of neutralising antibodies induced by inoculation of laboratory animals with the vaccine may be accepted if there is an established correlation with development of protective immunity.

Particular viral antigens associated with the induction of neutralising antibodies and protection against challenge can be quantified in killed vaccines using specific MAbs in ELISA, such as neutralising MAbs to the S protein of TGEV (43).

REFERENCES


Chapter 2.8.11. — Transmissible gastroenteritis


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**NB:** There is an OIE Reference Laboratory for Transmissible gastroenteritis (see Table in Part 3 of this *Terrestrial Manual* or consult the OIE Web site for the most up-to-date list: www.oie.int).
SECTION 2.9.

OTHER DISEASES

CHAPTER 2.9.1.

BUNYAVIRAL DISEASES OF ANIMALS
(excluding Rift Valley fever)*

SUMMARY

A part of the large complex group of arboviruses is the Bunyaviridae family with over 300 members distributed over five genera. Two of the genera of veterinary importance in the family are Nairovirus, which contains the ruminant pathogen Nairobi sheep disease (NSD) virus, and the largest genus, Orthobunyavirus, which is subdivided into 48 serogroups. This genus contains only a few viruses that are significant pathogens of animals, among them Cache Valley virus (CVV) and Akabane virus. Although they have been placed in different antigenic groups, these two viruses have a tropism for fetal tissues and are responsible for embryonic and fetal losses and multiple congenital deformities in domestic ruminants. Members of the Nairovirus and Orthobunyavirus genera are single-stranded, enveloped spherical or pleomorphic RNA viruses, 80–110 nm in diameter. A third member of the Bunyaviridae family that is of veterinary importance is Rift Valley fever, a member of the Phlebovirus genus, and is discussed in Chapter 2.1.14.

Identification of the agent:

Cache Valley virus (CVV), a member of the Bunyamwera virus serogroup of the Orthobunyaviridae genus, can be isolated from the blood of febrile or viraemic adult animals. Attempts at isolation from the fetus at birth are generally unsuccessful due to virus clearance by the fetal immune response. Cell lines derived from monkey or baby hamster kidney are employed for isolation of the virus or alternatively, intracerebral inoculation of infant mice may be used. Virus or antigen is identified by fluorescence (FAT), immunohistochemistry (IHC) or neutralisation tests. Group- and virus-specific polymerase chain reaction (PCR) techniques have been developed for the Orthobunyaviruses.

Akabane virus can be isolated from the blood of viraemic animals and occasionally from fetal material. Cell lines of monkey, baby hamster and mosquito are used. The virus produces deformities in the developing chicken embryo. Yolk sac inoculation, as well as intracerebral inoculation of suckling mice, is used. Virus or antigen is identified by FAT, IHC or neutralisation tests. Nested and multiplex real-time reverse-transcriptase PCR techniques have been developed for Akabane and related viruses.

Nairobi sheep disease (NSD) virus is best isolated from plasma from febrile animals, mesenteric lymph nodes or spleen. Laboratory-reared sheep, 2–4-day-old unweaned mice inoculated intracerebrally, or cell cultures may be used for primary isolation. Sheep are the most sensitive animals for this, whereas a baby hamster kidney cell line and lamb or hamster kidney cell cultures are the most sensitive cells. Subinoculation of plasma from an experimentally infected sheep into cell cultures or mice is also recommended. Identification of the virus may be made by direct immunofluorescence of inoculated tissue cultures or of mouse brain smears. The agar gel

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1 The diseases in this section that are marked with an asterisk are included in some individual species sections of the OIE List, but these Terrestrial Manual chapters cover several species and thus give a broader description.
immunodiffusion test can also be used to demonstrate the presence of NSD antigen in tissues but will cross react with other viruses of the Nairovirus genus. Infected tissue cultures or mouse brain suspensions may be used as sources of complement-fixing or enzyme-linked immunosorbent assay (ELISA) antigens.

**Serological tests:** For CVV and Akabane virus, haemagglutination inhibition, complement fixation (CF) and serum neutralisation tests are used to detect antibodies. An ELISA, based on one used for Rift Valley fever, has also been described. A competitive ELISA specific for Akabane has been published. For NSD the most suitable test is the indirect fluorescent antibody test. CF and indirect haemagglutination tests have also been used to confirm NSD outbreaks in the field. Virus neutralisation tests give equivocal results, a feature that also occurs with other members of the Nairovirus group. ELISAs are now being evaluated for NSD. Infected spleen may be used as a source of antigen in immunodiffusion tests.

**Requirements for vaccines and diagnostic biologicals:** No vaccine is currently available for CVV. Vaccines for domestic use against Akabane virus have been produced in both Japan and Australia. For NSD, an experimental attenuated live virus vaccine has been investigated, and a killed tissue culture vaccine has been shown to be immunogenic.

### A. INTRODUCTION

**Cache Valley virus**

Cache Valley virus (CVV) is a teratogenic *Orthobunyavirus* of North America affecting mainly sheep. A recent survey of cattle indicated that up to 28% were positive for specific antibodies to CVV (35). It is a member of the Bunyamwera serogroup of the *Orthobunyavirus* genus, family Bunyaviridae (19) and is also the most common of the *Orthobunyaviruses* of North America (4). CVV was first isolated from a mosquito pool in Utah, United States of America (USA) in 1956 (20), but was only linked to disease during an epizootic of neonatal loss and malformed lambs in a sheep flock in Texas in 1987 (9). The virus has also been isolated from a horse and a clinically healthy cow.

Serological surveys have shown a widespread prevalence of antibodies in domestic and wild ruminants and horses. Seroprevalence to CVV is high in deer, and the 1–3 day viraemia is sufficient to infect vectors allowing deer to act as amplifying hosts (2). Vectors include both *Culicoides* midges and mosquitoes of the *Aedes*, *Anopheles*, *Coquillettidia* and *Culiseta* groups.

CVV infection of adult animals is largely subclinical, and experimentally infected ewes show only a transient febrile response, but with a detectable viraemia. Human disease has been reported on two occasions (5, 36).

CVV was the first North American *Orthobunyavirus* to be linked to fetal arthrogryposis and hydrancephaly, however other related viruses have been shown experimentally to have the same potential. Fetal infection with CVV is age dependent in its outcome. Malformations take place between 27 and 45 days gestation, with infection at 28–36 days giving rise to central nervous system (CNS) and musculoskeletal defects, and infection at 37–42 days giving rise to musculoskeletal deformities only. Infection after 50 days gestation does not result in lesions and after 76 days the fetus is immunocompetent and antibodies are produced. Most CVV fetal deaths occur between 27 and 35 days gestation. The fetus is, however, susceptible at any age demonstrating the tropism of many *Orthobunyaviruses* for fetal tissues (6).

Gross pathology of the musculoskeletal system includes arthrogryposis of one or more limbs, torticollis, scoliosis of the vertebral column and muscular hypoplasia. CNS lesions include hydrancephaly, hydrocephalus, porencephaly, microencephaly, cerebral and cerebellar hypoplasia and micromelia (6, 18). Dead embryos and stillborn or mummified lambs with no obvious defects are also found. Anasarca is seen, as is oligohydramnion. This reduction in amniotic fluid is thought to contribute to restriction of fetal movement and thus to the skeletal deformities seen. Limb defects are also due to neurodegenerative changes seen histopathologically as areas of necrosis and loss of paraventricular neuripil in the brain together with a reduction in the number of motor neurons. Skeletal muscle changes involve poorly developed myotubular myocytes (18).

**Akabane virus**

Akabane virus is a teratogenic *Orthobunyavirus* widely distributed across the world but not in New World countries. It affects mainly cattle. It is a member of the Akabane serogroup, *Orthobunyavirus* genus, family Bunyaviridae (24). Other *Orthobunyaviruses* that are potential pathogens are Aino (Shuni virus serogroup), Peaton (Shamonda virus serogroup), Douglas (Sathuperi virus serogroup) and Tinaroo (Akabane virus
serogroup). Akabane virus is however, the best studied and most pathogenic of the Orthobunyaviruses and a major cause of arthrogryposis and hydranencephaly.

Akarabane virus was first isolated in Japan in 1959, initially from a mosquito pool and then a pool of Culicoides mites. This was followed in 1972 by isolations from Culicoides in Australia and mosquito pool isolations in Africa. Akabane virus antibodies have been demonstrated in sera from cattle, sheep, goats, horses, buffalo and camels. Many indigenous game species in Africa south of the Sahara have Akabane virus neutralising antibodies. The range of Akabane virus includes the Middle East, Asia, Cyprus and Africa, but it is in Australia and Japan where regular epizootics of Akabane virus disease occur. Conditions favourable to such outbreaks are susceptible animals in early pregnancy and a sudden increase in vector populations, particularly when the virus has been absent from the area for a number of years.

Akarabane virus infection in adult animals is usually subclinical, but encephalomyelitis has been recently associated with Akabane virus infection in adult cattle (28). Cattle seroconvert after a 3–4-day viraemia.

In endemic areas, antibody in the female animal prevents fetal infection, but Akabane virus is capable of establishing a long-term infection of the placenta in susceptible cattle and sheep. This takes place between 30 and 70 days gestation in the ewe and between 30 and 150 days gestation in the cow. Akabane virus has a predilection for brain, spinal cord and muscle cells where non-inflammatory necrosis interferes with morphogenesis.

Akarabane virus infection has been studied experimentally in sheep and goats with the production of arthrogryposis/hydroencephaly, kyphosis, scoliosis, micro- and porencephaly, stillbirths and abortions (34). Natural infection of the ovine fetus has been described in Australia where perinatal lamb mortality and congenital microencephaly were most often seen.

Experimental Akabane virus studies have been carried out in pregnant cattle and it was shown that the type of abnormality is dependent on the gestational age of the fetus with hydroencephaly seen at 76–104 days and arthrogryposis at 103–174 days gestation (25). This time differential in appearance of abnormalities is clearly seen in bovine fetuses, whereas in sheep with a shorter gestation period, brain and skeletal lesions appear concurrently in the same fetus. The sequence of events during an epizootic of Akabane virus-induced fetal loss are the birth of uncoordinated calves, followed by those with arthrogryposis and dysplastic muscle changes, and lastly those with hydrocephalus and other severe CNS lesions. These events may be preceded by stillbirths and abortions (37). Akabane virus is responsible for severe neural and muscular abnormalities and lesions are characterised by a nonpurulent encephalomyelitis, focal cerebral degenerative encephalomyelopathy porencephaly, microencephaly, hydrocephalus, loss of ventral horn motor neurons and axons, depletion of myelin in spinal cord motor tracts, necrosis and polymyositis in the myotubules with parenchymal degeneration of skeletal muscles. Spinal cord abnormalities include scoliosis, and kyphosis and arthrogryposis may affect almost any skeletal joint.

- Nairobi sheep disease

Nairobi sheep disease (NSD) is a disease of sheep and goats caused by a Nairovirus of the family Bunyaviridae (11). It is characterised by a mortality rate, which may range between 40% and 90%, and should always be suspected when animals have recently been moved from an area free from the disease into one where it is endemic. Outbreaks also follow incursions of ticks into previously free areas, particularly following heavy rains (12). The clinical signs are similar in both sheep and goats, although there are differences in susceptibility among the various breeds and strains in their response to infection with NSD virus, some being more susceptible than others. Cattle and game are refractory to infection with NSD virus (43). The incubation period for the disease varies from 2 to 5 days, when a temperature reaction of 41–42°C develops. There is hyperventilation accompanied by severe depression, anorexia and a disinclination to move. Animals stand with lowered head, and show a conjunctivitis and soro-sanguinous nasal discharge. Some of the superficial lymph nodes, such as the prescapular and/or precrural, become palpable. Diarrhoea usually develops within 36–56 hours of the onset of the febrile reaction. This is at first profuse, watery and fetid, later haemorrhagic and mucoid, and accompanied by colicky pains and tenesmus. Abortion is a common sequel to the infection. Examination of the predilection sites for the attachment of ticks, such as the ears, head and body, is likely to reveal the presence of the Ixodid tick Rhipicephalus appendiculatus.

Deaths can occur in peracute cases within 12 hours of the onset of the fever and at any time during the febrile reaction, while the animal is acutely ill. Further deaths then follow the fall in temperature for a further 3–7 days, associated with severe diarrhoea and dehydration.

The gross pathology of NSD can be misleading, for most deaths are likely to occur during the period of viraemia, when the only signs are likely to be lymphadenitis with petechial and ecchymotic haemorrhages on the serous surfaces of the alimentary tract, spleen, heart and other organs. None of these signs allows a specific diagnosis of NSD to be made or even suspected, for they are shared with many other febrile diseases of sheep in NSD-endemic areas. Diseases with which NSD may be confused include Rift Valley fever, peste des petits ruminants,
rinderpest, salmonellosis and heartwater. Later in the course of the disease, a haemorrhagic gastroenteritis becomes more obvious, with haemorrhages on the mucosa of the abomasum, especially along the folds, in the region of the ileo-caecal valve, and most commonly in the colon and rectum. Zebra striping of the latter is often seen. The gall bladder is usually enlarged and haemorrhagic. Inflammatory lesions with haemorrhage may be seen in the female genital tract, if there has been abortion. However, in many animals dying from NSD, there may be none of these gastroenteric lesions, and a tentative diagnosis based on post-mortem signs can rarely be made. Common histopathological lesions are myocardial degeneration, nephritis and necrosis of the gall bladder.

The post-mortem signs following the early stage of NSD death are the nonspecific changes of congestion and petechial and ecchymotic haemorrhages on serous surfaces, on lymph nodes, the spleen and other organs such as the kidney, lungs and liver. Following death at later stages, haemorrhagic gastroenteritis becomes apparent, with ulceration of the abomasum, duodenum, caecum and colon. The virus is principally transmitted by the tick *Rhipicephalus appendiculatus*, and any infestation with such parasites should arouse suspicion of the presence of the disease. NSD virus may also be transmitted by other species of the genus *Rhipicephalus* and by the bont tick *Amblyomma variegatum*.

NSD is an apparently rare zoonotic agent in the field, causing mild influenza-like disease in humans. Laboratory infection has been associated with fever and joint pains (43).

**B. DIAGNOSTIC TECHNIQUES**

1. **Identification of the agent**

   - **Cache Valley virus**

      At birth CVV cannot be isolated from the fetus but has been isolated from mosquito pools and the blood of viraemic adult animals. This has been done on tissue culture using hamster and monkey kidney cell lines including baby hamster kidney (BHK), African green monkey kidney (Vero) and LLC-MK2. Virus can be isolated from a febrile animal using a 10% buffy coat suspension in minimal essential medium (MEM) and co-cultivation with Vero cells in MEM supplemented with 2% fetal bovine serum.

      Virus isolation is also commonly done in newborn or weaned mice by intracerebral or intraperitoneal inoculation.

      Many *Orthobunyaviruses* have been sequenced as they are medically important pathogens associated with encephalitis in humans in both North and South America. Polymerase chain reaction (PCR) technology has been applied to mosquito-pool surveillance, instead of traditional isolation in infant mice, and sensitivity is reported to be one positive mosquito in a pool of 100, which is undetectable by traditional plaque titration in cell culture (21).

      Group-specific and virus-specific primers have been designed, and using the reverse-transcription PCR (RT-PCR) the Bunyamwera (BUN) and California (CAL) serogroup viruses can be distinguished. Using a nested RT-PCR technique the CAL and most of the BUN serogroup viruses can be distinguished from other *Orthobunyavirus* genus members (26, 31).

   - **Akabane virus**

      Diagnosis of infection is rarely made by virus isolation, but rather by histopathology and serology. Virus has however been isolated from viraemic sentinel animals using plasma or buffy coat suspensions from vector pools and occasionally from fetal material. RT-PCR has been described for the detection of Akabane virus and differentiation from Aino. Use of this assay could contribute to diagnosis, but the diversity of the *Orthobunyavirus* genus will require validation to confirm specificity of the test as there is evidence of reassortment.

      Suckling mice, 1–2 days old, may be used and inoculated intracerebrally with 0.01 ml of a clarified 10% suspension of the test material. A sensitive system for recovery of this serogroup of viruses is intravenous inoculation of embryonating chick eggs (ECE), followed by passage of harvested material in insect cells (C6/36) then mammalian cell lines (22, 42). Virus isolation in tissue culture is frequently done using Vero, BHK-21 and HmLu-1 cell lines. If C6/36 mosquito cells are used, cultures are left stationary for 7 days and material is repassaged on to a hamster or Vero cell line where cytopathic changes in the cultures become visible.

      Methods employed for specific identification of Akabane virus using monospecific antibodies have included virus neutralisation, and immunofluorescence (3, 22). Antigen detection in formalin-fixed material by peroxidase staining of bovine and ovine fetal material also in naturally infected newborn calves (33). Nucleic acid detection methods have also been developed for the differentiation of Aino and Akabane viruses using a nested RT-PCR technique followed by restriction enzyme digests to differentiate Akabane and Tinaroo viruses in the same
serogroup and Aino and Peaton viruses in the Shuni and Shamonda serogroups (1). A multiplex real time RT-PCR has also been described using Taq Man probes which is said to reliably identify akabane and Aino viruses accurately (38).

- **Nairobi sheep disease virus**

NSD virus may be isolated from material collected from field cases by the use of laboratory animals or cell cultures (16). Safety precautions against aerosol infections should be taken when working with this agent. Uncoagulated blood, mesenteric lymph nodes and spleen tissue submitted with frozen gel packs are the optimal samples to be collected from febrile or dead animals. The plasma can be used directly as inoculum, and the lymph nodes or spleen should be homogenised to make an approximate 10% (w/v) suspension in a transport medium. This medium can be Hanks’ medium with 0.5% lactalbumin hydrolysate or 0.75% bovine serum albumin, and containing penicillin (500 International Units/ml), streptomycin sulphate (500 µg/ml), and mycostatin (50 units/ml) or fungizone (2.5 µg/ml).

A recommended initial procedure is to inoculate an NSD-susceptible sheep held in isolation with 1–2 ml of the tissue suspensions or plasma. Any pyrexia and clinical disease that develops permits a tentative diagnosis of NSD and, at the same time, furnishes excellent samples for virus isolation. This is especially valuable where the original field samples have been transported in hot climates where some loss of virus has inevitably occurred. Sheep are at least 100 times more sensitive than mice to NSD virus infection.

Infant mice, 2–4 days old, can be inoculated intracerebrally with 0.01 ml of a 1/10 dilution of plasma or of the tissue suspension. Two litters, each of 8–10 suckling mice, should be used for each sample and one blind passage is made routinely. The mice become debilitated and die within 5–9 days post-inoculation. Their brains should be harvested aseptically, pooled and diluted 1/100 for passage into additional mice.

Cell cultures may be used in conjunction with mouse inoculation for the primary isolation of NSD virus, as they have shown levels of sensitivity similar to that of the intracerebral inoculation of unweaned mice. The BHK-21-C13 cell line is especially valuable; the Vero cell line (37) and primary and secondary lamb or hamster kidney cells have also been used. Most strains of NSD virus produce a cytopathic effect (CPE) on first passage in BHK cells; others produce a more obvious CPE only after subinoculation. The appearance of a CPE is not such a regular finding with lamb testis and kidney cells, although it is usually seen on the second passage in lamb kidney cells. Tube cultures should be used both with and without flying cover-slips, or if plastic bottles are used for isolation, micowell slide cultures should also be prepared. Approximately 0.2 ml should be inoculated and a period of 1–2 hours allowed for adsorption. The CPE becomes evident in roller cultures as foci of granular rounded cells after 24–48 hours in BHK cells, and in a further 24–48 hours in other cell types. The CPE is not specific for NSD virus, which is identified in cover-slip cultures by immunofluorescence or by staining with haematoxylin and eosin. The latter method reveals pleomorphic eosinophilic intracytoplasmic inclusions peculiarly of a spindle form; other inclusions are bipolar, or surround the nucleus.

The virus can be specifically identified by immunofluorescence staining, which may be positive as early as 24–48 hours post-inoculation when no CPE has yet become evident. Conjugates for direct immunofluorescence may be prepared from hyperimmune mouse ascitic fluids, and from immune rabbit or sheep antisera by standard methods. Some cross-fluorescence may occur with other Nairoviruses at low dilutions of the conjugate, but these viruses are not normally associated with disease in sheep or goats.

The agar gel immunodiffusion test (AGID) can be a valuable primary diagnostic tool for the detection of NSD antigen in tissues. The test can be carried out in laboratories without tissue culture facilities and at field investigation laboratories. The spleen and mesenteric lymph nodes are the tissues of choice to be used in the test. Aliquots of 0.5–1 g should be homogenised with sterile sand in a pestle or a homogeniser to give 10–20% suspensions in phosphate buffered saline (PBS) or saline. The suspension should be centrifuged for 10–15 minutes at approximately 1000 g and the supernatant fluid is used in the test. This test can also be used for the identification of NSD virus antigen in mouse brain harvested from experimentally infected mice (see above). Rabbit hyperimmune serum against NSD can be prepared by repeated inoculation of NSD-infected mouse brain. A mouse brain suspension at 2–5% (w/v) is prepared as above and centrifuged at 3–5000 g for 15 minutes. Aliquots are then mixed with an equal volume of Eg Titermax adjuvant. Various inoculation regimes may be used but 1-ml volumes may be given subcutaneously and/or intramuscularly at 7-day intervals for 3–5 weeks, or at multiple inoculation sites in 0.1-ml volumes for a similar period. Serum should be collected 5–7 days after the last injection and stored in aliquots at –20°C.

Difco Noble or other suitable agar may be used in the test, using 0.85% sodium chloride at pH 7.2. Slides are prepared to give an agar layer approximately 2 mm in depth. Six wells should be placed hexagonally around a central well. The hyperimmune rabbit serum is placed in the central well and positive control antigen in wells 1 and 4. The tissue under test is placed in wells 2 and 5. Negative control tissue is placed in wells 3 and 6. Wells containing test tissue that give a precipitin line of continuity with the line formed between the positive antigen and the hyperimmune serum are considered to be positive.
Mouse brain suspensions or infective tissue culture fluids can be used as antigens for complement fixation (CF) tests for virus identification. Both have proved satisfactory after partial purification with fluorocarbon; the mouse brain can also be used in the form of a suspension in a borate buffer solution.

An enzyme-linked immunosorbent assay (ELISA) antigen for virus identification purposes can be prepared from an infected tissue culture in a bottle. The cells are removed using a pipette fitted with a rubber bulb when approximately 20% of the monolayer is showing CPE. They are sedimented and washed three times in borate saline buffer, pH 9. The cells are then lysed and solubilised with SDS (sodium dodecyl sulphate) and 1% Triton X100, diluted approximately 1/5 in borate saline buffer and sonicated to provide an antigen for the ELISA. A control negative antigen is prepared in the same manner from uninfected cells. These are adsorbed directly on to ELISA plates and the test is carried out with NSD immune and normal serum with both antigens.

2. Serological tests

These include haemagglutination inhibition (HI), CF and virus neutralisation (VN) tests and ELISA.

- **Cache Valley virus**
  
  a) **Virus neutralisation test**

  VN tests for CVV used to be done by a plaque reduction neutralisation method but are now usually performed using inhibition of CPE on Vero cells in microtitre plates (7).

  - **Test procedure**
    i) Inactivate test sera at 56°C for 30 minutes in a water bath.
    ii) Make serial twofold dilutions of the sera in MEM from 1/2 to 1/16 and incubate at 37°C for 60 minutes with an equal volume of 100 TCID$_{50}$ (tissue culture infective dose) per ml of virus. Standard controls are prepared in a similar manner.
    iii) Discard the medium in a 96-well flat-bottomed cell-culture grade microtitre plate containing a preformed 24-hour Vero monolayer.
    iv) Add the serum/virus mixtures to the plate, 50 µl per well, using three wells per dilution.
    v) Back titrate the virus used in the test, making three tenfold dilutions using 50 µl per well and four wells per dilution.
    vi) Cover the plates and incubate for a further 60 minutes at 37°C.
    vii) Add 50 µl MEM maintenance medium to each well.
    viii) Incubate the plates at 37°C for 6 days in a humidified CO$_2$ incubator.
    ix) Read the plates microscopically, evaluate the CPE and determine the 50% end points.
    x) The virus control should give a value of 100 TCID$_{50}$ and there should be no neutralisation by the negative control serum at the lowest dilution tested. The positive control should give a titre within an expected range of its predetermined mean.

  b) **Enzyme-linked immunosorbent assay**

  An ELISA, modified and based on the one for Rift Valley fever described by Meegan et al. (30), has been used for CVV serological surveys. Modifications include a 1/400 dilution of mouse ascitic fluid to coat the plates, followed by a 1/25 dilution of a sucrose/acetone mouse brain antigen in a sandwich ELISA format. The diluent used is PBS with 0.5% Tween 20, 5% equine serum and 500 µg dextran sulphate per ml. A horseradish peroxidase conjugate detection system and an ABTS (2,2’-azino-di-[3-ethyl-benzthiazoline]-6-sulphonic acid) substrate are used (30).

  c) **Other tests**

  Not all members of the Bunyamwera group produce haemagglutinins, but a HI test has been described for CVV using a sucrose/acetone suckling mouse brain antigen and goose erythrocytes at pH 6.2. The test is said to lack sensitivity compared with a VN test, providing only 50% detection of antibodies. The CF test is little used because of extensive cross-reactivity within the Bunyamwera group.

- **Akabane virus**

  a) **Haemagglutination inhibition test**

  The HI test is modified after Clarke & Casals 1958 (8) and better haemagglutination is achieved with an increased NaCl molarity. The test is also pH dependant. Sera are pretreated with kaolin or acetone and
then heat inactivated at 56°C for 30 minutes. The test is performed using four units of sucrose/acetone-
extracted mouse brain antigen, 0.3% red blood cells and borate buffer, pH 9 (23).

b) Virus neutralisation test

VN tests have been described using HmLu-1 cells in tube cultures or Vero and BHK cells in flat-bottomed
96-well microtitre plates (10, 42). Two techniques have been described with a serum/virus incubation period of 1 hour or incubation overnight before the addition of the cells.

- Test procedure
  i) Inactivate the test sera at 56°C for 30 minutes in a water bath.
  ii) Prepare serial twofold dilutions of the sera in Eagles medium from 1/2 to 1/16 in a 96-well flat-
      bottomed microtitre plate using duplicate wells and 25 µl per well. Standard controls are prepared in a
      similar manner.
  iii) Add 25 µl per well of virus in Eagles medium diluted to provide 200 TCID50 per 50 µl.
  iv) Cover and incubate at room temperature for 1 hour.
  v) Include a back titration of virus in triplicate, making three tenfold dilutions using 25 µl per well.
  vi) Add 100 µl per well Vero cells in Eagles medium with 2% serum at 5 × 10^5 cells/ml.
  vii) Incubate the plates at 34–37°C for 5 days in a humidified CO2 incubator.
  viii) Read the plates microscopically and calculate the titre as the reciprocal of the highest serum dilution
       completely inhibiting the CPE.
  ix) The virus and serum controls should give the expected results.

Where overnight incubation is used, duplicate twofold serial dilutions of inactivated serum are mixed with
100 TCID50 of virus using 100-µl volumes in each case. Following incubation for 1 hour at 37°C and
overnight at 4°C, 50 µl BHK cells is added to the test. The plate is examined at 3 and 5 days incubation at
37°C and checked for CPE.

c) Enzyme-linked immunosorbent assay

Akabane virus ELISAs, using both IgG and IgM, have been described. Coating antigen is 10^6 TCID50 per ml
of virus grown on HmLu-1 cells diluted in a 0.05 M carbonate/bicarbonate buffer, pH 9.6. The wash medium
is PBS containing Tween 20 and alkaline phosphatase. Rabbit anti-bovine IgG and IgM conjugates are
used (40).

A similar ELISA using horseradish peroxidase rabbit anti-bovine IgG conjugate has also been described.

A competitive ELISA with a 98% specificity has also been developed (39).

d) Complement fixation test

The CF test is not described here as it is a group specific test and is rarely used anymore.

- Nairobi sheep disease

a) Indirect fluorescent antibody test

The indirect fluorescent antibody test (FAT) is the most suitable test for use with members of the Nairovirus
serogroup. There are, however, some cross-reactions, particularly with Dugbe virus and also with other
members of the group, such as Congo–Crimean haemorrhagic fever virus (13). The NSD antibody titres by
this method range from 1/640 to 1/10,240, and such titres are not obtained with immune sera to other
members of the group (14).

The method has been used in epidemiological studies and to study the response to experimental vaccines.
There do not appear to be any serological differences among the 40–50 isolates that have been examined.
An NSD I-34 strain² was the virus usually used to prepare antigen, and this has been adapted to grow in
BHK-21-C13 cells, after a series of passages.

² The I-34 strain was a virulent NSD isolate made in Kenya that was used extensively as a reference strain at the Kabete
Laboratory – Kenya Agriculture Research Institute, P.O. Box 58137, Kabete, Nairobi, Kenya.
The virus antigen in the cell substrate of choice may be grown in loose cover-slips, multiwell slides, Teflon-coated slides or microtitre plates for the test. A method using Teflon-coated slides is described.

- **Preparation of antigen slides**
  i) Wash and sterilise Teflon-coated slides. This is done briefly with a hot detergent that is used for tissue culture glassware in the laboratory, then three rinses in tap water for 30 minutes, each followed by similar rinses in distilled/deionised water. Slides are then placed in 70% alcohol for 10 minutes, removed with a sterile forceps and wrapped in greaseproof paper. They will then be found to be sterile, but further sterilisation in a microwave for two cycles of 5 minutes each is recommended.
  ii) Place these slides in sterile dishes using a sterile forceps; a square polystyrene type is better than the round variety.
  iii) Mix a suspension of BHK cells containing approximately 25,000 cells/ml in BHK growth medium (usually Eagles for BHK cells), and add 1000 TCID$_{50}$ of NSD I-34 strain per ml. Mix by pipetting. Prepare some uninfected negative control slides.
  iv) Add the infected cells in 50 µl volumes (for the 12-well size) or as appropriate to the size of the Teflon wells. Replace the cover on the dishes and put into a humidified CO$_2$ incubator or anaerobic container.
  v) Leave overnight for the monolayer to form. Then remove the plates from the incubator to a laminar flow cabinet, and flood with maintenance medium using a pipette to cover the slides to a depth of 2–3 mm. Return to the incubator.
  vi) Harvest the antigen slides just as foci of CPE become detectable. This will be in 36–56 hours (more accurate determination of the optimal harvesting time may be made by fixing and staining one slide after 24, 36 and 48 hours).
  vii) The slides are washed three times in PBS and dried. They are then fixed with dry heat (minimum 80°C) or with ice-cold acetone for 10 minutes. The slides are wrapped and may be stored at 4°C for 2–3 months, or at –20°C for 1–2 years. Slides stored at –20°C must be brought to 4°C overnight before use.

Similar procedures may be followed to prepare antigen on flying cover-slips or multiwell culture slides. When using Nunc tissue culture multiwell plates, however, fixation should be with 75% acetone.

- **Test procedure**
  i) Hydrate the slides by adding a drop of PBS to the wells with a Pasteur pipette. Number the slides according to the number of sera to be tested. Include in the series control positive and negative sera with infected and uninfected cell cultures.
  ii) Discard the PBS and add the serum dilutions 1/80–1/2560 in a predetermined manner to wells 1 to 6. It is preferable to duplicate each dilution on the same side.
  iii) Place the slides in dishes and hold at 37°C in a humid incubator for 40 minutes.
  iv) Wash the slides in racks in three changes of PBS, 5 minutes per wash.
  v) Add the fluorescein-conjugated anti-species conjugate (usually anti-sheep or anti-goat) at a predetermined working dilution; one drop can be added to each well with a Pasteur or other pipette.
  vi) Incubate as before for 30 minutes.
  vii) Wash three times in PBS and dry the slides.
  viii) Examine the slides by fluorescent microscopy. NSD virus antigen is found in the cell cytoplasm, and foci of bundles of fluorescing BHK cells will be seen. The antigen is seen mainly in fine fluorescent particles, but larger irregularly shaped antigen clumps occur, often surrounding the nucleus, or in spindle-like masses filling the cytoplasm to the pole of the cells. These particles will not be seen with negative sera or in the uninoculated control culture.
  ix) Sera that show this fluorescence at dilutions of 1/640 or 1/1280 are indicative of recent infection with NSD (14).

**b) Other tests**

CF tests are complicated by the marked anticomplementary activity of many sheep sera.

Immunodiffusion tests have been used successfully with crude antigens prepared from infected sheep tissue, tissue culture fluids or mouse brain material. Hyperimmune sera can be prepared in sheep, mice or rabbits for use in the test, using infected spleen taken from sheep dying from NSD as the source of antigen for immunisation.
An ELISA using a partially purified tissue culture antigen has been described for antibody testing and is suitable for use in serological surveys. The indirect FAT test should, however, be used to check doubtful results (32).

Monoclonal antibodies to the antigens of NSD virus strain I-34 have been developed and are being evaluated for their application as diagnostic reagents.

RNA probes have also been developed from the S (small) and M (medium) genome segments of Dugbe virus and have been used to demonstrate that the NSD serogroup of the genus Nairovirus is more closely related to the Crimean–Congo haemorrhagic fever serogroup than any of the remaining serogroups (29, 41). These probes also have the potential to be applied as diagnostic tools.

C. REQUIREMENTS FOR VACCINES AND DIAGNOSTIC BIOLOGICALS

- **Cache Valley virus**

Due to the sporadic nature of disease outbreaks, no vaccine has been developed.

- **Akabane virus**

Major epizootics of Akabane virus disease have only been reported from Japan and Australia, albeit at irregular intervals, but vaccination is seen to have merit in preventing fetal loss.

Vaccines for domestic use have been produced in Japan and Australia.

An inactivated vaccine is used in Japan for immunising cattle and goats. It is a formalin-inactivated intramuscular preparation with an aluminium phosphate gel adjuvant. Two 3-ml doses are given at a 4-week interval and yearly boosters are recommended. It is safe for use in pregnant animals. In field trials 88% of animals developed high VN antibodies after the first inoculation and there was a 100% response after the second dose (27). Similarly, in Australia an inactivated vaccine has been produced for intramuscular use giving two doses at a 4-week interval just before mating.

In Japan, a live Akabane virus vaccine has is commercially available. A 1-ml dose is administered subcutaneously to cattle before the haematophagous arthropod vectors become active. Pregnant cattle and calves have been inoculated subcutaneously, intramuscularly and intracerebrally; no leucopenia, viraemia or pyrexia were observed and a good VN antibody response was produced. A live Akabane virus vaccine, safe in cattle, was tested in pregnant ewes. During the trials, some ewes became viraemic and virus was found in the organs of several fetuses. Although no fetal deformities were produced, the vaccine is deemed unsuitable for use in sheep.

- **Nairobi sheep disease**

Epidemiological investigations have shown that in a state of enzootic stability, no problems are encountered with NSD. The disease arises from animal movements from free areas into endemic areas and can be avoided when such areas have been defined. Ecological changes that permit spread of the vector tick will result in extensions of these areas.

Experimental vaccines have been prepared for such situations. One vaccine consisted of virus attenuated by 35 passages in adult mice, but such vaccines can produce severe reactions in some breeds of sheep, and are not considered to be safe for general use. A similar vaccine was developed in Entebbe by further mouse brain passages, but this has not been further developed for use in the field in Uganda or elsewhere.

A tissue-culture-adapted strain of NSD virus has been grown to high titre in cultures grown in roller bottles. When precipitated with methanol, inactivated, and administered with an adjuvant, this was found to give good protection following two inoculations given at an interval of 14 days. Neither of these vaccines is routinely produced, for there has been little demand for their use from the field (15, 17).

REFERENCES


* *
Chapter 2.9.2.
Camelpox

Summary

Camelpox is a widespread infectious viral disease of Old World camelids. New World camelids are also susceptible. It occurs throughout the camel-breeding areas of Africa, north of the equator, the Middle East and Asia, causing economic impact through loss of production and sometimes death. The camelpox virus belongs to the family Poxviridae, subfamily Chordopoxvirinae, genus Orthopoxvirus. The disease is characterised by fever, local or generalised pox lesions on the skin and in the mucous membranes of the mouth, respiratory and digestive tracts. The clinical manifestations range from inapparent infection to mild, moderate and, less commonly, severe systemic infection and death. The disease occurs more frequently and more severely in young animals. Transmission is by either direct contact between infected and susceptible animals or indirect infection via a contaminated environment. The role of insects in transmission has been suspected because the disease is often observed after rainfall. Camelpox virus is very host specific and does not infect other animals. Only one suspected case of human camelpox involving mild skin lesions has been described, underlining that camelpox is of no public health importance.

Identification of the agent:

The presumptive diagnosis of camelpox infection is based on clinical signs. However, infections of camels with contagious ecthyma (orf), papilloma virus and reaction to insect bites are considered differential diagnoses in the early clinical stages and in mild cases of camelpox. Several diagnostic methods are available and, where possible, more than one should be used to make a confirmatory diagnosis of disease.

The fastest method of laboratory confirmation of camelpox is by the demonstration of the characteristic, brick-shaped orthopox virions in skin lesions, scabs or tissue samples using transmission electron microscopy (TEM). Camelpox virus is distinct from the ovoid-shaped parapox virus, the aetiological agent of the principle differential diagnosis: camel orf. However, both viruses may be seen simultaneously on TEM as infection with both viruses has been reported to occur.

Camelpox can be confirmed by demonstration of the camelpox antigen in scabs and pox lesions in tissues by immunohistochemistry. It is a relatively simple method that can be performed in laboratories where TEM is not available. In addition, the paraffin-embedded samples can be stored for a long period of time, enabling future epidemiological, retrospective studies.

Camelpox virus may be propagated on the chorioallantoic membrane (CAM) of embryonated chicken eggs. After 5 days, characteristic lesions can be observed on the CAM. Camelpox virus shows typical cytopathic effect on a wide variety of cell cultures. Intracytoplasmic eosinophilic inclusion bodies, characteristic of poxvirus infection, may be demonstrated in infected cells using haematoxylin and eosin staining. The presence of viral nucleic acid may be confirmed by polymerase chain reaction, and different strains of camelpox virus may be identified using DNA restriction enzyme analysis. An antigen-capture enzyme-linked immunosorbent assay (ELISA) for the detection of camelpox virus has been described.

Serological tests: A wide range of serological tests are available to identify camelpox. The tests used for the detection of the antibodies against camelpox virus include neutralisation, agar gel precipitation, haemagglutination, haemagglutination inhibition, complement fixation, fluorescent antibody and antibody-capturing ELISA.

Requirements for vaccines and diagnostic biologicals: Both attenuated and inactivated vaccines are commercially available. Vaccination with live attenuated vaccine provides protection for at least 6 years and with inactivated vaccine for 12 months. There are no standardised requirements for diagnostic biologicals.
A. INTRODUCTION

Camelpox occurs in almost every country in which camel husbandry is practised apart from the introduced dromedary camel in Australia and tylopods (llama and related species) in South America. Outbreaks have been reported in the Middle East (Bahrain, Iran, Iraq, Oman, Saudi Arabia, United Arab Emirates and Yemen), in Asia (Afghanistan and Pakistan), in Africa (Algeria, Egypt, Ethiopia, Kenya, Mauritania, Morocco, Niger, Somalia and Sudan) (9, 18) and in the southern parts of Russia and India. The disease is endemic in these countries and a pattern of sporadic outbreaks occurs with a rise in the seasonal incidence usually during the rainy season.

Camelpox is caused by Orthopoxvirus cameli virus which belongs to genus Orthopoxvirus within family Poxviridae. Based on sequence analysis, it has been determined that the camelpox virus is the most closely related to variola virus, the aetiologial agent for smallpox. Camels have been successfully vaccinated against camelpox with vaccinia virus strains. The average size of the virion is 265–295 nm. Orthopoxviruses are enveloped, brick-shaped and the outer membrane is covered with irregularly arranged tubular proteins. A virion consists of an envelope, outer membrane, two lateral bodies and a core. The nucleic acid is a double-stranded linear DNA. Virus replicates in the cytoplasm of the host cell, in so-called inclusion bodies. Camelpox virus haemagglutinates cockerel erythrocytes. However, the haemagglutination may be poor (4). Camelpox virus is ether resistant and chloroform sensitive (4, 15). The virus is sensitive to pH 3–5 and pH 8.5–10 (4). Poxviruses are susceptible to various disinfectants including 1% sodium hypochlorite, 1% sodium hydroxide, 1% peracetic acid, formaldehyde, 0.5–1% formalin and 0.5% quaternary ammonium compounds. The virus can be destroyed by autoclaving, boiling for 10 minutes and is killed by ultraviolet rays (245 nm wave length) in a few minutes (3).

The incubation period is usually 9–13 days (varying between 3 and 15 days). Clinical manifestations of camelpox range from inapparent and mild local infections, confined to the skin, to moderate and severe systemic infections, possibly reflecting differences between the strains of camelpox (16). The disease is characterised by fever, enlarged lymph nodes and skin lesions. Skin lesions appear 1–3 days after the onset of fever, starting as erythematous macules, developing into papules and vesicles, and later turning into pustules. Crusts develop on the ruptured pustules. These lesions first appear on the head, eyelids, nostrils and the margins of the ears. In severe cases the whole head may be swollen. Later, skin lesions may extend to the neck, limbs, genitalia, mammary glands and perineum. In the generalised form, pox lesions may cover the entire body. Skin lesions may take up to 4–6 weeks to heal. In the systemic form of the disease, pox lesions can be found in the mucous membranes of the mouth, respiratory and digestive tracts (7, 16).

The animals may show salivation, lacrimation and a mucopurulent nasal discharge. Diarrhoea and anorexia may occur in the systemic form of the disease. Pregnant females may abort. Death is usually due to secondary infections and septicaemia (16).

Histopathological examination of the early skin nodules reveals characteristic cytoplasmic swelling, vacuolation and ballooning of the keratinocytes of the outer stratum spinosum. The rupture of these cells produces vesicles and localised oedema. Perivascular infiltration of mononuclear cells and variable infiltration of neutrophils and eosinophils occurs. Marked epithelial hyperplasia may occur in the borders of the skin lesions (20).

There are only a few detailed pathological descriptions of internal camelpox lesions. The lesions observed on post-mortem examination of camels that die following severe infection with camelpox are multiple pox-like lesions on the mucous membranes of the mouth, respiratory and digestive tract. The size of the lesions in the lungs may vary in diameter between 0.5 and 1.3 cm, occasionally up to 4–5 cm. Smaller lesions may have a haemorrhagic centre. The lung lesions are characterised by hydropic degeneration, proliferation of bronchial epithelial cells, and infiltration of the affected areas by macrophages, necrosis and fibrosis (6, 13, 17).

The morbidity rate of camelpox is variable and depends on whether the virus is circulating in the herd. Serological surveys taken in several countries reveal a high prevalence of antibodies to camelpox (16). The incidence of disease is higher in males than females, and the mortality rate is greater in young animals than in adults (7). The mortality rate in adult animals is between 5% and 28% and in young animals between 25% and 100% (9).

Transmission is by either direct contact between infected and susceptible animals or indirect infection via a contaminated environment. The infection is usually achieved by inhalation or through skin abrasions. Virus is secreted in milk, saliva, and ocular and nasal discharges. Dried scabs shed from the pox lesions may contain live virus for at least 4 months and contaminate the environment. The role of an arthropod vector in the transmission of the disease has been suspected. Camelpox virus has been detected by transmission electron microscopy (TEM) and virus isolation from the camel tick, Hyalomma dromedarii, collected from animals infected with camelpox virus. The increased density of the tick population during the rainy season may be responsible for the spread of the disease (17). However, other potential vectors may be involved, such as biting flies and mosquitoes.
Chapter 2.9.2. — Camelpox

It has been suggested that different strains of camelpox virus may show some variation in their virulence (16). Restriction enzyme analysis of viral DNA allows isolates to be compared. However, no major differences from the vaccine strain have so far been demonstrated (17).

Immunity against camelpox is both humoral and cell mediated. The relative importance of these two mechanisms is not fully understood, but it is believed that circulating antibodies do not reflect the immune status of the animal (16). Life-long immunity follows after natural infection. Live, attenuated vaccine provides protection against the disease for at least 6 years, probably longer (19). Inactivated vaccine provides protection for 1 year only.

The camelpox virus is very host specific and does not infect other animal species, including cattle, sheep and goats. Field reports of mild skin lesions in humans associated with camelpox have been made (3), but it appears that only one suspected case of human camelpox has been described (7), underlining that camelpox is of no public health importance.

B. DIAGNOSTIC TECHNIQUES

During the viremic stage of the disease (within the first week of the occurrence of clinical signs) camelpox virus can be isolated in cell culture from heparinised blood samples, or viral DNA can be detected by the polymerase chain reaction (PCR) from blood in EDTA (ethylene diamine tetra-acetic acid). The blood samples should be collected in a sterile manner by venepuncture. Blood samples, with anticoagulant for virus isolation from the Buffy coat, should be placed immediately on ice and processed as soon as possible. In practice, the samples can be kept at 4°C for up to 2 days prior to processing, but should not be frozen or kept at ambient temperatures.

Blood obtained for serum samples should be collected in plain tubes with no anticoagulant. The blood tubes should be left to stand at room temperature for 1–2 hours until the clot begins to contract, after which the blood is centrifuged at 1000 g for 10–15 minutes. Separated serum can be collected with a pipette and held at 4°C for a short period of time or stored at –20°C.

A minimum of 2 g of tissue from skin biopsies and organs should be collected for virus isolation and histopathology. For the PCR, approximately 30–50 mg of tissue sample should be placed in a cryotube or similar container, kept at 4°C for transportation and stored at –20°C until processed. Tissue samples collected for virus isolation should be placed in a virus transport medium, such as Tris-buffered tryptose broth, kept at 4°C for transportation and stored at –80°C until processed. Material for histology should be placed immediately after collection into ten times the sample volume of 10% formalin. The size of the samples should not exceed 0.5 cm × 1–2 cm. Samples in formalin can be transported at room temperature.

1. Identification of the agent

a) Transmission electron microscopy

TEM is a rapid method to demonstrate camelpox virus in scabs or tissue samples. However, a relatively high concentration of virus in the sample is required for positive diagnosis and camelpox virus cannot be differentiated from other orthopoxvirus species. However, currently, TEM is the best method for distinguishing clinical cases of camelpox and orf caused by camelpox and parapox viruses respectively, although the viruses can be differentiated by serological techniques and by PCR (9).

The size of a sample should be at least 30–50 mg. Mince the scabs or tissue sample with a disposable blade or sterile scissors and forceps. Grind the sample in a five-fold volume of phosphate buffered saline (PBS) with antibiotics (such as 10² International Units [IU] penicillin and 10 mg streptomycin per ml) using a mortar and pestle with sterile sand. Transfer the sample into a centrifuge tube and freeze and thaw two to three times to release the virus from the cells. Vortex the samples while thawing. Place the tubes on ice and sonicate once for 30 seconds at 80 Hz. Centrifuge at 1000 g for 10 minutes to remove the gross particles and collect the supernatant (12, 14).

- Test procedure

Place 10 µl of above-mentioned supernatant on poly-L-lysine-covered grids and incubate at room temperature for 5 minutes. Remove the fluid with a chromatography filter paper. Add one drop of 2% phosphotungstic acid (diluted in sterile water and pH adjusted to 7.2 with NaOH) to the grid, incubate at room temperature for 5 minutes and air dry. Examine the grid by TEM (12, 14).

Camelpox virus has a typical brick-shaped appearance with irregularly arranged, tubular surface proteins. Parapoxviruses are slightly smaller, ovoid-shaped and the surface proteins are regularly arranged.
b) Virus isolation in cell cultures

Camelpox virus can be propagated in a large variety of cell cultures including the following cell lines: Vero, MA-104 and MS monkey kidney, and, baby hamster kidney (BHK) and the following primary cell cultures: lamb testis, lamb kidney, camel embryonic kidney, calf kidney, and chicken embryo fibroblast (4, 15).

The samples are prepared for virus isolation as described above in Section B.1.a.

• Test procedure

Incubate 400 µl of the supernatant for 1 hour at room temperature and overnight at 4°C. Filter the supernatant through a 0.45 µm filter and inoculate into a 25 cm² flask of confluent cells. Flush the filter with 0.5 ml of the maintenance medium used in the cell culture and incubate the flasks at 37°C for 1 hour. Add 6–7 ml of fresh medium into the flask and continue the incubation for about 10 days. If there is any reason to suspect fungal contamination, the contaminated medium must be discarded and 5 µg/ml of amphotericin B added to a new medium. The flasks must be monitored daily for 10–12 days.

Characteristic, plaque-type cytopathic effect (CPE) showing foci of rounded cells, cell detachment, giant cell formation and syncytia may appear as soon as 24 hours post-inoculation. Syncytia may contain up to 20–25 nuclei (15). The growth of camelpox virus on a cell culture can be confirmed by TEM, PCR or antigen-capture enzyme-linked immunosorbent assay (ELISA) (5).

c) Virus isolation on chorioallantoic membrane of embryonated chicken eggs

Camelpox virus can be isolated on the chorioallantoic membrane (CAM) of 11–13-day old embryonating chicken eggs. The eggs should be incubated at 37°C degrees and after 5 days, the eggs containing living embryos are opened and the CAM examined for the presence of characteristic pock lesions: dense, greyish-white pocks. Camelpox virus does not cause death in inoculated embryonated chicken eggs. The maximum temperature for the formation of pock lesions is 38.5°C degrees. If the eggs are incubated at 34.5°C the pocks are flatter and a haemorrhagic centre may develop (15).

d) Immunohistochemistry

Immunohistochemistry for the detection of the infectious agent of camelpox is a relatively fast method and can be used instead of electron microscopy for establish a tentative diagnosis (11). Almost any polyclonal antibody against vaccinia virus is likely to produce reasonable results in this test because of the wide homology between vaccinia and camelpox viruses (11).

• Test procedure

The following procedure for immunohistochemistry is described by Kinne et al. (6) and Pfeffer et al. (14). The entire skin pustule should be collected for the immunohistochemical examination. Fix the tissue in 10% formalin, dehydrate through graded alcohols and embed in paraffin wax according to standard histopathological procedures. Cut approximately 3 µm sections and place on the glass slides. Treat the deparaffinised and dehydrated sections with 3% H₂O₂, prepared in distilled water, for 5 minutes and wash with PBS. Incubate the slides for 60 minutes at 37°C with anti-vaccinia virus monoclonal antibody 5B4, diluted 1/500. Remove the monoclonal antibody by washing twice with cold PBS. Incubate the slides for 30 minutes with anti-mouse antibodies labelled with biotin (ABC-kit, Dako, Glostrup, Denmark). Wash with PBS for 5 minutes and incubate with streptavidin-peroxidase for 30 minutes. Wash again with PBS for 5 minutes and add diaminobenzidine as chromogen for 10 minutes.

e) Polymerase chain reaction

The PCR is a fast and sensitive method for the detection of orthopoxviral DNA. A generic PCR assay, described by Meyer et al. (10), allows the detection and differentiation of species of the genus Orthopoxivirus because of the size differences of the amplicons. Using the primer pair: 5’-AAT-ACA-AGG-AGG-ATC-3’ and 5’-CTT-AAC-TTC-TTC-TTC-3’, the gene sequence encoding the A-type inclusion protein (ATIP) will be amplified. The size of the PCR product, specific for the camelpox virus, is 881 bp.

• Test procedure

Suspend a small aliquot of crusted scabs in 90 µl of lysis solution (50 mM Tris/HCl, pH 8.0, 100 mM Na₂EDTA, 100 mM NaCl, 1% sodium dodecyl sulphate) and add 10 µl of proteinase K (20 mg/ml, Invitrogen). Digest the sample for 10 minutes at 37°C prior to the disruption of the scab or tissue with a microfuge tube pestle. Add another 350 µl lysis solution and 50 µl of proteinase K, mix gently and incubate for 3 hours at 37°C. Extract the lysed suspension with an equal volume of phenol/chloroform/isoamylalcohol (25/24/1) and centrifuge at 8000 g at 4°C for 1 minute. Collect the upper aqueous phase and mix it again with an equal volume of phenol/chloroform/isoamylalcohol (25/24/1). Centrifuge at 8000 g at 4°C for 1 minute and transfer the upper, aqueous phase to a new tube. Precipitate the DNA by adding 1/10 volume
of 3 M sodium acetate and two volumes of ice-cold absolute ethanol. Place the mixture at −70°C for 30 minutes or −20°C overnight. Centrifuge at 15,000 \( g \) for 5 minutes at 4°C. Discard the supernatant and wash the pellet with 0.5 ml of 70% ethanol. Centrifuge at 15,000 \( g \) for 5 minutes. Discard the supernatant and air-dry the pellets. Resuspend the pellets in 10 \( \mu l \) of nuclease-free water.

DNA amplification is carried out in a final volume of 50 \( \mu l \) containing 2 \( \mu l \) of each dNTP (10 mM), 5 \( \mu l \) of 10 \( \times \) PCR buffer, 1.5 \( \mu l \) of MgCl\(_2\) (50 mM), 1 \( \mu l \) of each primer, 2.5 U Taq DNA polymerase, 1 \( \mu l \) DNA template and an appropriate volume of nuclease-free water.

Incubate the samples in a thermal cycler: first cycle: 5 minutes at 94°C (initial denaturation step), second cycle: 1 minute at 94°C, 1 minute at 45°C, 2.5 minutes at 72°C. Repeat the second cycle 29 times. Last cycle: 10 minutes at 72°C (final elongation step) and hold at 4°C until analysis.

Mix 10 \( \mu l \) of a sample with loading dye solution and load in 1% agarose gel in TBE (Tris/Borate/EDTA) buffer containing ethidium bromide. Load a parallel lane with a 100 bp DNA-marker ladder. Separate the products at 100 V for 30–40 minutes and visualise using an UV transilluminator. Confirm the positive reactions according to the size.

A commercial PCR kit has been developed that allows detection of orthopoxvirus-DNA and contains a second ‘conventional’ amplification system, consisting of primers to the haemagglutinin (HA) gene of the orthopoxvirus. The amplicon can be sequenced and identified by comparison with already existing orthopoxvirus sequences.

2. Serological tests

All the viruses in the genus *Orthopoxvirus* cross-react serologically. However, within the genus only camelpox virus can cause pox-like lesions in camels. Parapox and camelpox viruses do not cross-react and so infections of camelpox and camel orf can be distinguished serologically. Most of the conventional serological tests are very time- and labour-consuming, which makes them not suitable for primary diagnosis. However, serological tests are a valuable tool for secondary confirmatory testing and retrospective epidemiological studies in those areas where vaccination against camelpox is not practised.

a) Serum neutralisation test

In this method the test sera are titrated against a constant titre of camelpox virus (100 TCID\(_{50}\) [50% tissue culture infectious dose]).

- **Test procedure**
  1. **Mark the microtitre plates.**
  2. **Dilute the test sera, positive and negative serum controls 1/5 in Dulbecco’s Modified Eagle’s Medium (DMEM).**
  3. **Inactivate the sera at 56°C for 30 minutes.**
  4. **Add the growth medium (DMEM containing 5–10% of fetal calf serum and antibiotics) into the wells:**
    - 100 \( \mu l \) into rows A to H, columns 2 to 6 (test serum, positive and negative serum controls) and rows A to D, columns 7 to 12 (virus control rows).
    - 200 \( \mu l \) into rows G to H, columns 7 to 12 (cell control rows).
  5. **Add the diluted and inactivated test sera, positive and negative control sera: 200 \( \mu l \)well into rows A to H, column 1 (duplicate rows for each sample).**
  6. **Collect 100 \( \mu l \) from the test serum wells (1/5 dilution) and prepare twofold dilutions (1/10, 1/20, 1/40, 1/80, 1/160) of the serum samples on the microtitre plate using a multichannel pipette. Discard 100 \( \mu l \) from the last wells.**
  7. **Prepare 100 TCID\(_{50}\)/100 \( \mu l \) dilution of the virus suspension with a known titre of over log\(_{10}\) 6 TCID\(_{50}\) to be used as a working virus seed.**
  8. **Prepare tenfold dilution series from working virus seed (10\(^{-1}\), 10\(^{-2}\), 10\(^{-3}\), 10\(^{-4}\)).**
  9. **Add 100 \( \mu l \) of each virus dilution into the virus control rows:**
    - Working virus seed in rows A to D, columns 7 and 8.
    - 10\(^{-1}\) dilution of the working virus seed into rows A to D, column 9.
    - 10\(^{-2}\) dilution of the working virus seed into rows A to D, column 10.
• $10^{-3}$ dilution of the working virus seed into rows A to D, column 11.
• $10^{-4}$ dilution of the working virus seed into rows A to D, column 12.

x) Add 100 µl of working virus seed into each well in test serum, positive and negative serum control rows.

xi) Incubate the microtitre plate at 37°C with 5% CO₂ for 1 hour.

xii) Add 80 µl of suitable cell suspension, such as lamb testis cells, at a concentration of 480,000 cells/ml into each well.

xiii) Incubate at 37°C with 5% CO₂.

xiv) Read the results using an inverted microscope. Examine the microtitre plates daily. Serum antibody titres are calculated and recorded when CPE is evident in the virus control wells. The test is valid if the virus titre is approximately 100 TCID₅₀. The antibody titre is determined by the Spearman–Kärber method.

b) Enzyme-linked immunosorbent assay for the detection of antibodies against camelpox virus

The following procedure for antibody-detecting ELISA test for *Orthopoxvirus cameli* is described by Azwai *et al.* (2) and Pfeffer *et al.* (14). The following description gives general guidelines for the test procedure.

• **Preparation of the antigen**
  i) Harvest the cell culture when 100% infected with camelpox virus. Freeze and thaw two to three times. Sonicate for 30 seconds at 80 Hz on ice to release the virus from the cells.
  ii) Centrifuge at 1000 g for 10 minutes and collect the supernatant.
  iii) Centrifuge the supernatant at 45,000 g at 4°C for 1 hour. Re-suspend the pellet in PBS.
  iv) Add NaCl to a final concentration of 330 mM and polyethylene glycol (PEG 6000) to a final concentration of 7%.
  v) Stir overnight at 4°C, centrifuge at 3000 g at 4°C for 10 minutes and wash the pellet twice with 15 mM NaCl.
  vi) Freeze and thaw, and treat with 1% non-ionic detergent (Nonidet P40, Sigma) at 37°C for 3 hours.
  vii) Freeze and thaw and centrifuge at 3000 g for 10 minutes at 4°C.
  viii) Collect the supernatant and dialyse at least three times against PBS.
  ix) Measure the protein concentration as described by Lowry (8).
  x) Store the aliquots at -20°C.

• **Preparation of rabbit anti-camel IgG horseradish-peroxidase conjugate**

Unfortunately rabbit anti-camel IgG horseradish-peroxidase conjugate is not commercially available. The method for producing monoclonal antibodies for camel IgM and IgG has been described by Azwai *et al.* (1). However, rabbit anti-camel IgG horseradish-peroxidase can be replaced with commercially available product in which the antibodies are raised against IgG of the New World cameldids and conjugated with fluorescein (Fluorescein Conjugated Camelid IgG, Kent Laboratories, Triple J Farms, USA).

  i) Precipitate camel sera twice adding saturated ammonium sulphate to a final concentration of 40% (v/v) (29.6% ammonium sulphate [w/v]) at room temperature. Centrifuge at 12,000 g for 15 minutes and dissolve in PBS, pH 7.2. Dialyse against several changes of PBS overnight.
  ii) Separate the immunoglobulins using gel filtration chromatography: an ACA-34 (LBK) column (2.6 x 100 cm) can be used to separate the salt precipitated immunoglobulins (IgM and IgG) by size. Elution can be effected with PBS at 20 ml/hour and 6 ml fractions can be collected. Determine the protein concentrations by absorbance at 280 nm.

• **Antiserum production**

Immunise rabbits with a subcutaneous injection of camel IgG emulsified in appropriate adjuvant. The animals should be immunised three times to booster antibody production. Collect the serum and store at −20°C until used.

• **Test procedure**

Non-activated, 96-well, microtitre ELISA plates, such as Immulon 2 supplied by Dynatech can be used.
i) Coat the microtitre plates with prepared antigen at 1 µg/ml in carbonate/bicarbonate buffer, 0.05 M, pH 9.6 (100 µl per well).

ii) Incubate the ELISA plates in a humid chamber (100% humidity) at 37°C for 1 hour and then overnight at 4°C.

iii) Wash off the unbound antigen with PBS containing 0.05% Tween 20 (PBS/Tween) three times.

iv) Add 100 µl of test and control serum at a predetermined optimal dilution in blocking buffer (PBS containing 0.05% Tween 20, and 1% fat-free milk powder) in duplicate wells.

v) Incubate the plates for 30 minutes at 37°C.

vi) Wash the plates three times with PBS/Tween.

vii) Dilute rabbit anti-camel IgG horseradish-peroxidase conjugate or fluorescein-conjugated camelid IgG at a predetermined working dilution in blocking buffer and add 100 µl into the wells.

viii) Incubate the plates at 37°C for 30 minutes.

ix) Wash the plates three times with PBS/Tween.

x) Make the reaction visible using 100 µl chromogen 3.3’5.5’-tetramethylbenzidine (TMB) per well and incubate 15 minutes at 37°C with shaking.

xi) Stop the reaction 10 minutes later by 2 M H₂SO₄ at a volume of 50 µl/well.

xii) Measure the values with a photometer at a wave length of 450 nm. Seropositivity can be calculated as values above the mean +2 standard deviations from negative control sera.

C. REQUIREMENTS FOR VACCINES AND DIAGNOSTIC BIOLOGICALS

A live attenuated vaccine, Ducapox, is manufactured by Highveld Biologicals, Onderstepoort, South Africa and an inactivated vaccine by Biopharma, Rabat, Morocco. A live attenuated vaccine gives long-term protection against camelpox (19). However, a booster vaccination is recommended for young animals vaccinated before the age of 6–9 months. When inactivated vaccine is used, the animals must be vaccinated annually. Guidelines for the production of the veterinary vaccines are given in Chapter 1.1.8 Principles of veterinary vaccine production.

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REFERENCES


Chapter 2.9.2. — Camelpox


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CHAPTER 2.9.3.

CAMPYLOBACTER JEJUNI AND CAMPYLOBACTER COLI

SUMMARY

Definition of the disease: Campylobacter jejuni and C. coli can colonise the intestinal tract of most mammals and birds and are the most frequently isolated Campylobacter species in humans with gastro-enteritis. Transmission from animals to humans is mainly through consumption and handling of animal food products but also direct contact with colonised animals may contribute to human campylobacteriosis. This chapter focuses on C. jejuni and C. coli in primary livestock production with regard to food safety.

Description of the disease: Campylobacter jejuni and C. coli do not cause clinical disease in adult animals except for sporadic cases of abortion in ruminants and very rare cases of hepatitis in ostriches. The faecal contamination of meat (especially poultry meat) during processing is considered to be a major source of human food-borne disease. In humans, extraintestinal infections, including bacteraemia, can occur and some sequelae of infection, such as polyneuropathies, though rare, can be serious.

Identification of the agent: In mammals and birds, detection of intestinal colonisation is based on the isolation of the organism from faeces, rectal swabs and/or caecal contents. Campylobacter jejuni and C. coli are thermophilic, Gram-negative, highly motile bacteria that, for optimal growth, require microaerobic environment and incubation temperatures of 37–42°C. Agar media containing selective antibiotics are required to isolate these bacteria from faecal/intestinal samples. Alternatively, their high motility can be exploited using filtration techniques for isolation. Enrichment techniques to detect intestinal colonisation are not routinely used. Preliminary confirmation of isolates can be made by light microscopy. The organisms in the log growth phase are short and S-shaped in appearance, while coccoid forms predominate in older cultures. Under phase-contrast microscopy the organisms have a characteristic rapid corkscrew-like motility. Phenotypic identification is based on reactions under different growth conditions. Biochemical and molecular tests can be used to confirm various Campylobacter species. Polymerase chain reaction assays also can be used for the direct detection of C. jejuni and C. coli.

Serological tests: serological assays are not routinely in use for the detection of C. jejuni/C. coli colonisation.

Requirements for vaccines and diagnostic biologicals: There are no effective vaccines available for the prevention of enteric Campylobacter infections in birds or mammals.

A. INTRODUCTION

1. Disease

Campylobacter jejuni and C. coli are generally considered commensals of livestock, domestic pet animals and birds. Large numbers of Campylobacter have been isolated from young livestock, including piglets, lambs and calves, with enteritis, but the organisms are also found in healthy animals. Outbreaks of avian hepatitis have been reported, but the pathogenic role of Campylobacter spp. is unclear. One possible exception is ostriches where Campylobacter-associated death and enteritis occurs in young birds. Campylobacter are the main cause of human bacterial intestinal disease identified in many industrialised countries (24). Over 80% of cases are caused by C. jejuni and about 10% of cases are caused by C. coli. In humans, C. jejuni/coli infection is associated with acute enteritis and abdominal pain lasting for 7 days or more. Although such infections are
generally self-limiting, complications can arise and may include bacteraemia, Guillain–Barré syndrome, reactive arthritis, and abortion (21). The primarily source of *C. jejuni/coli* infections in human is believed to be the handling and/or consumption of contaminated meat, especially poultry meat. However, contact with pets and livestock, the consumption of contaminated water or raw milk and travelling in high prevalence areas are also considered risks factors in human disease (8). The control of *Campylobacter* in the food chain has now become a major target of agencies responsible for food safety world-wide.

2. **Taxonomy**

In 1991 a revision of the taxonomy and nomenclature of the genus *Campylobacter* was proposed. According to Bergey's Manual, the genus *Campylobacter* comprises sixteen species and six subspecies. More recently, two additional species have been proposed (25). Members of the genus are typically Gram-negative, non-sporoforming, S-shaped or spiral shaped bacteria (0.2–0.8 µm wide and 0.5–5 µm long), with single polar flagella at one or both ends, conferring a characteristic corkscrew-like motility. These bacteria require microaerobic conditions, but some strains also grow aerobically or anaerobically. They neither ferment nor oxidise carbohydrates. Some species, particularly *C. jejuni*, *C. coli* and *C. lari*, are thermophilic, growing optimally at 42°C. They can colonise mucosal surfaces, usually the intestinal tract, of most mammalian and avian species tested. The species *C. jejuni* includes two subspecies (*C. jejuni* subsp. *jejuni* and *C. jejuni* subsp. *doylei*) that can be discriminated on the basis of several phenotypic tests (nitrate reduction, selenite reduction, sodium fluoride, and safranine) and growth at 42°C (subsp. *doylei* does not grow at 42°C) (9). Subspecies *jejuni* is much more frequently isolated then subspecies *doylei*.

**B. DIAGNOSTIC TECHNIQUES**

1. **Isolation and identification of the agent**

Two ISO (International Organization for Standardization) procedures for detection of *Campylobacter* exist, a horizontal method for detection of thermotolerant *Campylobacter* in food and animal feeding stuffs (10) and a procedure for the isolation of *Campylobacter* from water (11). However, neither of these standard methods may be optimal for the isolation of campylobacters from live animals. An appendix to ISO 10272 on this topic is currently being developed.

a) **Collection of samples**

i) **Poultry at the farm**

Poultry is frequently colonised with *C. jejuni* (65–95%) less often with *C. coli* and rarely with other *Campylobacter* species (16). Colonisation rates in broiler chickens are age-related. Most flocks are negative until 2 weeks of age. Once *Campylobacter* colonisation occurs in a broiler flock, transmission, via coprophagy, is extremely rapid and up to 100% of birds within a flock can become colonised within 72 hours. Samples from live birds, destined for the food chain, should therefore be taken as close to slaughter as possible (16). The majority of birds shed large numbers of organisms (>10⁶ colony-forming units/g faeces). Campylobacters can be isolated from fresh faeces/caecal droppings or cloacal swabs. For reliable detection of *Campylobacter* by culture, freshly voided faeces (preferably without traces of urine) should be collected. Such samples must be prevented from drying out before culture. When swabs are used, a transport medium such as Amies, Cary Blair or Stuart must be used.

ii) **Cattle, sheep and pigs at the farm**

Campylobacters are frequent colonisers of the intestine of livestock such as cattle, sheep and pigs (2, 26, 27). Cattle and sheep are found to be colonised mainly with *C. jejuni*, *C. coli*, *C. hyointestinalis*, and *C. fetus*, whereas pigs are predominantly colonised by *C. coli*. In young animals, the numbers are higher than in older animals. In older animals, the organisms can be intermittently detected in faeces, probably due to low numbers or due to intermittent shedding. Fresh samples have to be taken (rectal samples if possible) and they should be prevented from drying out. When swabs are used, a transport medium (like Amies, Cary Blair or Stuart) must be used.

iii) **At slaughter**

In poultry, the caeca are usually used for the detection of *Campylobacter*. They can be cut with sterile scissors from the remaining part of the intestines and submitted intact to the laboratory in a plastic bag or Petri-dish.

Samples from cattle, sheep and pigs are collected from the intestines by aseptically opening the gut wall or by taking rectal swabs.
b) Transportation and treatment of samples

i) Transport

Campylobacters are remarkably sensitive to environmental conditions, including dehydration, atmospheric oxygen, sunlight and elevated temperature. Transport to the laboratory and subsequent processing should therefore be as rapid as possible, preferably the same day, but within at least 2 days. The samples must be protected from light.

No recommendation on the ideal temperature for transportation can be made, but it is clear that freezing or high temperatures can reduce viability. High temperatures (>20°C), low temperatures (<0°C) and fluctuations in temperature must be avoided. When the time between sampling and processing is longer, storage at 4°C (±2°C) is advised.

ii) Transport media

Swabs: When samples are collected in swabs, the use of commercially available transport tubes, containing a medium, such as Amies, is recommended. This medium may be plain agar or charcoal-based. The function of the medium is not for growth of Campylobacter spp., but to protect the swab contents from drying and the toxic effects of oxygen.

When only small amounts of faecal/caecal samples can be collected and transport tubes are not available, shipment of the specimen in transport medium is recommended. Several transport media have been described: Cary-Blair, modified Cary-Blair, modified Stuart medium, Campythioglycolate medium, alkaline peptone water and semisolid motility test medium. Good recovery results have been reported using Cary-Blair (13, 20).

iii) Maintenance of samples

On arrival at the laboratory, samples should be processed as soon as possible, preferably on the day of arrival but no longer than 3 days after collecting the samples. To avoid temperature variation, samples should only be refrigerated when they cannot be processed on the same day, otherwise they should be kept at room temperature. When samples are submitted or kept in the laboratory at 4°C, they should be allowed to equilibrate to room temperature before processing to avoid temperature shock.

c) Isolation of Campylobacter

For the isolation of Campylobacter from faecal/caecal or intestinal samples, no pretreatment is needed; samples can be plated on to selective medium or the filtration method on non-selective agar can be used. In the case of caecal samples, caeca are aseptically opened by cutting the end with a sterile scissors and squeezing out the material to be processed. Enrichment is recommended to enhance the culture sensitivity of potentially environmentally stressed organisms or in the case of low levels of organisms in faeces, for example from cattle, sheep or pigs. However, enrichment from the latter samples is not carried out routinely and in research setting only.

i) Selective media for isolation

Many media can be used in the recovery of Campylobacter spp. Modified charcoal, cefoperazone, desoxycholate agar (mCCDA), is the recommended medium, although alternative media may be used. A detailed description on Campylobacter detection by culture and the variety of existing media is given by Corry et al. (6, 7). The selective media can be divided into two main groups: blood-containing media and charcoal-containing media. Blood components and charcoal serve to remove toxic oxygen derivatives. Most media are commercially available. The selectivity of the media is determined by the antibiotics used. Cefalosporins (generally cefoperazone) are used, sometimes in combination with other antibiotics (e.g. vancomycin, trimethoprim). Cycloheximide (actidione) and more recently amphotericin B are used to inhibit yeasts and molds (15). The main difference between the media is the degree of inhibition of contaminating flora. All the selective agents allow the growth of both C. jejuni and C. coli. There is no medium available that allows growth of C. jejuni and inhibits C. coli or vice versa. To some extent, other Campylobacter species (e.g. C. lari, C. upsaliensis, C. helveticus, C. fetus and C. hyointestinalis) will grow on most media, especially at the less selective temperature of 37°C.

Examples of selective blood-containing solid media:

- Preston agar
- Skirrow agar
- Butzler agar
- Campy-cefex
Examples of charcoal-based solid media:
- mCCDA (modified charcoal cefoperazone deoxycholate agar), slightly modified version of the originally described CCDA (4, 5)
- Karmali agar or CSM (charcoal-selective medium) (12)
- CAT agar (cefoperazone, amphotericin and teicoplanin), facilitating growth of C. upsaliensis (1).

ii) Passive filtration
Passive filtration, a method developed by Steele and McDermott (22) obviates the need for selective media; thus it is very useful for the isolation of antimicrobial-sensitive Campylobacter species. As the method does not use expensive selective media, it may be used in laboratories with fewer resources. For passive filtration, faeces are mixed with PBS (approximately 1/10 dilution) to produce a suspension. Approximately 100 µl of this suspension are then carefully layered on to a 0.45 or 0.65 µm filter, which has been previously placed on top of a non-selective blood agar plate. Care must be taken not to allow the inoculum to spill over the edge of the filter. The bacteria are allowed to migrate through the filter for 30–45 minutes at 37°C or room temperature. The filter is then removed, the fluid that has passed through the filter is spread with a sterile glass or plastic spreader, and the plate is incubated microaerobically at 42°C.

iii) Incubation
- Atmosphere
Microaerobic atmospheres of 5–10% oxygen, 5–10% carbon dioxide are required for optimal growth (7, 25). Appropriate atmospheric conditions may be produced by a variety of methods. In some laboratories, (repeated) gas jar evacuations followed by atmosphere replacement with bottled gases are used. Gas generator kits are available from commercial sources. Variable atmosphere incubators are more suitable if large numbers of cultures are undertaken.
- Temperature
Media may be incubated at 37°C or 42°C, but it is common practice to incubate at 42°C to minimise growth of contaminants and to select for optimal growth of C. jejuni/ C. coli. The fungistatic agents cycloheximide or amphotericin are added in order to prevent growth of yeasts and mould at 37°C (5). In some laboratories, incubation takes place at 41.5°C to harmonise with Salmonella and Escherichia coli O157 isolation protocols (10).
- Time
Campylobacter jejuni and C. coli usually show growth on solid media within 24–48 hours at 42°C. As the additional number of positive samples obtained by prolonged incubation is very low, 48 hours of incubation is recommended for routine diagnosis (5).

d) Confirmation
A pure culture is required for confirmatory tests, but a preliminary confirmation can be obtained by direct microscopic examination of suspect colony material.
- Identification on solid medium: On Skirrow or other blood-containing agars, characteristic Campylobacter colonies are slightly pink, round, convex, smooth and shiny, with a regular edge. On charcoal-based media such as mCCDA, the characteristic colonies are greyish, flat and moistened, with a tendency to spread, and may have a metal sheen.
- Microscopic examination of morphology and motility: material from a suspect colony is suspended in saline and evaluated, preferably by a phase-contrast microscope, for characteristic, spiral or curved slender rods with a corkscrew-like motility. Older cultures show less motile coccoid forms.
- Detection of oxidase: take material from a suspect colony and place it on to a filter paper moistened with oxidase reagent. The appearance of a violet or deep blue colour within 10 seconds is a positive reaction. If a commercially available oxidase test kit is used, follow the manufacturer’s instructions.
- Microaerobic growth at 25°C: Inoculate the pure culture on to a non-selective blood agar plate and incubate at 25°C in a microaerobic atmosphere for 48 hours.
- Aerobic growth at 41.5°C: Inoculate the pure culture on to a non-selective blood agar plate and incubate at 41.5°C in an aerobic atmosphere for 48 hours.
- Latex agglutination tests for confirmation of pure cultures of C. jejuni/ C. coli (often also including C. lari) are commercially available.
Chapter 2.9.3. — Campylobacter jejuni and Campylobacter coli

e) **Identification of Campylobacter to the species level**

Among the Campylobacter spp. growing at 42°C, the most frequently encountered species from samples of animal origin are C. jejuni and C. coli. However, low frequencies of other species have been described. Generally, C. jejuni can be differentiated from other Campylobacter species on the basis of the hydrolysis of hippurate as this is the only hippurate-positive species isolated from veterinary or food samples. The presence of hippurate-negative C. jejuni strains has been reported (23). Table 2 gives some basic classical phenotypic characteristics of the most important thermophilic Campylobacter species (10). Sensitivity to nalidixic acid used to be one of the most commonly tested characteristics, but nowadays may give difficulties in interpretation, both due to an increase in nalidixic acid-resistant strains of C. jejuni and C. coli and to the isolation of nalidixic acid-sensitive genogroups of C. lari. More extensive speciation schemes have been described in the literature (18, 25). Speciation results should be confirmed using defined positive and negative controls.

The confirmatory tests for the presence of thermophilic campylobacters and the interpretation (10) are given in Table 1. Confirm results of confirmation tests using positive and negative controls.

**Table 1. Confirmatory tests for thermophilic Campylobacter**

<table>
<thead>
<tr>
<th>Confirmatory test</th>
<th>Result for thermophilic Campylobacter</th>
</tr>
</thead>
<tbody>
<tr>
<td>Morphology</td>
<td>Small curved bacilli</td>
</tr>
<tr>
<td>Motility</td>
<td>Characteristic (highly motile and cork-screw like)</td>
</tr>
<tr>
<td>Oxidase</td>
<td>+</td>
</tr>
<tr>
<td>Aerobic growth at 41.5°C</td>
<td>–</td>
</tr>
<tr>
<td>Microaerobic growth at 25°C</td>
<td>–</td>
</tr>
</tbody>
</table>

**Table 2. Basic phenotypic characteristics of selected thermophilic Campylobacter species**

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>C. jejuni</th>
<th>C. coli</th>
<th>C. lari</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hydrolysis of hippurate</td>
<td>+</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Hydrolysis of indoxyl acetate</td>
<td>+</td>
<td>+</td>
<td>–</td>
</tr>
</tbody>
</table>

Key: + = positive; – = negative; S = sensitive; R = resistant

i) **Detection of hippurate hydrolysis:** Suspend a loopful of growth from a suspect colony in 400 µl of a 1% sodium hippurate solution (care should be taken not to incorporate agar). Incubate at 37°C for 2 hours, then slowly add 200 µl 3.5% ninhydrin solution to the side of the tube to form an overlay. Re-incubate at 37°C for 10 minutes, and read the reaction. Positive reaction: dark purple/blue. Negative reaction: clear or grey. If commercially available hippurate hydrolysis test disks are used, follow the manufacturer’s instructions.

ii) **Detection of indoxyl acetate hydrolysis:** Place a suspect colony on an indoxyl acetate disk and add a drop of sterile distilled water. If indoxyl acetate is hydrolysed a colour change to dark blue occurs within 5–10 minutes. No colour change indicates hydrolysis has not taken place. If commercially available indoxyl acetate hydrolysis test disks are used, follow the manufacturer’s instructions.

Biochemical speciation may be supplemented or even replaced with molecular methods. A variety of DNA probes and polymerase chain reaction (PCR)-based identification assays has been described for Campylobacter species (18, 25). On et al. (19) evaluated the specificity of 11 PCR-based identification assays for C. jejuni and C. coli.

f) **Molecular detection of Campylobacter**

PCR-based methods for the detection of Campylobacter in animal faecal samples and enriched meat samples have been previously described in the literature (17). One of these assays is in use in Denmark for routine screening of cloacal swabs from broilers at the slaughterhouse (3, 14).

g) **Antigen-capture-based tests**

Enzyme immunoassays are available for the detection of Campylobacter in human stool samples only.
2. Serological tests

There are no serological assays in routine use for the detection of colonisation of \textit{C. jejuni}/\textit{C. coli} in livestock.

C. REQUIREMENTS FOR VACCINES AND DIAGNOSTIC BIOLOGICALS

There are no vaccines specifically developed for \textit{C. jejuni} or \textit{C. coli} in animals or birds.

REFERENCES


Chapter 2.9.3. — Campylobacter jejuni and Campylobacter coli


* *

NB: There is an OIE Reference Laboratory for Campylobacteriosis (see Table in Part 3 of this Terrestrial Manual or consult the OIE Web site for the most up-to-date list: www.oie.int).
CHAPTER 2.9.4.

CRYPTOSPORIDIOSIS

SUMMARY

Cryptosporidiosis is caused by protozoan parasites of the genus Cryptosporidium, in which there are 18 'valid' species. In livestock, C. parvum, C. andersoni, C. baileyi, C. meleagridis and C. galli have been reported to cause morbidity and outbreaks of disease. Laboratory identification is required to confirm diagnosis. Cryptosporidium parvum cryptosporidiosis causes scour in young, unweaned mammalian livestock, however, weaned and adult animals can also become infected. Signs range from a mild inapparent infection to severe scouring, and the young, old or immunocompromised are most susceptible. Mortality is low. Weaned and adult animals normally do not exhibit signs of disease, but will excrete oocysts that may contaminate the environment. Cryptosporidium andersoni cryptosporidiosis affects the digestive glands of the abomasum of older calves and adult cattle. Some infected animals exhibit reduced weight gain, but do not develop diarrhoea. Cryptosporidium baileyi, C. meleagridis and C. galli cryptosporidiosis are diseases of birds. Cryptosporidium baileyi affects primarily the bursa of Fabricius and cloaca of gallinaceous birds, C. meleagridis affects primarily the ileum of turkey poults and C. galli infects the surface, ductal, and glandular epithelium of the proventriculus of adult hens and some wild birds.

Identification of the agent: There is no prescribed test for cryptosporidium infection. The demonstration of cryptosporidium species oocysts or cryptosporidium antigen in a properly collected and submitted sample is sufficient for a positive diagnosis. Diagnosis is established microscopically, with the acid-fast Ziehl–Neelsen or auramine phenol methods using unconcentrated or concentrated faecal smears. Microscopy-based methods for detecting oocysts and enzyme-linked immunosorbent assays for detecting cryptosporidium antigens are relatively insensitive, but are sufficiently sensitive for detecting clinical cases. Neither tinctorial- nor fluorescence-based stains can determine the species of cryptosporidium present if the oocysts fall within the size range of 4–6 µm. These methods can detect oocysts in clinically ill animals, but sometimes are not sufficiently sensitive to detect infection in clinically normal animals. Nucleic acid detection tests have a greater sensitivity. The polymerase chain reaction (PCR) and restriction fragment length polymorphism (RFLP) and/or sequencing can be used to determine some or all cryptosporidium species/genotypes or subtypes. Those typing and subtyping systems used for veterinary (and human) samples should also be used for environmental samples, to avoid any confusion arising from using different systems during the investigation of disease outbreaks with both veterinary and public health implications. However the sensitivity of subtyping systems will need to be increased so that they can be used with clinical and environmental samples containing small numbers (<10) of oocysts. Current limitations of discriminatory species subtyping systems are that they are only applicable to C. parvum and C. hominis. Discriminatory subtyping systems for non-'parvum' and non-'hominis' pathogens (including the majority of livestock pathogens) have yet to be developed.

Specimens for primary diagnosis should be collected during acute infection, and should be processed as soon as possible, ideally, within 24 hours. Transportation to the laboratory should be in accordance with the International Air Transport Association regulations, which are summarised in Chapter 1.1.1 Collection and shipment of diagnostic specimens.

Demonstration of Cryptosporidium oocysts or Cryptosporidium-specific antigen in faecal samples is the most appropriate test for most applications. Most infections that cause morbidity and/or mortality in mammalian livestock are likely to be caused by C. parvum cryptosporidiosis. Cryptosporidium bovis is a highly prevalent species that infects primarily post-weaned calves. Cryptosporidium baileyi, C. meleagridis and C. galli cryptosporidiosis cause morbidity and/or
mortality in avian livestock. The species of Cryptosporidium responsible can be determined by PCR-RFLP and/or sequencing of cryptosporidium oocyst DNA. There are no international standards for the preparation of purified oocysts, antisera, antigens, monoclonal antibodies or hybridomas, although a variety of purified oocysts and coproantigen detection kits using monoclonal antibodies are available commercially.

**Serological tests:** Cryptosporidiosis is often a disease of the newborn and unless there has been exclusion of exposure to infectious oocysts, serological tests do not offer any benefit.

**Requirements for vaccines and diagnostic biologicals:** There is no control programme for cryptosporidiosis, neither is there a rigorously tested and accepted vaccine available.

**A. INTRODUCTION**

Originally described in 1907, Cryptosporidium spp. were regarded as commensals until their association with diarrhea in young turkeys (*C. meleagridis*) in the 1950s, and with large outbreaks of diarrhea in calves (*C. parvum*) in the 1970s. Cryptosporidium is an important pathogen of livestock and human beings, and since the 1980s, *C. parvum* cryptosporidiosis has been recognised as a common cause of acute self-limiting gastroenteritis in immunocompetent hosts. Fayer (9) provides a good account of the biology of *Cryptosporidium*.

Cryptosporidiosis is caused by protozoan parasites of the genus *Cryptosporidium* (family Cryptosporididae, order Eucoccidiorida, subclass Coccidiasina, class Sporozoasida, phylum Apicomplexa). Although more than 20 ‘species’ of this coccidian parasite have been described on the basis of the animal hosts from which they were isolated, host specificity as a criterion for speciation appears to be ill-founded as some ‘species’ lack such specificity. Species definition and identification of this genus is constantly changing, with the addition of ‘new’ species based primarily on molecular criteria. Currently, there are 18 ‘valid’ species (Table 1) namely: *C. hominis* found primarily in humans (previously known as *C. parvum* Type 1), *C. parvum*, found in humans and other mammals (previously known as *C. parvum* Type 2), *C. andersoni* and *C. bovis* in cattle, *C. canis* in dogs, *C. musis* in mice, *C. felis* in cats, *C. wrairi* in guinea-pigs, *C. suis* in pigs, *C. fayeri* in red kangaroo (31), *C. macropodum* in grey kangaroo (28), *C. meleagridis* in turkeys and humans, *C. baileyi* in chickens, *C. galli* in adult hens and some wild birds (26, 27), *C. varani* in emerald monitor lizards, *C. serpentis* in snakes and lizards, and *C. molnari* in fish (9). In livestock, *C. parvum*, *C. andersoni*, *C. baileyi* and *C. meleagridis* have been reported to cause morbidity and outbreaks of disease.

*Cryptosporidium bovis* has been described recently (10). Previously identified as *Cryptosporidium* genotype Bovine B (GenBank AY120911), *C. bovis* oocysts are morphologically indistinguishable from *C. parvum* oocysts (Table 1). *Cryptosporidium bovis* is a highly prevalent species that infects primarily post-weaned calves (10, 11). *Cryptosporidium bovis* oocysts were not infectious for neonatal BALB/c mice or for two experimentally exposed lambs (<1 week of age), but were infectious for two calves that were previously infected with *C. parvum*. *Cryptosporidium bovis* was detected in calves, 2–7 months of age, none of which had diarrhoea; *C. bovis* has also been detected in a 2-week-old lamb.

In addition to the 18 valid species, there are over 40 *Cryptosporidium* genotypes (9, 48). Some of these are likely to become recognised as species as further research is carried out. The *Cryptosporidium* cervine, skunk, chipmunk genotype I and the *C. hominis* monkey genotypes (Table 2) have been described in humans. Laboratory identification is required to confirm diagnosis.

In human and non-human hosts, molecular methods including the polymerase chain reaction (PCR), restriction fragment length polymorphism (RFLP) and DNA sequencing have demonstrated a broader range of cryptosporidium species than previously thought. *Cryptosporidium meleagridis*, *C. canis*, *C. musis*, *C. felis* and *C. suis* has been described in immunocompetent and immunocompromised human patients, as well as *C. hominis* and *C. parvum*. For example, *C. meleagridis* oocysts purified from human faeces are indistinguishable from *C. parvum* by conventional methods (described in this chapter), but show genetic identity to *C. meleagridis* from turkeys at a variety of separate genetic loci. There is some evidence that *C. andersoni* can also infect humans. The most significant zoonotic threat for humans is from *C. parvum* and *C. meleagridis*.

The discovery of sequence-based differences within various genes (ribosomal RNA [rRNA], *Cryptosporidium* heat-shock protein 70, actin, *Cryptosporidium* oocyst wall protein [COWP], *Cryptosporidium* thrombospondin-related adhesive protein-1 & -2) and between individual isolates within a ‘previously valid’ species has resulted in revision of the taxonomy of the genus. Some of the 40+ *Cryptosporidium* genotypes currently described may represent different species (9, 48). Therefore, as for *Cryptosporidium* species, the current classification of *Cryptosporidium* genotypes will be subject to change.
Chapter 2.9.4. – Cryptosporidiosis

Table 1. Some differences among species within the genus Cryptosporidium

<table>
<thead>
<tr>
<th>Species</th>
<th>Oocyst dimensions (µm)</th>
<th>Site of infection</th>
<th>Major host</th>
<th>Infectious to humans</th>
</tr>
</thead>
<tbody>
<tr>
<td>C. hominis</td>
<td>4.5 × 5.5</td>
<td>Small intestine</td>
<td>Humans</td>
<td>Yes</td>
</tr>
<tr>
<td>C. parvum</td>
<td>4.5 × 5.5</td>
<td>Small intestine</td>
<td>Neonatal mammalian livestock, humans</td>
<td>Yes</td>
</tr>
<tr>
<td>C. suis</td>
<td>5.05 × 4.41</td>
<td>Small intestine</td>
<td>Pigs</td>
<td>Yes</td>
</tr>
<tr>
<td>C. felis</td>
<td>4.5 × 5.0</td>
<td>Small intestine</td>
<td>Cats</td>
<td>Yes</td>
</tr>
<tr>
<td>C. canis</td>
<td>4.95 × 4.71</td>
<td>Small intestine</td>
<td>Dogs</td>
<td>Yes</td>
</tr>
<tr>
<td>C. meleagridis</td>
<td>4.5–4.0 × 4.6–5.2</td>
<td>Intestine</td>
<td>Turkeys</td>
<td>Yes</td>
</tr>
<tr>
<td>C. muris</td>
<td>5.5 × 7.4</td>
<td>Stomach</td>
<td>Rodents</td>
<td>Yes</td>
</tr>
<tr>
<td>C. andersoni</td>
<td>5.6 × 7.4 (5.0–6.5 × 8.1–6.0)</td>
<td>Stomach</td>
<td>Cattle</td>
<td>No</td>
</tr>
<tr>
<td>C. wrairi</td>
<td>4.0–5.0 × 4.8–5.6</td>
<td>Small intestine</td>
<td>Guinea-pigs</td>
<td>No</td>
</tr>
<tr>
<td>C. bovis</td>
<td>4.7–5.3 × 4.2–4.8</td>
<td>Small intestine</td>
<td>cattle</td>
<td>No</td>
</tr>
<tr>
<td>C. baileyi</td>
<td>4.6 × 6.2</td>
<td>Trachea, bursa of Fabricius, cloaca</td>
<td>Poultry</td>
<td>No</td>
</tr>
<tr>
<td>C. fayeri</td>
<td>4.5–5.1 × 3.8–5.0 (mean 4.9 × 4.3)</td>
<td>Intestine</td>
<td>Red kangaroo (Macropus rufus)</td>
<td>No</td>
</tr>
<tr>
<td>C. macropodum</td>
<td>Grey kangaroo (Macropus giganteus)</td>
<td></td>
<td></td>
<td>No</td>
</tr>
<tr>
<td>C. galli</td>
<td>8.0–8.5 × 6.2–6.4</td>
<td>Proventriculus</td>
<td>Finches, chicken</td>
<td>No</td>
</tr>
<tr>
<td>C. serpentis</td>
<td>5.6–6.6 × 4.8–5.6</td>
<td>Stomach</td>
<td>Reptiles</td>
<td>No</td>
</tr>
<tr>
<td>C. varanii</td>
<td>6.3 × 5.5</td>
<td>Intestine</td>
<td>Emerald monitor lizard (Varanus prasinus)</td>
<td>No</td>
</tr>
<tr>
<td>C. molnari</td>
<td>4.72 × 4.47</td>
<td>Intestine</td>
<td>Fish (gilthead seabream)</td>
<td>No</td>
</tr>
<tr>
<td>C. scophthalmi</td>
<td>3.7–5.0 × 3.0–4.7 (mean 4.44 × 3.91)</td>
<td>Intestine, very seldom in the stomach</td>
<td>Fish (turbot)</td>
<td>No</td>
</tr>
</tbody>
</table>

For many of the Cryptosporidium species in Table 1, oocyst size and shape are similar. This makes species identification based on oocyst morphometry at the light microscope level difficult if not impossible, due to size overlap.

Table 2. Some Cryptosporidium genotypes, infection site, and their infection status relative to humans

<table>
<thead>
<tr>
<th>Cryptosporidium genotypes</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Intestinal</strong></td>
</tr>
<tr>
<td>Bear</td>
</tr>
<tr>
<td>Deer mice</td>
</tr>
<tr>
<td>Goose (×2)</td>
</tr>
<tr>
<td>Monkey*</td>
</tr>
<tr>
<td>Ostrich</td>
</tr>
<tr>
<td>Raccoon</td>
</tr>
<tr>
<td>Snake</td>
</tr>
</tbody>
</table>
**Table 2 cont. Some Cryptosporidium genotypes, infection site, and their infection status relative to humans**

<table>
<thead>
<tr>
<th>Cryptosporidium genotypes</th>
<th>Gastric</th>
<th>Lizard</th>
<th>Tortoise</th>
<th>Woodcock</th>
</tr>
</thead>
<tbody>
<tr>
<td>C. galli Finch</td>
<td>C. muris Japanese field mouse genotype</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Key: * infectious to humans. (Data from references 9, 47, 48).

1. **Clinical signs**

Cryptosporidium parvum cryptosporidiosis is a cause of scour in young, unweaned farmed livestock including calves, lambs, goat kids and alpaca. Endogenous stages infect enterocytes of the distal small intestine, caecum and colon. Villous atrophy, shortening of microvilli and sloughing of enterocytes are the major pathological changes associated with disease, and affected animals usually recover within 2 weeks of showing signs of illness. Scouring is most common in young animals but weaned and adult animals can also become infected. Signs can range from a mild to inapparent infection in older animals to severe scouring in young animals. Mortality is low unless occurring as a mixed infection with other enteric pathogens such as rotavirus. Older animals can remain infected and excrete oocysts that can be transmitted to other susceptible hosts.

Cryptosporidium parvum infections of cattle can cause varying degrees of dehydration, dullness, anorexia, fever and loss of condition. Mortality may be high. Rarely do they cause the acute dehydration, collapse and high mortality seen with enterotoxigenic Escherichia coli or rotavirus, which can occur at a similar time. Oocysts can be detected in clinically normal and clinically ill hosts. Scouring calves and lambs can excrete between 10^9 and 10^8 oocysts per g of faeces. Infected adult cattle excrete far fewer oocysts, yet subclinical infections of adult cattle can generate similar numbers of oocysts over a 12-month period.

Cryptosporidium andersoni colonises the digestive glands of the abomasum of older calves and adult cattle. The microvilli of peptic glands are destroyed by endogenous stages, which may account for the elevated concentrations of plasma pepsinogen detected in infected hosts. Some infected animals exhibit reduced weight gain compared with uninfected controls. Infected cattle do not develop diarrhoea, but can excrete oocysts for several months.

Cryptosporidium is a primary pathogen in chicken, turkeys and quail, causing respiratory and/or intestinal disease, leading to morbidity and mortality. Currently, two named species (21) infect chicken and turkeys (C. baileyi and C. meleagridis), and a third, unnamed species infects quail (Cryptosporidium sp.). Cryptosporidium spp. are common intestinal infections in broiler chicken in the USA and Japan. Cryptosporidium baileyi cryptosporidiosis is a disease of the epithelial lining of the bursa of Fabricius and cloaca of chicken, although the trachea and the conjunctiva are lesser sites of infection. Cryptosporidium baileyi intestinal cryptosporidiosis of chicken does not normally result in gross lesions or result in overt signs of disease. Villous atrophy, shortening of microvilli and enterocyte detachment are the major pathological changes associated with disease. Cryptosporidium baileyi respiratory cryptosporidiosis of chicken can result in severe morbidity and, on occasion, mortality. Initially, severe disease is accompanied by sneezing and coughing, followed by head extension to facilitate breathing. Epithelial cell deciliation and hyperplasia, mucosal thickening and discharge of mucocellular exudate into the airways are major pathological changes associated with disease in young broilers. Severe signs of respiratory disease can last up to 4 weeks post-infection (7). Cryptosporidium baileyi cryptosporidiosis of turkeys is similar to that of chicken. Chicken isolates of C. baileyi cause infection in other birds.

Cryptosporidium meleagridis cryptosporidiosis is a disease of the ileum of turkey and other poults and human beings. Cryptosporidium meleagridis cryptosporidiosis can cause severe diarrhoea in turkey poults. Villous atrophy, crypt hyperplasia and shortening of microvilli are major pathological changes associated with disease (7). Transmission of a turkey isolate of C. meleagridis to chicken and domestic ducks has been reported.

Cryptosporidium galli cryptosporidiosis is a disease of adult hens and some wild and exotic birds (26, 27, 30). Unlike the life cycle stages of either C. meleagridis or C. baileyi, the life cycle stages of C. galli are limited to the epithelial cells of the proventriculus. Histology reveals Cryptosporidium parasites in the proventriculus (surface, ducal, and glandular epithelium) (3, 26, 27, 30). Clinical signs include puffed plumage with head held under the wing, responsiveness to external stimuli, and failure to thrive (30). Histopathology of haematoxylin and eosin stained sections from finches demonstrated necrosis and hyperplasia of proventricular glandular epithelial cells, and a mixed inflammatory cell infiltration into the lamina propria of the proventriculus associated with large numbers of Cryptosporidium oocysts attached to the surface of glandular epithelial cells (30). Cryptosporidium galli oocysts (8.0–8.5 × 6.2–6.4 μm) are larger than those of C. baileyi.
Respiratory and intestinal cryptosporidiosis have been reported in commercially grown quail caused by *Cryptosporidium* of an inadequately described species (*Cryptosporidium* sp.) whose oocysts are smaller than those of *C. baileyi* and are not infectious to chicken or turkeys. Pathological changes are similar to those described for *C. baileyi* respiratory and intestinal cryptosporidiosis of chicken (7).

2. Infectious dose

The infectious dose for *C. parvum* varies from isolate to isolate and from host species to host species. For mouse (CD-1 strain) neonates the ID$_{50}$ (median infectious dose) is between 87 and 60 oocysts (19). Ten oocysts produced infection in two out of two primates tested, and five oocysts produced clinical disease in gnotobiotic lambs. The infectious dose for cattle is not known, but is thought to be small. Whether *C. parvum* isolates vary in their ability to colonise different host species is unknown. In healthy adult human volunteers, the ID$_{50}$ is also dependent on both the isolate and the host immune status. In human volunteer infectivity studies, *C. parvum* isolates differ in their ID$_{50}$, their attack rate, and the duration of diarrhoea they induce. The ID$_{50}$ of the *C. parvum* UCP isolate (Ungar *Cryptosporidium parvum*, human derived, from Dr B. Ungar, USA) is 1042 oocysts; the *C. parvum* IOWA isolate (bovine derived, from Ames, Iowa, USA) is 132 oocysts; and the *C. parvum* TAMU isolate (equine derived, from Texas A & M University, USA) is 9 oocysts (25). Oral infection with 100 *C. baileyi* oocysts can result in intestinal cryptosporidiosis (7).

3. Transmission

Transmission can occur via any route by which material contaminated with viable oocysts excreted by infected individuals can be ingested. Practices likely to enhance the spread of cryptosporidiosis include indoor calving and lambing and the communal feeding and husbandry of neonates, where young susceptible animals are in close contact with each other and the faeces of infected animals. Similarly, the disposal of faeces, farmyard manure or other contaminated waste in land-based dumps, when followed by periods of heavy rainfall or melting snow can lead to *C. parvum* oocyst contamination of water courses. These courses may be used as a source of drinking water for other animals and for potable water for human consumption. Contaminated waste includes both the liquid and solid by-products of animal husbandry.

4. Maintenance of infection

A variety of wild mammals act as hosts to *C. parvum* (9, 39, 43, 47), particularly neonates, but little is known of the importance of their involvement in transmitting infection to, or maintaining infection, in domesticated species in farmyard environments. Their role in ‘on farm’ epidemiology in domesticated species is also uncertain. The methods used for diagnosing infection in small mammals and wildlife are the same as those described for farm animals. Oocysts are environmentally robust and can survive for long time periods (>6 months) in moist, cool microclimates. Evidence exists for transmission of cryptosporidiosis from clinically normal dams to suckling neonates, but, in general, the duration of the carrier state remains unknown. A variety of bird species act as hosts to *C. baileyi*.

**B. DIAGNOSTIC TECHNIQUES**

1. Identification of the agent

There is no prescribed test for diagnosing *Cryptosporidium* infection. The demonstration of *Cryptosporidium* species oocysts or *Cryptosporidium* antigen in a properly collected and handled sample is sufficient for a positive diagnosis, and the methods of choice for collection of the samples are non-invasive. There are no reproducible *in-vitro* culture techniques available to amplify parasite numbers prior to identification, therefore the detection of the oocyst (the transmissive stage), *Cryptosporidium* antigen and/or DNA from faeces, or other suitable body fluids are the methods of choice. In addition to these tests, haematoxylin and eosin can be used for histological confirmation of the diagnosis on post-mortem. Haematoxylin and eosin histology is useful for confirming diagnosis, is commonplace world-wide, and will not be described in this chapter.

Further analyses including species and/or *C. parvum* subtype identity, can be performed on *Cryptosporidium* DNA using molecular techniques, such as the PCR-RFLP and/or sequencing of products amplified from defined genetic loci. This not only confirms diagnosis, but also provides discrimination beyond that possible with morphology and morphometry using light microscopy.

For *Cryptosporidium* species that infect the gastrointestinal tract (Table 1), primary diagnosis is based on the demonstration of oocysts in faeces by conventional tinctorial stains, fluorescent/immunofluorescent stains or *Cryptosporidium* antigen(s) (copro-antigens) in faeces by enzyme-linked immunosorbent assay (ELISA) or immuno-chromatographic (IC) methods. The majority of diagnostic methods have been developed using *C. parvum* because of its commercial importance and availability. There is anecdotal evidence indicating that in a minority of samples, the methods described below may not detect all isolates. The methods described below are
expected to detect most *C. parvum* infections, but their usefulness for detecting non-*parvum* species from clinical material is less well understood.

Demonstration of *Cryptosporidium* oocysts or *Cryptosporidium*-specific antigen in faecal samples is the most appropriate test for most applications. Most infections that cause morbidity and/or mortality in mammals are likely to be due to *C. parvum* cryptosporidiosis. The species of *Cryptosporidium* responsible can be determined by PCR-RFLP or sequencing of *Cryptosporidium* DNA isolated from oocysts, later. There are no international standards for the preparation of purified oocysts, antisera, antigens, monoclonal antibodies (MAbs) or hybridomas, although a variety of purified oocysts and coproantigen detection kits using MAbs are available commercially.

a) **Laboratory diagnosis**

The diagnostic features of *C. parvum* oocysts viewed in suspension using Nomarski Differential Interference Contrast (DIC) microscopy are as follows. Oocysts are smooth, thick walled, colourless, have spherical or slightly ovoid bodies containing, when fully developed (sporulated), four elongated, naked (i.e. not within a sporocyst[s]) sporozoites and a cytoplasmic residual body. The modal size measurement of *C. parvum* oocysts is 4.5 × 5.0 µm (range 4–6 µm).

Diagnosis is normally established by conventional microscopic methods, and the modified Ziehl–Neelsen (mZN) or auramine phenol (AP) methods using unconcentrated faecal smears are frequently used (5, 6, 32, 37). Where low oocyst numbers are expected in samples, or purified oocysts are required for molecular investigations, concentrating oocysts in faecal samples can increase the sensitivity of detection. Sugar (e.g. Sheather), salt, zine sulphate or formalin-ether (formalin-ethyl acetate) solutions or specific concentration techniques, such as immuno-magnetic separation, are the best options for concentrating oocysts from faeces (32, 33, 37).

b) **Demonstration in faeces**

Stool samples from most clinically ill cases will contain large numbers of thick-walled oocysts and sufficient *Cryptosporidium* antigen, therefore, the use of standard staining and immunological techniques should result in a positive diagnosis. The numbers of clinically normal excretors is not known, and, given the insensitivity of conventional methods, low oocyst excretors may not be diagnosed using conventional techniques, as oocyst numbers may be below the limit of detection of these methods (37, 41, 42). In clinically ill animals, oocysts can normally be demonstrated in unconcentrated stool smears. The use of an oocyst concentration method can enhance the detection rate. Both flotation and sedimentation methods are suitable for concentrating *Cryptosporidium* spp. oocysts, and oocyst antigens can still be sought in faeces following these oocyst concentration procedures.

Oocysts might not be detectable in clinical samples from all cryptosporidiosis cases, and the absence of oocysts in repeated submissions of samples from symptomatic hosts does not necessarily indicate the absence of infection. In these instances, and particularly when clinical suspicion is high, oocyst negative stool samples should be subjected to antigen and/or PCR-based detection, as sufficient *Cryptosporidium* antigen or DNA from asexual life cycle forms should be present in faeces (37). This is a major advantage of copro-antigen detection immunoassays. Nucleic acid detection tests such as the PCR are being used increasingly as they offer both improved sensitivity and species/genotype/subtype identity. For PCR-based methods, nested PCR methods, being more sensitive than direct PCR methods, are likely to have a higher diagnostic index (37).

*Cryptosporidium* spp. positive faecal samples should be available to personnel who are familiarising themselves with staining and concentration techniques, and smears from positive faecal samples should be included each time a test is performed. Stool samples containing *C. parvum* oocysts can be stored at 4°C in either 2.5% K₂Cr₂O₇ or 10% formalin for reference purposes. Similarly, oocyst-positive faecal smears, air dried and fixed in absolute methanol, can be prepared in advance from previous positive samples for use as positive controls. Where bronchio-pulmonary involvement is suspected, similar tests can be performed on bronchial and pleural exudates or lavages.

Note that 2.5% K₂Cr₂O₇ can be inhibitory to PCR. Oocyst positive stool samples or partially purified oocysts stored in 2.5% K₂Cr₂O₇ and intended for nucleic acid amplification by PCR should be washed in deionised water to remove residual 2.5% K₂Cr₂O₇ prior to DNA extraction. A series of three washes each followed by centrifugation (3000 g for 10 minutes), removal of the supernatant and resuspension of the pellet in deionised water should minimise PCR inhibition by 2.5% K₂Cr₂O₇. It should be noted that inhibitory factors can still be present even after long-term (>6 months) storage.

c) **Laboratory staff and operator safety**

*Cryptosporidium* is included in laboratory risk Hazard Group 2 and all laboratory procedures that can give rise to infectious aerosols must be conducted in a biosafety cabinet. Specimens for *Cryptosporidium*
analyses can contain other pathogenic organisms and should be processed accordingly. In order to safeguard the health of laboratory workers, the safety procedures outlined in Chapter 1.1.2 Biosafety and biosecurity in the veterinary microbiological laboratory and animal facilities must be followed.

The laboratory should have an internal and external quality assurance programme in place as outlined in Chapter 1.1.3 Quality management in veterinary testing laboratories.

d) Collections and submission of samples

Where possible, specimens for primary diagnosis should be collected during acute infection, and should be processed as soon as possible. Ideally, transportation systems should be selected to ensure that specimens arrive at the laboratory within 24 hours. If prompt examination for Cryptosporidium cannot be carried out, the deterioration of protozoan morphology and their overgrowth by other microorganisms, particularly yeasts, can be reduced by the addition of 10% aqueous (v/v) formalin, although 10% formalin can interfere with PCR tests. Both oocyst morphology for microscopic identification and sporozoite DNA for PCR testing can usually be preserved for long periods at 4°C without formalinisation. Faeces can be stored frozen for over 2 years without affecting the ability to extract Cryptosporidium DNA for molecular analysis. In some instances, this can enhance DNA extraction rates, possibly due to the softening of the outer oocyst wall. Repeated freeze–thawing of frozen faecal samples is not recommended as the released DNA will degrade.

The procedures used for collection and transport of specimens are critically important for successful laboratory analyses. Specimens should be collected in a suitable leak-proof sample container and should be enclosed in secure primary and secondary packaging. Procedures for packaging and shipping of specimens must be as outlined in the International Air Transport Association’s Dangerous Goods Regulations (16). These regulations are summarised in Chapter 1.1.1 Collection and shipment of diagnostic specimens.

e) Threshold of detection in faeces

Most tinctorial and fluorescence methods for detecting oocysts, and ELISA and IC for detecting Cryptosporidium antigens, are relatively insensitive. These methods can detect oocysts in clinically ill animals, but may not be sufficiently sensitive to detect infection in clinically normal animals. Anusz et al. (2) reported a detection limit of \(10^6\) oocysts per ml of faeces using the Kinyoun modification of mZN on unconcentrated faecal smears. Concentrating oocysts in the sample can increase the sensitivity of detection. In oocyst-positive human stool samples, between \(1 \times 10^4\) and \(5 \times 10^4\) oocysts per g of unconcentrated stool are necessary to obtain a 100% detection efficiency using the Kinyoun mZN staining method (41). Variations in faecal consistency influence the ease of detection, with oocysts being more easily detected in concentrates made from watery, diarrhoeal specimens than from formed stool specimens (41). In addition to microscopic techniques, a number of antigen-capture ELISAs and ICs have been reported in the literature with detection limits in the region of \(3 \times 10^5–10^6\) oocysts per ml (2, 29, 37), which indicates that they do not appear to offer increased sensitivity over microscopical methods.

In bovine faecal samples, oocysts were not detected in samples seeded with 10,000 C. parvum oocysts per g following formol–ether sedimentation and examination using AP or immunofluorescence (IF) staining. When oocysts were concentrated using sucrose flotation, the threshold of detection was 4000 oocysts per g for both staining methods. After salt flotation, 4000 oocysts per g could be reliably detected by AP staining, but the detection limit was increased to 6000 oocysts per g using IF staining (41). Webster et al. (42) also compared microscopy with PCR and found that PCR coupled with immunomagnetic particle separation (IMS) of oocysts from faecal samples detected five oocysts per ml of diluted faeces, which corresponds to \(80–90\) oocysts per g. Even allowing for the dilution of formed faecal samples required for IMS, this represented an increase in sensitivity of several orders of magnitude over the conventional coprodiagnostic methods. Currently, a variety of sensitive, PCR-based tests are available (see Section B.1 Nucleic acid recognition methods).

• Preparation of unconcentrated faecal (or appropriate body fluid) smears (include a positive control slide each time this procedure is performed)
  • Test procedure
    i) Wear protective clothing and disposable gloves. Score the reference number of the specimen on a microscope slide with a diamond marker\(^1\), and use separate microscope slides for each specimen. Place 1 drop of saline (about 50 µl) in the centre of the slide.

\(^1\) Alternatively, a lead pencil can be used to mark the etched (frosted) portion of a frosted glass microscope slide.
ii) Remove a small sample of faeces (about 2 mg) with the tip of a clean applicator stick\(^2\) (or pipette after mixing thoroughly, if liquid) and emulsify the sample in saline by thorough mixing. For liquid stools (or other appropriate body fluid) dispense one drop directly on to the slide. In liquid stools, mucus strands and exudates or pus can be mixed with saline on the microscope slide. Liquid stools can be diluted with a drop of 150 mM saline solution.

iii) Prepare a medium to thick smear with areas of varying thickness. Ensure that the smear is of the correct transparency\(^3\).

iv) Air dry the smear at room temperature.

v) Fix the smear\(^4\) in methanol for 3 minutes.

f) Preparation of faecal (or appropriate body fluid) smears following concentration by flotation or sedimentation

No flotation or sedimentation method is specific for Cryptosporidium spp. oocysts. Flotation fluids are denser than the parasites to be concentrated and are formulated to a defined specific gravity using a suitable hydrometer available from most large laboratory suppliers. Parasites concentrated by flotation or sedimentation methods can be identified by all the methods described in this chapter. Flotation/sedimentation fluids can sometimes interfere with diagnostic tests. Excess sucrose can reduce both oocyst attachment to glass slides and subsequent antibody binding, prolonged exposure to NaCl can distort morphology and morphometry, and formalin can reduce the sensitivity of PCR reactions. When oocysts are concentrated, excess flotation/sedimentation fluid can be removed by washing the concentrate in water and re-centrifuging. The supernatant is then aspirated and discarded, care being taken not to disturb the pellet. These concentration methods are suitable for any appropriate body fluid that could contain oocysts.

1. Flotation

The flotation principle uses a liquid suspending medium, which is denser than the oocysts to be concentrated. Therefore, when mixed with flotation fluid, the oocysts rise to the surface and can be skimmed out of the surface film and detected using the chosen method. For a flotation fluid to be useful in diagnostics, when morphology and morphometry are the critical factors, the suspending medium must not only be heavier than the object to be floated but must not produce shrinkage sufficient to render the object undiagnosable (32). Sucrose flotation, zinc sulphate flotation and saturated salt flotation methods are all suitable for concentrating cryptosporidium oocysts. The following is a description of the methods used to prepare flotation solutions and to concentrate oocysts.

- **Sucrose flotation**

Sucrose solution (specific gravity 1.18) is prepared in a glass beaker by adding 256 g of sucrose to 300 ml of deionised water. The solution is gently heated (<60°C) and continuously stirred with the aid of a magnetic stirrer on a hot plate stirrer, until the sucrose has dissolved completely. The sucrose solution is either placed on ice or in a refrigerator until its temperature has adjusted to 4°C. The cold sucrose solution is transferred to a 500 ml measuring cylinder and its specific gravity is adjusted to 1.18 by adding sufficient cold, deionised water (4°C). The sucrose solution is transferred to a screw-cap glass bottle, labelled, dated, initialled and stored at 4°C until used.

- **Zinc sulphate flotation**

Zinc sulphate solution (specific gravity 1.18) is prepared in a glass beaker by adding 100 g of zinc sulphate to 300 ml of deionised water. The solution is gently heated (<60°C) and continuously stirred with the aid of a magnetic stirrer on a hot plate stirrer, until the zinc sulphate had dissolved completely. The zinc sulphate solution is transferred to a 500 ml measuring cylinder and its specific gravity is adjusted to 1.18 by adding sufficient cold, deionised water (4°C). The zinc sulphate solution is transferred to a screw-cap glass bottle, labelled, dated, initialled and stored at 4°C until used.

- **Salt flotation**

Saturated salt solution (specific gravity 1.2) is prepared by adding approximately 200 g of sodium chloride to 200 ml of deionised water. The solution is gently heated (<60°C) and continuously stirred with the aid of a magnetic stirrer on a hot plate stirrer. Small amounts of sodium chloride

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\(^2\) For formed stools, the sample should include portions from the surface and from within the stool.

\(^3\) Moderately thick smears are recommended for this procedure. If the smear is too thin or thick, oocysts will be missed. An acceptable thickness can be achieved when either the hands of your watch or the print on this page can just be read when viewed through the preparation.

\(^4\) Air-dried, methanol-fixed smears can be kept at room temperature for >6 months before staining.
(approximately 10 g) are added at 10-minute intervals until the solution becomes saturated. The saturated salt solution is then decanted into a clean glass bottle and either placed on ice or in a refrigerator until its temperature had adjusted to 4°C. The cold saturated salt solution is then transferred into a 500 ml measuring cylinder and its specific gravity is adjusted to 1.2 by adding cold, deionised water (4°C). The saturated salt solution is transferred to a screw-cap glass bottle and labelled, dated, initialled and stored at 4°C until used.

Brine is a concentrated aqueous NaCl solution, which has a specific gravity between 1.120 and 1.200 depending on the impurity of the salt used. While suitable for concentrating *Cryptosporidium* spp. oocysts, some protozoan cysts can become badly shrivelled or open up in this flotation fluid. The optimal time to examine specimens obtained from brine flotation is between 5 and 20 minutes after their recovery following flotation.

Centrifugal flotation has also been used to recover *Cryptosporidium* oocysts (and a various other parasite cysts and ova) from faeces. Most centrifugal flotation methods are based on modifications of the Clayton-Lane technique, whereby oocysts are concentrated by flotation and collected as a hanging drop on the underside of a glass cover-slip placed onto the positive meniscus of the flotation fluid. Centrifugation is used to separate particles which are denser than the flotation fluid from oocysts and particulates which will float on the surface of the flotation fluid. The inclusion of a centrifugation step speeds up separation of oocysts from other particulates (and hence, the concentration of oocysts) and minimises the risk of the flotation fluid adversely affecting the morphometry or morphology of the oocysts. The operator should note the health and safety issues, including lacerations and puncture wounds, associated with handling cover-slips.

**Concentration of *Cryptosporidium* spp. oocysts by flotation**

- **Test procedure**
  i) Wear protective clothing and disposable gloves. Transfer approximately 1–2 g of faeces\(^5\) with an applicator stick to 3 ml of flotation fluid in a 12 ml test tube and mix thoroughly. If the stool is liquid, mix thoroughly, and dispense approx. 1–2 ml fluid into the test tube.
  ii) Add, with gentle stirring, sufficient flotation fluid to form a positive meniscus at the rim of the test tube. Remove any large particles from the surface and if necessary, add more flotation fluid to maintain this positive meniscus.
  iii) Leave for 20 minutes, then, taking great care not to disturb the positive meniscus gently remove the meniscus with a disposable pipette and dispense gently on to a microscope slide\(^6\).
  iv) Air dry the smear at room temperature.
  v) Fix the smear\(^7\) in methanol for 3 minutes.

**Concentration of *Cryptosporidium* spp. oocysts by centrifugal flotation**

- **Test procedure**
  i) Wear protective clothing and disposable gloves. Transfer approximately 1–2 g of faeces\(^8\) with an applicator stick to 3 ml of flotation fluid in a 12 ml centrifuge tube and mix thoroughly. If the stool is liquid, mix thoroughly, and dispense approx. 1–2 ml fluid into the centrifuge tube.
  ii) Add, with gentle stirring, sufficient flotation fluid to form a positive meniscus at the rim of the centrifuge tube. Remove any large particles from the surface and if necessary, add more flotation fluid to maintain this positive meniscus.
  iii) Place the centrifuge tube in a bench top centrifuge with swing out buckets, and place a 22 mm × 22 mm glass cover-slip onto the rim of the centrifuge tube, so that it flattens the positive meniscus. Add a balance tube if necessary, and centrifuge at 1100 \(g\)\(^9\) for 5 minutes.
  iv) Once the centrifuge stops, pick up the glass cover-slip between index finger and thumb at opposing corners of the cover-slip. A hanging drop will be present on the underside of the cover-slip. Carefully place the cover-slip, with the hanging drop lowermost, onto a glass microscope slide.

\(^5\) For formed stools, the sample should include portions from the surface and from within the stool.

\(^6\) Moderately thick smears are recommended for this procedure. If the smear is too thin or thick, oocysts will be missed. An acceptable thickness can be achieved when either the hands of your watch or the print on this page can just be read when viewed through the preparation. Score the reference number of the specimen on a microscope slide with a diamond marker, and use separate microscope slides for each specimen. Alternatively, a lead pencil can be used to mark the etched (frosted) portion of a frosted glass microscope slide.

\(^7\) Air-dried, methanol-fixed smears can be kept at room temperature for >6 months before staining.

\(^8\) For formed stools, the sample should include portions from the surface and from within the stool.

\(^9\) Centrifugation at speeds higher than 1100 \(g\) for longer (>5 minutes) periods of time is not advised as some parasites may deform and/or rupture and collapse.
2. **Centrifugal sedimentation**

Parasites will settle more rapidly if the stool suspension is subjected to centrifugation, however, food particles will also sediment more rapidly and can mask the presence of parasites in the film examined. To overcome this potential problem, larger food particles can be removed prior to centrifugation by filtering the emulsified stool through a sieve with an aperture size large enough for parasites to pass through, but which retains the larger food particles. As this process is more efficient than sedimentation by gravity, a smaller faecal sample (500 mg–1 g: the size of a pea) is sufficient for examination. Although centrifugation concentrates the material more quickly, faecal debris, which can obscure parasites, remains present. The efficiency of detection is increased by adding formalin for fixation and preservation of parasites, and ether to remove fats and oils. Both 10% formalin and ether are bactericidal. After centrifugation, a fatty plug, which may adhere to the inner walls of the tube, can be seen at the interface of the two liquids. The ether layer, the fatty plug and the formalin below it are discarded and the whole pellet is retained for examination.

Many modifications to this procedure have been advocated, and the following protocol is typical of the method used in diagnostic laboratories. Less distortion of protozoan cysts occurs with this method than with zinc sulphate flotation. This method achieves a concentration of 15–50-fold, dependent on the parasite sought, and provides a good concentrate of protozoan cysts and helminth eggs, which are diagnostically satisfactory. All steps that can generate aerosols (excluding centrifugation) should be performed in an operator protection safety cabinet.

**Concentration of Cryptosporidium spp. oocysts by (formol/ether) centrifugal sedimentation**

- **Test procedure**
  1. Wear protective clothing and disposable gloves. Sample approximately 500 mg–1 g faeces with an applicator stick and place in a clean 12–15 ml centrifuge tube containing 7 ml of 10% formalin. If the stool is liquid, dispense about 750 µl into the centrifuge tube.
  2. Break up the sample thoroughly and emulsify using the applicator stick.
  3. Filter the resulting suspension through a sieve into a beaker, then pour the filtrate back into the same centrifuge tube.
  4. Add 3 ml of diethyl ether (or ethyl acetate) to the formalinised solution, seal the neck of the tube with a rubber bung (or a gloved thumb over the top of the tube) and shake the mixture vigorously for 30 seconds. Invert the tube a few times during this procedure and release the pressure developed gently by removing the rubber bung (or your thumb) slowly.
  5. Centrifuge the tube at 1100 g for 2 minutes.
  6. Loosen the fatty plug with a wooden stick by passing the stick between the inner wall of the tube and the plug. Discard the plug and the fluid both above and below it by inverting the tube, allowing only the last one or two drops to fall back into the tube. Discard this fluid, containing diethyl ether and formalin, into a marked re-sealable liquid waste container.
  7. Re-suspend the pellet by agitation. Pour the whole, or the majority of the re-suspended pellet on to a microscope slide, or transfer the re-suspended contents on to a microscope slide with a disposable pipette, and air dry.

A commercial device for concentrating helminth ova, larvae and protozoan cysts and oocysts using the formalin-ether method is available. Sold as the Fecal Parasite Concentrator, it is an enclosed system, and consists of two polypropylene tubes, a flat-bottomed tube used for emulsifying the stool, and a conical tube used for centrifugation, with an interconnecting sieve. The comprehensive method states that both fresh and preserved (10% formalin, merthiolate-iodine-formalin, polyvinyl alcohol, and sodium acetate-formalin) stool specimens may be used.

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10 This is the size of a pea.
11 The sample should include portions from the surface and from within a formed stool.
12 425 µm aperture size, 38 mm diameter is equivalent to 36 mesh British Standard (BS 410-86) or 40 mesh American Standard (ASTM E11-81). The skirt of the sieve should fit neatly into the rim of the beaker. Debris trapped on the sieve is discarded by inverting the sieve and passing a stream of tap water through the mesh. Both the sieve and the beaker should be washed thoroughly in running tap water between each sample.
13 Ethyl acetate, although less flammable than diethyl ether is nevertheless flammable, therefore the procedure should be performed in well ventilated areas, ensuring that they contain no naked flames. Avoid prolonged breathing or skin contact.
14 Centrifugation at speeds higher than 1100 g for longer (>5 minutes) periods of time is not advised as some parasites may deform and/or rupture and collapse.
15 Too large a pellet is indicative of one or more of the following: centrifuging above the recommended speed and/or time, insufficient shaking (step iv), taking too large a faecal sample.
16 FPC, Evergreen Scientific, 2300 East 49th Street, P.O. Box 58248, Los Angeles, California 90058, USA; http://www.evergreensci.com/micro/hfpc.htm
g) Conventional staining methods

Both mZN and AP are effective for detecting Cryptosporidium oocysts in faeces (5, 6, 32, 37). mZN-stained slides should be screened under the ×40 objective lens and putative oocysts confirmed and measured under the ×100 objective lens (morphology and morphometry) using a bright-field microscope with a ×10 eyepiece. AP-stained slides require to be read using an epifluorescence microscope equipped with a fluorescein isothiocyanate (FITC) filter set (excitation 490 nm; emission 510 nm). A UV filter set (excitation 355 nm, emission 450 nm) can assist in visualising AP-stained sporozoites. AP-stained slides can be screened under the ×20 objective lens and oocysts with typical morphology can be confirmed under the ×40 objective lens. The ×100 objective lens must be used for all morphometric (size) measurements. AP-stained oocysts visualised under either the FITC or UV filters can be measured by slowly increasing the voltage (light intensity) of the bright-field light source so that both fluorescent and bright-field images can be seen concurrently. The object can then be measured with the eye-piece graticule.

- Calibration of size using the eye-piece and stage micrometers

Diagnosis of intact organisms often necessitates the measurement of the size and shape (morphometry) of the organism in question, in order to ensure that it falls within the accepted range of standard parameters (e.g. size and shape) for the species in question. At the light microscope level, measurement of objects <1 mm is achieved by means of a stage micrometer used in conjunction with an eye-piece micrometer. Objects are measured in Systeme International (SI) units, and the standard unit of measurement for conventional microscopy is the micron (µ = 0.001 mm).

The stage micrometer consists of a 76 × 26 mm glass slide that has a millimetre scale, graduated in microns permanently mounted on it. The eye-piece micrometer is a disc of transparent glass or plastic bearing a graduated scale, which is placed in one of the eye-pieces of a binocular microscope. The scale is usually 1 cm in length and is subdivided into millimetre intervals. When the microscope is focused on the object to be measured, both the scale on the eye-piece micrometer and the image of the object are seen simultaneously in focus. The standard scale on the stage micrometer is usually 1 or 2 mm.

When measurements are to be made, the appropriate objective lens, which is dependent on the magnification required, is chosen, and the number of divisions corresponding to the length or breadth of the image of the object is read on the scale of the eye-piece micrometer. The observed measurement is translated into real length (which corresponds to the number of eye-piece micrometer divisions representing the chosen parameter to be measured) by substituting the stage micrometer for the object and determining the number of divisions on the eye-piece micrometer corresponding to a definite number of divisions of the millimetre scale on the stage micrometer, under the same magnification.

Remember that your calculation, in real length, of the value of the division on the eye-piece micrometer scale will only be valid for the magnification of the objective chosen. You will have to recalculate the value of a division on the eye-piece micrometer for each objective of differing magnification on the microscope.

Because morphometry is a significant component of diagnostic parasitology, repetitive measurements of similar objects present in a single sample, or of various objects of varying sizes in sequential samples, necessitating the use of a variety of magnifications, will have to be undertaken. By determining the micrometer value of the eye-piece scale for each objective used, the constant interchange of objects and stage micrometer can be overcome. This enables rapid calculation of morphometry, in millimetres, or fractions thereof, to be undertaken with any of the objective lenses available.

The graticule is placed in the eye-piece by unscrewing the lower component of the eye-piece and placing it into the open tube. It must be seated correctly before the lower component is screwed back on to the eye-piece. Ensure that the diameter of the graticule is similar to the internal diameter of the lower lens tube. Do not touch the surface of the graticule – hold it by its edges. Make sure it is dust and grease free. The eye lens is focused on to the graticule by adjusting it until the scale on the graticule is critically sharp.

The determination is carried out as follows:

i) Insert the eye-piece micrometer, with its scale already in focus, into the microscope, making sure that the graticule scale is the right way up.

ii) Select the lowest power objective lens (e.g. ×10 objective) and focus the microscope on the stage micrometer, rotating the eye-piece and positioning the stage micrometer until the scales of the eye-piece micrometer and the stage micrometer lie parallel and close to or overlapping each other.

iii) Count the number of intervals on the stage micrometer that correspond exactly to a whole number of divisions on the eye-piece micrometer.
iv) Divide the value observed on the stage micrometer by the number of divisions counted on the eye-
piec micrometer scale to determine the value of each division on the eye-piece micrometer scale.

v) Repeat the above for each objective lens.

vi) Keep a permanent record of the calculation of the value for each of the divisions on the eye-piece
micrometer for each objective lens in close proximity to or attached on to the body of the microscope
(e.g. a piece of cardboard stuck to the front of the microscope).

- Example

For each objective lens:
x eye-piece divisions = y µm (on the stage micrometer)
1 eye-piece division = y/x µm

- Microscopical examination of a sample

The sample must be examined in a systematic manner. Observation should commence using the lowest
suitable objective, ensuring that the entire sample is viewed. A suggested scheme is as follows: commence
in the upper left-hand corner of the sample, working across the slide from left to right, one field width at a
time, until the upper right-hand edge of the sample is reached. Move down one field height and continue
working across the slide from right to left, field by field, until the left-hand edge of the sample is reached.
Continue in this manner until the end of the sample (lower right-hand corner) is reached. During this period
of observation, the fine focus should be adjusted continuously so that the depth of the sample is also
scanned. When a suspicious object is located, it is inspected under high power magnification and either
verified or disregarded. If the magnification of the image of the object is insufficient to be able to visualise
definitive morphological characteristics under the high (dry) objective, the immersion lens (×100) must be
used. Wet mounts can be sealed with nail varnish or a proprietary permanent sealant.

Neither tinctorial- nor fluorescence-based stains can determine the species of cryptosporidium present if the
oocysts fall within the size range of 4–6 µm (see Table 1). For mammalian livestock, the consensus opinion
is that the majority of infections are likely to be due to C. parvum, therefore, a preliminary diagnosis of
C. parvum cryptosporidiosis can be made. However, the presence of oocysts of the size range 4–6 µm
does not necessarily indicate that the infectious species is C. parvum. Similarly, for birds, a preliminary
diagnosis of C. baileyi, C. meleagridis or C. galli cryptosporidiosis can be made depending on infection site
and oocyst size. Molecular identification of species/genotype/subtype can be performed later.

- Reporting results of microscopical examination

Negative specimens should be reported as ‘NO Cryptosporidium oocysts seen’.
Positive specimens should be reported as ‘Cryptosporidium oocysts seen’.

A scoring system for positive samples can be used, based on the number of oocysts observed under the
×40 objective lens. However, microscopic examination cannot be considered as a quantitative
determination as oocyst numbers vary considerably during the course of infection.

+ = less than 5 oocysts per slide
++ = 1 to 10 oocysts per field of view
+++ = 11 or more oocysts per field of view

- Modified Ziehl–Neelsen (mZN)

Strong carbol fuchsin: Dissolve 20 g basic fuchsin in 200 ml absolute methanol and mix on a magnetic
stirrer until dissolved. Add 125 ml liquid phenol (GPR [80% w/w in distilled water]) carefully until well mixed,
and make up to the final volume with 1675 ml deionised water. Mix thoroughly. Filter before use through
Whatman No.1 filter paper to remove debris and store in a stock reagent bottle. Label, date and initial.
Store the stock reagent in a dark cupboard at room temperature.

Commercial supplies are also available. Often the concentration of basic fuchsin can vary within the
acceptable range of 1–3%.

1% acid methanol: Carefully add 20 ml hydrochloric acid (GPR/SLR) to 1980 ml of absolute methanol and
mix. Transfer to a stock reagent bottle, and label, date and initial.

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17 Notice that the value calculated in millimetres for each of the divisions on the eye-piece micrometer will be different for
objectives of different magnifications, with values calculated for real length being smaller for each of the divisions of the
eye-piece micrometer with increasing magnification.
0.4% malachite green: Add 2 g malachite green to 480 ml deionised water and mix on a magnetic stirrer. Filter through Whatman No.1 filter paper into a stock reagent bottle, label, date and initial.

- **Test procedure**

Include a positive control slide each time you perform this procedure.

  i) Wear protective clothing and disposable gloves. Fix the air-dried smear or concentrate in methanol for 3 minutes.
  
  ii) Immerse the slide in cold strong carbol-fuchsin and stain for 15 minutes.
  
  iii) Rinse the slide thoroughly in tap water.
  
  iv) Decolourise in 1% acid methanol for 10–15 seconds.
  
  v) Rinse the slide in tap water.
  
  vi) Counterstain with 0.4% malachite green for 30 seconds.
  
  vii) Rinse the slide in tap water.
  
  viii) Air-dry the slide. (The smear can be examined with or without a cover-slip. A little immersion oil is spread over the smear which is then viewed with either dry or oil immersion lenses, without the addition of a cover-slip. An alternative method is to add a cover-slip and mounting medium and then examine the smear.)
  
  ix) Examine for the presence of oocysts by scanning the slide using the ×40 objective lens of a bright-field microscope. Confirm the presence of oocysts under the oil immersion objective lens.
  
  x) Measure the size and shape of the red-stained bodies.

- **Diagnostic features of Cryptosporidium spp. oocysts stained with mZN**

Cryptosporidium spp. oocysts stain red on a pale green background. The degree and proportion of staining varies with individual oocysts. In addition, the internal structures take up the stain to varying degrees. Some may appear amorphous while others may contain the characteristic crescentic forms of the sporozoites. Cryptosporidium parvum oocysts appear as discs, 4–6 µm in diameter. Yeasts and faecal debris stain a dull red. Some bacterial spores may also stain red, but these are too small to cause confusion.

- **Auramine-phenol**

Auramine phenol (AP): Dissolve 3 g phenol in 100 ml deionised water and slowly add 0.3 g Auramine O. Filter through Whatman No. 1 filter paper into a stock reagent bottle. Label, date and initial the stock reagent. Store at room temperature in a light-proof glass bottle with an airtight stopper. Commercially available stains, such as Lempert’s reagent, are also acceptable.

3% Acid methanol: Carefully add 60 ml hydrochloric acid (GPR/SLR) to 1940 ml absolute methanol and mix. Transfer to a stock reagent bottle, and label, date and initial.

0.1% potassium permanganate: Add 0.5 g potassium permanganate to the 499.5 ml deionised water and mix using a magnetic stirrer. Filter through Whatman No. 1 filter paper into a stock reagent bottle, and label, date and initial.

- **Test procedure**

Include a positive control slide each time you perform this procedure.

  i) Wear protective clothing and disposable gloves. Fix air-dried smears or concentrate in absolute methanol for 3 minutes.
  
  ii) Immerse the slides in AP stain for 10 minutes.
  
  iii) Rinse in tap water to remove excess stain.

---

18 Moderately thick smears are recommended for this procedure.

19 Over-destaining must be avoided.

20 Isospora spp. oocysts stain red and appear as large elongated ovoid bodies, tapered at the end and containing either a granular zygote or two sporoblasts. Cyclospora spp. oocysts stain pinkish red and appear as circular discs (8–10 µm in diameter) containing a central morula. The degree and proportion of staining varies with individual oocysts. The unsporulated oocyst is seen generally in stool samples.

21 Moderately thick smears are recommended for this procedure.
Chapter 2.9.4. — Cryptosporidiosis

iv) Decolourise with 3% acid alcohol for 5 minutes.

v) Counterstain in 0.1% potassium permanganate for 30 seconds.

vi) Air dry slide at room temperature22 (see Modified Ziehl–Neelsen (mZN) step viii, above).

vii) Examine for the presence of oocysts, using an epifluorescence microscope equipped with FITC filters, by scanning the slide under the ×20 objective lens. Confirm the presence of oocysts under the ×40 objective lens.

viii) Measure the size and shape of the fluorescent bodies23.

• Diagnostic features of Cryptosporidium spp. oocysts stained with AP

Cryptosporidium spp. oocysts appear ring or ovoid shaped and exhibit a characteristically bright apple-green fluorescence against a dark background. Cryptosporidium parvum oocysts are ring or doughnut shaped, measuring 4–6 µm in diameter. If available, view the preparation under a UV filter (excitation 355 nm, emission 450 nm), as sporozoites are more readily seen under the UV rather than the FITC filter set. Under the UV filter, oocysts appear light green and sporozoites appear yellow green.

• Culture

There is no reproducible method for culturing cryptosporidium from body fluids. In-vitro cell culture systems have been described for semi-purified, infectious oocysts, but they have not been tested in sufficient depth with enough isolates or inhibitory materials to be recommended for routine purposes.

• Immunological methods

Three approaches to the immunological detection of Cryptosporidium oocysts have proven useful, and a variety of commercial kits are available. Each has a similar level of sensitivity, and either unconcentrated or concentrated stool samples can be used depending on the likely number of oocysts in the sample. Immunofluorescence-based kits, using a fluorescein isothiocyanate-conjugated anti-Cryptosporidium MAb that recognises surface exposed epitopes of oocysts (FITC-C-MAbs) are more specific for, and can be more sensitive at, detecting Cryptosporidium oocysts in faecal smears than conventional tinctorial stains. Compared with conventional tinctorial stains, antibody-based detection kits (immunofluorescence, ELISA and immunochromatography) appear to be expensive, considering that many report a similar detection threshold.

a) Direct immunofluorescence

In direct immunofluorescence, a FITC-labelled MAb reactive with genus-specific, surface-exposed epitopes on cryptosporidium oocysts binds to oocysts present in the sample. UV excitation using a FITC filter system (maximum excitation wavelength 490 nm, mean emission wavelength 530 nm) causes the labelled oocysts to exhibit a bright apple-green fluorescence. Materials provided with commercial kits vary but C. parvum oocyst positive and negative controls, FITC-labelled anti-cryptosporidium MAb (provided at the working dilution), and glycerol-based mounting medium containing a photo-bleaching inhibitor are normally included. Known negative and positive samples must always be included in each test.

Air-dried faecal smears or faecal concentrates are fixed in absolute methanol (or acetone, depending on the manufacturers’ instructions) and air dried. Often the slides provided in the kit are well slip slides into which both sample and kit reagents are dispensed and retained. The manufacturers’ instructions must be followed. It is false economy to dilute kit reagents to increase the testing volume. The FITC-labelled anti-Cryptosporidium genus-specific MAb (Cryptosporidium MAb), at the predetermined working dilution, is applied over the fixed, air-dried specimen(s) and the slide(s) are incubated horizontally in the dark in a humidified chamber. Excess antibody is removed by gentle rinsing, and the excess moisture drained. Mounting medium is placed over the specimen and a cover-slip is applied to the sample, ensuring that no air bubbles are trapped over the specimen. If mounting medium is not supplied, a mixture of 50% non-fluorescent glycerol: 50% phosphate buffered saline (PBS) (v/v) is suitable. Samples are scanned using ×20 objective lens, oocysts are confirmed using the ×40 objective lens, and the number of oocysts present is determined. Numbers can be recorded as identified previously. In the absence of a manufacturer’s method, the following method will produce satisfactory results.

The nuclear fluorogen, 4’6-diamidino-2-phenyl indole (DAPI; [C₁₆H₁₅N₅.2HCl, FW 350.2]), can be used to highlight sporozoite nuclei within fluorescent oocysts providing further confirmatory morphological information (13, 34). DAPI is a non-specific DNA intercalator, therefore DNA of other cellular interferents

22 Do not blot slides dry, as some blotting papers contain fluorescent fibres.
23 Putative oocysts are measured by slowly increasing the voltage (light intensity) of the bright-field light source so that both fluorescent and bright-field images can be seen concurrently. Objects can then be measured with the eye-piece graticule.
including bacteria and yeasts will also be stained. DAPI at a working strength of 0.4 µg/ml is particularly useful when oocysts are sought in non-faecal samples (e.g. water and foodstuffs). DAPI intercalates with the nuclei of the sporozoites within viable or non-viable oocysts and causes them to fluoresce sky blue.

A blue filter block (excitation 490 nm; emission 510 nm) is used to visualise FITC-C-mAb localisation and an ultra-violet (UV) excitation (excitation 355 nm, emission 450 nm) is used to determine the presence of DAPI stained sporozoite nuclei.

- **Test procedure**

Include a positive and negative control slide each time this procedure is performed. Positive and negative slides are supplied with the majority of immunofluorescence kits, and *C. parvum* oocysts can be purchased from commercial suppliers

Sources other than *C. parvum* may be available from diagnostic veterinary laboratories or research institutes or facilities. Local distributors may also supply oocysts obtained from commercial sources.

i) Wear protective clothing and disposable gloves. Fix air-dried faecal smears or faecal concentrates in absolute methanol for 5 minutes.

ii) Dispense 50 µl of anti-cryptosporidium MAb at its working dilution on to the well of each slide. Ensure complete coverage of the well.

iii) Place the prepared slide(s) in a humidity chamber with the slide(s) elevated above the absorbent material used to generate humidity. Ensure that the absorbent material is moist.

iv) Place the humidity chamber in an incubator at approximately 37°C for the period of time prescribed by the manufacturer (normally 30–60 minutes).

v) Using a Büchner-type aspirator gently aspirate excess MAb from each well. Tilt the slide to an angle of about 45°C from the horizontal towards the operator, and aspirate the fluid that collects at the bottom of the well(s) by placing the tip of an aspirator close to, but not touching, the fluid. The suction at the aspirator tip will draw the fluid to waste. Repeat this procedure for each slide well containing a sample.

vi) Dispense 50 µl of PBS to each well and allow to stand for 2 minutes at room temperature.

vii) Gently aspirate the PBS from each well as described in step v. Apply a further 50 µl of PBS to each well and allow to stand for a further 2 minutes, before gently aspirating the PBS as described.

viii) Apply 50 µl of a 1/5000 DAPI in PBS solution to each well and allow to stand for 2 minutes at room temperature. The working solution of DAPI is prepared by diluting a 2 mg/ml DAPI stock solution by 1/5000 in PBS (150 mM, pH 7.2). The working solution should be prepared for each day it is required. A stock solution of DAPI (2 mg/ml in methanol) can be stored at 4°C in the dark, indefinitely.

ix) Gently aspirate off the DAPI solution from each well as described in step v.

x) Apply 50 µl of deionised water to each well and leave to stand for 1–3 seconds at room temperature, then gently aspirate the deionised water from each well as described in step v.

xi) Dispense 50 µl of mounting medium to the centre of each well of each slide, then gently apply a cover-slip on to the microscope slide.

xii) Allow the cover-slip to settle into place before scanning the slide.

xiii) Scan the preparation for oocysts under the ×20 and confirm under the ×40 objective of an epifluorescence microscope equipped with an FITC filter set. Measure oocysts under the ×100 objective. If necessary, slides can be stored at room temperature, in the dark, until read.

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24 For example from Kate Miller, Sterling Parasitology Laboratory, University of Arizona, Dept of Veterinary Science and Microbiology, Building 90, Room 306, Tucson, Arizona, 85721, USA: millerk@email.arizona.edu or Geoff and Sue Pritchard, Bunch Grass Farm, 1301 Drury Road, Deary, Idaho, 83823, USA: pritchard@turbonet.com

25 Alternatively, allow the methanol to evaporate to dryness at room temperature.

26 Volumes, times, etc. can vary according to manufacturers’ instructions. Always follow the manufacturers’ instructions.

27 These temporary mounts can be made semi-permanent by sealing around the edges of the cover-slip with clear nail varnish. Allow the cover-slip to settle into place for approximately 30 minutes to 1 hour before sealing with nail varnish. Using the brush supplied with the nail varnish, carefully apply the nail varnish around the perimeter of the settled cover-slip, using the width of the brush as the guide to the width of nail varnish applied. Ensure even coverage around the cover-slip perimeter. Do not leave any gaps. Allow the nail varnish to dry at room temperature before labelling the slide(s) appropriately with their unique identifier number. If required, use a scalpel to carefully shave/scrape any excess dried and hardened nail varnish.

28 Putative oocysts are measured by slowly increasing the voltage (light intensity) of the bright-field light source so that both fluorescent and bright-field images can be seen concurrently. Objects can then be measured with the eye-piece graticule.
- Diagnostic features of Cryptosporidium spp. oocysts stained by FITC-labelled anti-cryptosporidium MAb

Cryptosporidium spp. oocysts are round or slightly ovoid objects that exhibit a bright apple-green fluorescence under the FITC filter set. Their measurements (measured length × breadth) are presented in Table 1. Often the fluorescence has an increased intensity around the entire circumference of the oocyst, with no visible breaks in oocyst wall staining. If Evans’ blue, which reduces nonspecific fluorescence, is included in the kit, the background fluorescence will be red. Nonspecific fluorescence is yellow. Always refer to the positive control to ensure that the size, shape and colour of the putative oocyst is consistent with those of the positive control. DAPI intercalates with the nuclei of the sporozoites within viable or non-viable oocysts and causes them to fluoresce sky blue. Under the x100 oil immersion lens, a sporozoite nucleus is spherical to subspherical, measuring approximately 1 micron in diameter. In the event of an oocyst being distorted, the demonstration of up to four fluorescent nuclei in an object of a comparable size to an oocyst will assist in its identification (13, 34).

b) Detection of Cryptosporidium antigens by enzyme linked immunosorbent assay

In the ELISA, the presence of Cryptosporidium antigens in faeces (coproantigen) is sought. Depending on the commercial kit, Cryptosporidium coproantigens are captured and developed using a mixture of monoclonal and polyclonal antibodies. With the exception of increased throughput and automation, coproantigen detection kits do not offer increased sensitivity beyond the methods described.

Commercially available sandwich ELISA antigen detection kits contain anti-Cryptosporidium-coated well strips for capturing Cryptosporidium coproantigens, anti-Cryptosporidium antibodies for developing the reaction that is conjugated to an enzyme (frequently horseradish peroxidase), substrate, chromogen/substrate development system and stopping solution (which inhibits further enzyme catalysis when added to the reaction mixture). These have been developed to detect C. parvum antigens in stool samples, but they may also be capable of detecting common epitopes from non-C. parvum infections. Known negative and positive samples are included in commercial kits. Commercial kits normally contain all the necessary reagents to perform the analysis and the manufacturers’ instructions must be followed. It is false economy to dilute kit reagents to increase testing capacity. A comprehensive method and a formula for calculating the cut-off value and assigning positive or negative status to samples are usually included. Kit reagents are normally stored at 4°C when not in use. All reagents should reach room temperature before being used. The diagnostician should always determine whether any contraindications apply to the use of a commercial test and any stool/sample fixative used. Because of the variation in the methods described for different commercial kits, no method for ELISA or IC coproantigen detection is included in this chapter.

c) Detection of Cryptosporidium antigens by immunochromatography

Rather than relying on molecular diffusion to dictate the rate of antigen binding by the capture antibody as in the ELISA format, which normally takes about an hour per reaction, in lateral flow immunochromatography (IC), the speed of antigen binding to the solid phase-bound capture antibody is increased by a wicking action. This draws all fluids rapidly through a membrane enclosed in the immunochromatography cassette and reduces the time required for analysis from hours to minutes or seconds. Soluble Cryptosporidium antigens in the test sample are drawn through the membrane and come into contact with, and bind to, immobilised antibodies raised against Cryptosporidium antigens, which dramatically increases the speed of antigen–antibody interaction. Positive reactions are qualitative and are seen as a band of colour at a specific location on the membrane, normally identified by a line on the cassette. The assay format can vary between commercial kits. As for antigen detection by ELISA, the diagnostician should always determine whether any contraindications apply to the use of a commercial test and any fixative used.

IC is a convenient alternative method for detecting Cryptosporidium antigen in stool samples and specificity is reported to be high (98–100%). Debate continues as to whether IC (or indeed ELISA) has reduced, equal or better sensitivity than oocyst-staining methods. As with ELISA, IC assays can be invaluable in cases of infection in the absence of detectable oocysts. Because of the variation in the methods described for different commercial kits, no method for IC coproantigen detection is included in this chapter.

- Nucleic acid recognition methods

PCR is more sensitive than conventional and immunological assays for detecting oocysts in faeces, although the sensitivity of published methods can range between 1 and 10^6 oocysts. These techniques are often restricted to specialist laboratories. Care is necessary when choosing primers, as some primers amplify genus-specific DNA whereas others amplify species-specific DNA. Prior to routine adoption in clinical laboratories, both the variability between methods and the recognised difficulties in amplifying nucleic acids from faecal specimens by PCR must be overcome.
Faecal samples can contain many PCR inhibitors. In addition to bilirubin and bile salts, complex polysaccharides are also significant inhibitors. For Cryptosporidium, boiling faecal samples in 10% polyvinylpolypyrrolidone (PVPP) before extraction can reduce inhibition.

The most robust information regarding species/genotype/subtype information has been derived from the study of three genetic loci (two 18S rRNA [18, 24, 45, 46] and the Cryptosporidium Oocyst Wall Protein [COWP] [15, 38]) gene fragments by PCR-RFLP and/or sequencing amplicons.

PCR amplification of Cryptosporidium DNA using the 18S rRNA primers (CPB-DIAGF/R) of Johnston et al. (18) yields products that vary in length from 428 bp to 455 bp. The Johnston et al. primers (18) are included because they have been evaluated for cross reactions against a total of 23 microorganisms and the primers have been shown to work in a variety of matrices. The Ward et al. (40) modification of the Johnson et al. (18) reverse primer (substitution of CPB-DIAGR with PW99R [TAA-GGA-ACA-ACC-TCC-ATC], which produces an amplicon of approximately 420 bp) was shown by the authors to be more sensitive than CPB-DIAGF/R (18) in both direct and nested PCR assays. Further corroboration is required in different matrices before PW99R (40) can be fully recommended as a replacement for CPB-DIAGR. The nested (Nichols–Johnson; 24) 18S rRNA assay has also been shown to be sensitive. A multi-locus approach to characterising Cryptosporidium isolates is essential. A multiplex allele-specific PCR (MAS-PCR) based on the dihydrofolate reductase gene sequence differentiates C. hominis (357 bp) from C. parvum (190 bp) in a one-step reaction, which can be distinguished on agarose gel, without the requirement for endonuclease digestion and RFLP analysis, thus reducing assay time considerably (12). MAS-PCR is as sensitive as other diagnostically used assays targeting the 18S rRNA gene for C. hominis and C. parvum detection, and can detect more mixed (C. hominis and C. parvum) species infections than 18S rRNA gene assays (12). However, MAS-PCR failed to detect the DNA of C. felis, C. canis, C. muis and Cryptosporidium pig genotype I in human samples (17) and its usefulness for determining the range of pathogenic species found in livestock samples remains to be determined.

Currently, there is no ‘standard’ genetic locus recommended for species identity, but RFLP or sequencing of 18S rRNA gene loci are widely used (44) and provide information about more species than the COWP gene locus. For detecting small numbers of oocysts (<100) consistently, a nested PCR is required. Two 18S rRNA PCRs can offer better information than PCR-RFLP, but sequencing is more expensive and takes longer than PCR-RFLP. The most robust information regarding species/genotype/subtype information has been derived from the study of three genetic loci (two 18S rRNA [18, 24, 45, 46] and the Cryptosporidium Oocyst Wall Protein [COWP] [15, 38]) gene fragments by PCR-RFLP and/or sequencing amplicons.

A single-tube nested PCR-RFLP assay (15) amplifying a fragment of the gene coding for COWP distinguishes between C. hominis and C. parvum. This assay is recommended over and above that in reference 38 as it is more sensitive and offers a solution to the contamination frequently experienced in nested PCRs due to re-amplification of PCR products. In this single-tube nested PCR, the inner and outer primers are added to the initial reaction mixture. Optimisation of primer set concentrations and annealing temperatures result in the preferential amplification of one product size only, defined by the inner primers.

No recommended method for extracting Cryptosporidium DNA from oocysts exists, and the sensitivity of most methods described has not been addressed fully. Cryptosporidium DNA can be extracted either following partial purification of oocysts using one of the flotation/sedimentation techniques described above, or from oocysts in faeces following zirconia bead extraction (20). If concentration by formol–ether sedimentation is the routine laboratory test, oocyst concentrates suitable for lysis and amplification by PCR can be made by substituting deionised water for the 10% formalin used in the method described. DNA loss can be a consequence of subsequent DNA purification using commercial purification columns, but normally there should be an adequate number of oocysts present in the sample to extract sufficient Cryptosporidium DNA for PCR-RFLP/sequencing analysis. The selection of a suitable DNA extraction technique is the most important step in determining the final sensitivity of oocyst DNA detection.
Table 3. Structural analysis of the 18S rRNA gene defined by CPB-DIAGR primers after simultaneous digestion with the restriction enzymes VspI or AseI and Dral (reproduced from ref. 24)

<table>
<thead>
<tr>
<th>Cryptosporidium species (amplicon length in bp)</th>
<th>Number of VspI/AseI sites (AT↓TAAT)</th>
<th>Number of Dral sites (TT↓AAA)</th>
<th>Fragments' length in bp</th>
</tr>
</thead>
<tbody>
<tr>
<td>C. hominis (438)</td>
<td>2</td>
<td>None</td>
<td>222; 112; 104</td>
</tr>
<tr>
<td>C. parvum (435)</td>
<td>2</td>
<td>None</td>
<td>219; 112; 104</td>
</tr>
<tr>
<td>C. muris (431 or 432)</td>
<td>1</td>
<td>None</td>
<td>319; 112</td>
</tr>
<tr>
<td>C. feliis (455)</td>
<td>2</td>
<td>1</td>
<td>189; 112; 104; 50</td>
</tr>
<tr>
<td>C. baileyi (428)</td>
<td>2</td>
<td>1</td>
<td>128; 112; 104; 84</td>
</tr>
<tr>
<td>C. meleagridis (434)</td>
<td>3</td>
<td>None</td>
<td>171; 112; 104; 47</td>
</tr>
</tbody>
</table>

Table 4. Cryptosporidium spp. and genotypes determined by RFLP of the amplicons defined by the CPB-DIAG primers following digestion with enzymes VspI or AseI, SspI and Ddel, according to available GenBank complete/partial sequences of the 18S rRNA gene

<table>
<thead>
<tr>
<th>Cryptosporidium species (amplicon length in bp)</th>
<th>VspI / AseI</th>
<th>SspI</th>
<th>Ddel</th>
<th>GenBank Accession No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>C. hominis (438)</td>
<td>222, 104,112</td>
<td>264,111, 40,12, 11</td>
<td>204, 166,68</td>
<td>L16997</td>
</tr>
<tr>
<td>C. parvum (435)</td>
<td>219, 104,112</td>
<td>264, 108, 40, 12, 11</td>
<td>201, 166, 68</td>
<td>L16996</td>
</tr>
<tr>
<td>C. muris (432)</td>
<td>320, 112</td>
<td>395, 37</td>
<td>224,166,42</td>
<td>AF093498</td>
</tr>
<tr>
<td>C. andersoni (431)</td>
<td>319, 112</td>
<td>394, 37</td>
<td>265, 166</td>
<td>L19069</td>
</tr>
<tr>
<td>C. feliis (455)</td>
<td>239, 104,112</td>
<td>401, 40, 14</td>
<td>221, 166, 68</td>
<td>AF087577</td>
</tr>
<tr>
<td>C. baileyi (428)</td>
<td>212, 104,112</td>
<td>264, 164</td>
<td>262, 166</td>
<td>L19068</td>
</tr>
<tr>
<td>C. meleagridis (434)</td>
<td>171, 104,112, 47</td>
<td>264, 119, 40, 11</td>
<td>200, 166, 68</td>
<td>AF112574</td>
</tr>
<tr>
<td>C. serpentis (430)</td>
<td>318, 112</td>
<td>380, 36, 14</td>
<td>264, 166</td>
<td>AF093502</td>
</tr>
<tr>
<td>C. wrairi (435)</td>
<td>219, 104,112</td>
<td>264, 109, 40, 11, 11</td>
<td>201, 166, 68</td>
<td>AF115378</td>
</tr>
<tr>
<td>Cryptosporidium pig (435)</td>
<td>219, 104,112</td>
<td>375, 40, 11, 9</td>
<td>201, 166, 68</td>
<td>AF108861</td>
</tr>
<tr>
<td>C. saurophilum (432)</td>
<td>216, 108,112</td>
<td>264, 109, 40, 19</td>
<td>198, 166, 68</td>
<td>AF112573</td>
</tr>
<tr>
<td>Cryptosporidium mouse (439)</td>
<td>175, 104,112, 48</td>
<td>264, 112, 40, 12, 11</td>
<td>205, 166, 68</td>
<td>AF108863</td>
</tr>
<tr>
<td>Cryptosporidium ferret (438)</td>
<td>174, 103,113, 48</td>
<td>264, 111, 40, 23</td>
<td>204, 166, 68</td>
<td>AF112572</td>
</tr>
<tr>
<td>Cryptosporidium dog (429)</td>
<td>213, 104,112</td>
<td>264, 105, 40, 20</td>
<td>195, 166, 68</td>
<td>AF112576</td>
</tr>
<tr>
<td>Cryptosporidium koala (436)</td>
<td>220, 104,112</td>
<td>264, 109, 63</td>
<td>202, 166, 68</td>
<td>AF108860</td>
</tr>
<tr>
<td>Cryptosporidium kangaroo (436)</td>
<td>220, 104,112</td>
<td>373, 63</td>
<td>202, 166, 68</td>
<td>AF112570</td>
</tr>
<tr>
<td>Cryptosporidium monkey (436)</td>
<td>220, 104, 112</td>
<td>264, 109, 52, 11</td>
<td>202, 166, 68</td>
<td>AF112569</td>
</tr>
</tbody>
</table>
Table 5. Cryptosporidium spp. and genotypes determined by RFLP of the amplicons defined by the XR2 / XF2 primers following digestion with enzymes AseI and SspI (Reproduced from ref. 45)

<table>
<thead>
<tr>
<th>Cryptosporidium species</th>
<th>Vsp</th>
<th>Ssp</th>
</tr>
</thead>
<tbody>
<tr>
<td>C. hominis</td>
<td>561, 104, 102, 70</td>
<td>449, 254, 111, 12, 11</td>
</tr>
<tr>
<td>C. parvum gene type A</td>
<td>628, 104, 102</td>
<td>449, 254, 108, 12, 11</td>
</tr>
<tr>
<td>C. parvum gene type B</td>
<td>625, 104, 102</td>
<td>449, 254, 119, 9</td>
</tr>
<tr>
<td>C. muris</td>
<td>731, 102</td>
<td>448, 385</td>
</tr>
<tr>
<td>C. andersoni</td>
<td>731, 102</td>
<td>448, 385</td>
</tr>
<tr>
<td>C. felis</td>
<td>476, 182, 104, 102</td>
<td>426, 390, 33, 15</td>
</tr>
<tr>
<td>C. baileyi</td>
<td>620, 104, 102</td>
<td>572, 254</td>
</tr>
<tr>
<td>C. serpentis</td>
<td>729, 102</td>
<td>414, 370, 33, 14</td>
</tr>
<tr>
<td>C. wrairi</td>
<td>628, 104, 102</td>
<td>449, 254, 109, 11, 11</td>
</tr>
<tr>
<td>Cryptosporidium pig</td>
<td>632, 104, 102</td>
<td>453, 365, 11, 9</td>
</tr>
<tr>
<td>C. saurophilum</td>
<td>628, 104, 102</td>
<td>418, 255, 109, 33, 19</td>
</tr>
<tr>
<td>Cryptosporidium mouse</td>
<td>457, 175, 104, 102</td>
<td>449, 254, 112, 12, 11</td>
</tr>
<tr>
<td>Cryptosporidium ferret</td>
<td>457, 174, 104, 102</td>
<td>449, 254, 111, 12, 11</td>
</tr>
<tr>
<td>Cryptosporidium kangaroo, koala</td>
<td>631, 104, 102</td>
<td>441,254, 109, 33</td>
</tr>
<tr>
<td>Cryptosporidium dog</td>
<td>633, 102, 94</td>
<td>417, 254, 105, 33, 20</td>
</tr>
<tr>
<td>Cryptosporidium monkey</td>
<td>559, 104, 102, 70</td>
<td>461, 254, 109, 11</td>
</tr>
</tbody>
</table>

The following method is effective in extracting DNA from small numbers (~10+) of partially purified oocysts, and is used in the author’s laboratory (22–24). Partially purified oocysts are suspended in 100 µl of lysis buffer (50 mM Tris/HCl, pH 8.5, 1 mM ethylene diamine tetra-acetic acid, pH 8, 0.5% sodium dodecyl sulphate [SDS], Sigma-Aldrich) and subjected to 15 freeze–thaw cycles (1 minute in liquid nitrogen; 1 minute 65°C). Samples are then transferred to a 55°C water bath, proteinase K (at a final concentration of 200 µg/ml) is added, and the samples are incubated for 3 hours. Proteinase K is heat denatured (90°C, 20 minutes), samples are chilled on ice for 1 minute, centrifuged (16,000 g, 5 minutes) then 70 µl of supernatant is removed for PCR amplification. SDS is inhibitory to Taq polymerase at concentrations as low as 0.01%, therefore, neutralisation of SDS in the extracted DNA is necessary. The addition of 2% Tween 20 will neutralise up to 0.05% SDS.

Reagents for PCR reactions are dispensed in 0.5 ml thin-walled tubes. Each tube contains 90 µl of pre-mixed reagents (200 µM of each of the four dNTPs, 200 nM each of primers CPB-DIAGR and CPB-DIAGF, bovine serum albumin at a final concentration of 400 µg/ml, MgCl₂ at 3.5 mM, 2.5 U of Taq polymerase in PCR buffer and Tween 20 at a final concentration of 2% to inactivate 0.05% SDS). Finally, 10 µl of DNA template is introduced below approximately 40 µl of mineral oil. Samples are subjected to 39 amplification cycles, and products are visualised following ethidium bromide staining of 1.4% agarose gels (24).

The primers and step cycle protocols for amplifying either 18S rRNA gene fragment (24, 45, 46) or the COWP gene fragment (15) are given in Table 6.

- **Reporting results of PCR-RFLP/sequencing examination**

  Negative specimens should be reported as ‘NO Cryptosporidium DNA detected’.

  Positive specimens should be reported as ‘Cryptosporidium DNA detected’ inserting the species/genotype(s)/subtype(s) identified (see Tables 3–5) after identifying the respective species from the RFLP profiles presented in Table 2.
Table 6. Step cycle PCR protocols for 18S rRNA (refs 24, 45 & 46) and single-tube nested COWP (ref. 15) PCR

<table>
<thead>
<tr>
<th>Primers</th>
<th>Step cycle protocol</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>CPB-DIAGF</td>
<td>80°C, 5 minutes; 98°C, 30 seconds</td>
<td>24</td>
</tr>
<tr>
<td>AAG-CTC-GTA-GTT-GGA-TTT-CTG</td>
<td>55°C, 30 seconds; 72°C, 1 minute; 94°C, 30 seconds</td>
<td>39 cycles</td>
</tr>
<tr>
<td>CPB-DIAGR</td>
<td>72°C, 10 minutes</td>
<td>39 cycles</td>
</tr>
<tr>
<td>TAA-GGT-GCT-GAA-GGA-GTA-AGG</td>
<td>4°C</td>
<td>39 cycles</td>
</tr>
<tr>
<td>XF1 (outer)</td>
<td>94°C, 3 minutes</td>
<td>45, 46</td>
</tr>
<tr>
<td>TTC-TAG-AGC-TAA-TAC-ATG-CG</td>
<td>94°C, 35 seconds; 55°C, 45 seconds; 72°C, 1 minute</td>
<td>35 cycles</td>
</tr>
<tr>
<td>XR1 (outer)</td>
<td>72°C, 7 minutes</td>
<td>35 cycles</td>
</tr>
<tr>
<td>CCC-ATT-TCC-TTC-GAA-ACA-GGA</td>
<td>4°C</td>
<td>1 cycle</td>
</tr>
<tr>
<td>XF2 (inner)</td>
<td>4°C soak</td>
<td>1 cycle</td>
</tr>
<tr>
<td>oocry3 (outer)</td>
<td>94°C, 1 minute; 67°C, 1 minute; 72°C, 1 minute</td>
<td>20 cycles</td>
</tr>
<tr>
<td>oocry4 (outer)</td>
<td>94°C, 1 minute; 54°C, 1 minute; 72°C, 1 minute</td>
<td>35 cycles</td>
</tr>
<tr>
<td>CCA-TGA-TGT-CCT-GAA-TTT-TGT-A</td>
<td>72°C, 10 minutes</td>
<td>1 cycle</td>
</tr>
<tr>
<td>oocry1 (inner)</td>
<td>4°C</td>
<td>soak</td>
</tr>
<tr>
<td>CCT-GGA-TAT-CTC-GAC-AAT</td>
<td>Oocry2 (inner)</td>
<td>soak</td>
</tr>
<tr>
<td>GCG-AAC-TAA-CTC-GAT-TCT-CT</td>
<td>GCG-AAC-TAA-CTC-GAT-TCT-CT</td>
<td>soak</td>
</tr>
</tbody>
</table>

a) Quantitative real-time PCR

Quantitative real-time PCR methods use patented technologies (e.g. TaqMan™, Applied Biosystems, LightCycler™, Roche Molecular Biochemicals). For both technologies, fluorogenic dyes are incorporated into the amplicon during PCR, thus amplicon fluorescence increases as more PCR product is generated. Both systems can detect PCR products during the initial cycles of the PCR reaction when amplification is exponential, thus enabling quantitative analysis of fluorescent product. Quantitative real-time PCR methods have been used to identify different Cryptosporidium species by exploiting the genetic polymorphism of the 18S RNA gene for devising probes with differing melting temperatures, and for the quantitative detection of cryptosporidium oocysts in environmental water samples and sewage. The increased sensitivity of real-time PCR guarantees increased speed of detection and qualitative diagnosis while the quantitative nature of the assay will be invaluable in estimating levels of contamination. The ‘closed-tube’ assay format reduces the danger of contamination from ‘carry over’ (36). Quantitative real-time PCR should be a useful tool for the future, once issues surrounding matrix inhibition are overcome. Currently, there are no standardised quantitative real-time PCR methods.

b) Typing and subtyping for disease and source tracking

The value of characterising the genetic diversity of Cryptosporidium at different levels of specificity and the importance of appropriate nucleic acid analysis cannot be over-emphasised. Molecular tools for inter- and intra-species discrimination differ (4) and the publication of complete genomic sequences of C. parvum (1) and C. hominis (49) has assisted in developing appropriate typing and subtyping tools (4). Genetic markers differ in their information content and the nature of the DNA fragment selected for detecting and characterising cryptosporidium and Giardia should be considered carefully (see Table 4 below). In order to detect species, analysis of highly or moderately conserved coding regions (e.g. 18S ribosomal DNA, structural and house-keeping genes) is necessary, whereas investigations into the transmission of genotypes and subtypes, identifying sources of infection and risk factors, requires more discriminatory fingerprinting techniques, which can identify individual isolates or clonal lineages (4, 35).

Typing and subtyping systems used for veterinary (and human) samples should also be used for environmental samples, particularly for source and disease tracking in order to avoid any confusion arising from using different systems for human, non-human hosts and environmental samples for veterinary and public health investigation of disease outbreaks (4, 35). Species determination should be based on the analysis of at least two loci since this provides more robust information. At least one locus should be 18S rRNA, and, where possible, another should be suitable for both species identification and further subtyping analysis. For Cryptosporidium, mini- and micro-satellite typing, GP60 sequencing and analysis of a double stranded RNA element have been used to subtype C. parvum and C. hominis, and may offer sufficient subspecies discrimination to address veterinary and public health investigations, either separately or in combination (reviewed in references 4 and 35). Further development of these subtyping systems is required e.g. gp60 sequencing shows variability within C. parvum populations but not C. hominis. The goal would be to find common isolate identifiers. There is also evidence that mixed species infections occur in both the
human and animal populations. It is therefore important that species and subtyping systems be capable of identifying mixed infections to give accurate diagnosis and information. Recent work has confirmed the utility of mini- and micro-satellite markers in the study of the population structure of Cryptosporidium, and in understanding transmission dynamics of infection (reviewed in references 4, 8 and 35). The most commonly used PCR assays for detecting and typing Cryptosporidium are listed in Table 7.

**Table 7.** List of targets, type of assay and main use of amplification-based techniques for Cryptosporidium (refs 4 & 35)

<table>
<thead>
<tr>
<th>Amplification target</th>
<th>Assay type</th>
<th>Main application</th>
</tr>
</thead>
<tbody>
<tr>
<td>18S rDNA</td>
<td>PCR, nested PCR, sequencing, PCR-RFLP, Real-time PCR, microarray</td>
<td>Species &amp; genotype identification</td>
</tr>
<tr>
<td>Hsp70</td>
<td>PCR, nested PCR, sequencing, Real-time PCR, microarray</td>
<td>Species &amp; genotype identification</td>
</tr>
<tr>
<td>COWP</td>
<td>PCR, nested PCR, sequencing, PCR-RFLP, microarray</td>
<td>Species &amp; genotype identification</td>
</tr>
<tr>
<td>Actin</td>
<td>PCR, nested PCR, sequencing,</td>
<td>Species &amp; genotype identification</td>
</tr>
<tr>
<td>j-tubulin</td>
<td>PCR, nested PCR, sequencing, PCR-RFLP</td>
<td>Species &amp; genotype identification</td>
</tr>
<tr>
<td>GP60</td>
<td>PCR, nested PCR, sequencing,</td>
<td>Subgenotype identification</td>
</tr>
<tr>
<td>Microsatellites</td>
<td>PCR, nested PCR, sequencing, fragment typing</td>
<td>Subgenotype identification</td>
</tr>
<tr>
<td>Minisatellites</td>
<td>PCR, nested PCR, sequencing, fragment typing</td>
<td>Subgenotype identification</td>
</tr>
<tr>
<td>Extra-chromosomal double stranded RNA</td>
<td>Reverse transcriptase, PCR, sequencing, heteroduplex mobility assays</td>
<td>Subgenotype identification</td>
</tr>
</tbody>
</table>

Key: RFLP = restriction fragment length polymorphism; Hsp70 = heat shock protein 70; COWP = Cryptosporidium oocyst wall protein; GP60 = glycoprotein 60.

2. Serological tests (and/or tests for cellular immunity where relevant)

Cryptosporidiosis is often a disease of the newborn and unless there is sufficient evidence to exclude exposure to infectious oocysts, serological tests do not offer any benefit. Serological tests can be used for seroepidemiological surveys of exposure: most are ELISA based, using various aqueous extracts of C. parvum oocysts (e.g. ref. 14). Tests for cellular immunity do not appear to offer specific benefit, and are not available.

C. REQUIREMENTS FOR VACCINES AND DIAGNOSTIC BIOLOGICALS

There is no control programme for cryptosporidiosis, neither is there a rigorously tested and accepted vaccine available.

REFERENCES


Chapter 2.9.4. – Cryptosporidiosis

43. WWW.KSU.EDU/PARASITOLOGY/ Click on ‘Host checklist for C. parvum’


* *

* *
CHAPTER 2.9.5

CYSTICERCOSIS*

SUMMARY

Cysticercosis of farmed and wild animals is caused by the larval stages (metacestodes) of cestodes (tapeworms), the adult stages of which occur in the intestine of humans and dogs or wild Canidae. Bovine cysticercosis (primarily in muscle) and porcine cysticercosis (primarily in muscle, the central nervous system and the liver) are caused by the metacestodes (cysticerci) of the human cestodes Taenia saginata and T. solium, respectively. Cysticerci of T. solium also develop in the central nervous system and musculature of humans. Cysticerci of T. asiatica occur in the liver and viscera of pigs. Cysticercosis and coenurosis of sheep and goats (in the muscles, brain, liver and peritoneal cavity) are caused by T. ovis, T. multiceps and T. hydatigena, adults of which occur in the intestines of dogs and wild canids.

Most adult and larval tapeworm infections cause little or no disease. Exceptions are severe, potentially fatal human neurocysticercosis (NCC) caused by T. solium, and occasionally neurocoenurosis caused by T. multiceps in humans. These parasites are also occasional causes of muscle or ocular signs in humans. ‘Gid’ caused by T. multiceps in ruminants can require slaughter of the animal. Acute T. multiceps coenurosis and T. hydatigena cysticercosis in sheep and goats is rare but may be fatal. Cysticercosis causes economic loss through condemnation of infected meat and offal.

Identification of the agent: Adult Taenia tapeworms are dorsoventrally flattened, segmented and large, reaching from 20 to 50 cm (species in dogs) to several metres (species in humans). Anteriorly, the scolex (head) has four muscular suckers and may have a rostellum, often armed with two rows of hooks, the length and number of these being relatively characteristic of a species. A neck follows the scolex, and this is followed by immature and then by mature reproductive segments, and finally gravid segments filled with eggs. Segment structure, although unreliable, can aid in the identification of the species. Taenia species cannot be differentiated by egg structure. Metacestodes consist of a fluid-filled bladder with one or more invaginated protoscoleces. These ‘bladderworms’ are each contained within a cyst wall at the parasite–host interface. This structure comprises the cisticercus or coenurus.

Adult Taenia are recognised at post mortem or by passage of segments or eggs. Metacestodes are grossly visible at post mortem and meat inspection, but light infections are often missed. NCC can be diagnosed by imaging techniques.

Immunological tests: Adult Taenia infections can be recognised by detection of Taenia coproantigen in faeces using an antigen-capture enzyme-linked immunosorbent assay, but the test does not differentiate species and is not commercially available. Use of species-specific probes remains experimental.

Serological tests: Tests for antibodies in serum are not used currently for the diagnosis of cysticercosis in animals except for epidemiological purposes. Diagnosis is by meat inspection. Antigens have been identified for the serological diagnosis of NCC in humans.

Requirements for vaccines and diagnostic biologicals: Vaccine antigens have been identified for the metacestodes, but not for the adult stages of T. ovis, T. multiceps, T. saginata and T. solium. A T. ovis vaccine is registered in New Zealand, but is not commercially available. A T. solium vaccine is undergoing the steps for practical production.
A. INTRODUCTION

The metacestodes (or larval cestodes) of *Taenia* spp. tapeworms are the cause of cysticercosis in various farmed and wild animals and in humans. Adult tapeworms are found in the small intestine of carnivore definitive hosts—humans, dogs, and wild canids. *Taenia saginata* of humans causes bovine cysticercosis, which occurs virtually world-wide, but particularly in Africa, Latin America, Caucasian and South/Central Asian and eastern Mediterranean countries and the infection occurs in many countries in Europe. *Taenia solium* of humans causes porcine cysticercosis and human neurocysticercosis (NCC). It is found principally in Mexico, Central and South America, sub-Saharan Africa, non-Islamic countries of Asia, including India and China where there are free-ranging, scavenging pigs. The cysticerci of *T. asiatica* of humans in South-East Asia occur in the liver of pigs. Dogs and wild canids are the definitive hosts of metacestodes of sheep, goats and other ruminants, which occur throughout most of the world, although *T. multiceps* has disappeared from the United States of America (USA) and New Zealand. *Taenia ovis* occurs in the muscles of sheep, *T. multiceps* in the brain (occasionally in the muscles) of sheep, goats, sometimes other ruminants and rarely humans, and *T. hydatigena* is found in the peritoneal cavity and on the liver of ruminants and pigs. Diagnosis in animals is usually based on the host and the location of the metacestode when identified at meat inspection or necropsy. Adults in definitive hosts are acquired by the ingestion of viable metacestodes in meat and offal that has not been adequately cooked or frozen to kill the parasite.

Gravid segments that are shed by the adult tapeworms migrate spontaneously from the anus to fall to the ground and release eggs on the ground, or the segments and free eggs are passed in the faeces. Eggs may also be disseminated from these sites by physical means or transport hosts, particularly flies. *Taenia solium* segments, however, are often passed in chains. Eggs are immediately infective when passed. Animals acquire infection from ingestion of segments and eggs contaminating herbage or in faeces. It is possible that pigs acquire *T. solium* by coprophagy of the faeces of pigs that have eaten segments. Humans may be infected with *T. solium* by eggs on vegetables, etc., that have been contaminated by faeces or dirty hands, by faeco-oral transmission or through retro-peristalsis and hatching of eggs internally. Routine diagnosis continues to be mainly based on the morphology of the adult tapeworm and the presence of eggs or segments in the faeces of infected definitive hosts.

B. DIAGNOSTIC TECHNIQUES

1. Identification of the agent

*Taenia saginata* (the beef tapeworm): The adult is large, 4–8 metres long and can survive many years, usually singly, in the small intestine of humans. The scolex (or head) has no rostellum or hooks. Useful diagnostic features are presented in Table 1 (16, 17, 24, 32, 34). Gravid segments usually leave the host singly and often migrate spontaneously from the anus.

The eggs are typical ‘taeniid’ eggs that cannot be differentiated from other *Taenia* or *Echinococcus* spp. eggs. *Taenid* eggs measure about 30–45 µm in diameter; contain an oncosphere (or hexacanth embryo) bearing three pairs of hooks; have a thick, brown, radially striated embryophore or ‘shell’ composed of blocks; and there is an outer, oval, membranous coat, the true egg shell, that is lost from faecal eggs. Metacestodes (*Cysticercus bovis*) of *T. saginata* usually occur in the striated muscles of cattle (beef measles), but also buffalo, reindeer and deer. They are oval, about 0.5–1 × 0.5 cm long, translucent and contain a single white scolex that is morphologically similar to the scolex of the future adult tapeworm. They are contained in a thin, host-produced fibrous capsule. Cysts occasionally are found in the liver, lung, kidney, fat and elsewhere.

*Taenia solium* (the pork tapeworm) is smaller than *T. saginata* being up to 3–5 metres. The scolex has an armed rostellum bearing two rows of hooks; the number and size of hooks can aid differentiation of *Taenia* spp. (Table 1). Gravid segments have 7–16 (<17) uterine branches and do not usually leave the host spontaneously, but passively in chains with the faeces.

Metacestodes (*C. cellulosae*) occur in the muscles and central nervous system of pigs (pork measles), bear and dogs and in the muscles, subcutaneous tissues and central nervous system of humans. Cysts are grossly similar to those of *T. saginata*, but may be larger than the *T. saginata* cyst. They have a scolex bearing a rostellum and hooks similar to the adult. Occasionally, in the brain of humans, they develop as racemose cysts up to 2 cm or more across that lack a scolex.
**Table 1. Useful features for identification of scoleces and segments of *Taenia* spp.**

<table>
<thead>
<tr>
<th>Parasite species</th>
<th>Number of hooks</th>
<th>Length of hooks (µm)</th>
<th>Number of testes</th>
<th>Layers of testes</th>
<th>Cirrus sac extends to longitudinal vessels</th>
<th>Number of uterine branches</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Lobes of ovary unequal in size. No vaginal sphincter. Testes extend to vitellarium, but not confluent behind.</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Lobes of ovary unequal in size. Well developed vaginal sphincter. Testes extend to posterior edge of ovary.</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Lobes of ovary equal in size. Pad of muscle on anterior wall of vagina. Testes extend to vitellarium, but not confluent behind.</td>
<td></td>
</tr>
<tr>
<td><em>T. saginata</em></td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>765–1200</td>
<td>No</td>
<td>14–32 that re-divide</td>
</tr>
<tr>
<td>without rostellum</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Ratio of uterine twigs to branches 2.3</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Lobes of ovary unequal in size with small Well developed vaginal sphincter. Testes extend to vitellarium, but not confluent behind.</td>
<td></td>
</tr>
<tr>
<td><em>T. solium</em></td>
<td>22–36</td>
<td>139–200</td>
<td>93–159</td>
<td>375–575</td>
<td>Yes</td>
<td>7–16 that re-divide</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Lobes of ovary unequal in size with small accessory lobe. No vaginal sphincter. Testes confluent behind vitellarium</td>
<td></td>
</tr>
<tr>
<td><em>T. asiatica</em></td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>868–904</td>
<td>No</td>
<td>16–32 that re-divide</td>
</tr>
<tr>
<td>with rostellum on some</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Ratio of uterine twigs to branches 4.4</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Ovary, vaginal sphincter and extent of testes as <em>T. saginata</em>. Posterior protuberances on some gravid segments</td>
<td></td>
</tr>
</tbody>
</table>

*Taenia asiatica* (Asian *Taenia*): Closely related to but genetically distinguishable from *T. saginata* (8), the adult in humans has an ovary, vaginal sphincter muscle and cirrus sac like those of *T. saginata*, but *T. asiatica* has a rostellum and posterior protuberances on segments and 16–32 uterine buds with 57–99 uterine twigs on one side. Segments are passed singly and often spontaneously.

The metacestodes (*C. viscerotropica*) are small, about 2 mm, and have a rostellum and two rows of primitive hooks, those of the outer row being numerous and tiny. They occur mainly in the parenchyma and on the surface of the liver of domesticated and wild pigs; they may be found on the omentum and, rarely, on the lungs and colonic serosa. Occasionally they are found in cattle, goats, and monkeys.

*Taenia ovis*: Adults in the intestine of dogs and wild canines reach 1–2 metres in length and have an armed rostellum (Table 1). Metacestodes (*C. ovis*) that occur in the musculature (skeletal and cardiac) of sheep and less commonly goats reach 3.5–1.0 × 0.2–0.4 cm. Commonly, the cysticerci are degenerate with a green or cream, caseous or calcified centre. A similar parasite occurs in wild canines and dogs and the muscles of reindeer and deer in northern areas.
**Taenia hydatigena**: Adults are 1–5 metres long, are found in the intestine of dogs and wild canines, and have an armed rostellum (Table 1). Metacestodes (C. tenuicollis) are large, from 1 cm up to 6–7 cm, and the scolex has a long neck. They are found attached to the omentum, mesentery and occasionally on the liver surface, particularly of sheep, but also of other domesticated and wild ruminants and pigs. A wolf and reindeer/deer cycle exists in northern latitudes, in which the metacestodes are found in the liver of the intermediate host; dogs are also infected as definitive hosts.

**Taenia multiceps**: Adults are 40–100 cm long in the intestine of canines and have an armed rostellum (Table 1). The metacestodes (Coenurus cerebralis) are large, white fluid-filled cysts that may have up to several hundred scolecites invaginated on the wall in clusters. Coenuri grow to 5 cm or so in size in the brain of sheep, the brain and intermuscular tissues of goats, and also the brain of cattle, wild ruminants and occasionally humans. In neural tissue the cysts are not encapsulated. The cysts induce neurological signs that in sheep are called 'gid', 'sturdy', etc.

### a) Diagnosis of adult parasites in humans or canine carnivores

All parasite or faecal material from humans with possible *T. solium* infections must be handled with suitable safety precautions to prevent accidental infection with the eggs. *Taenia multiceps* and *Echinococcus* spp. also infect humans and, as taeniid eggs in dogs cannot be differentiated to species or genus level, in areas where these are endemic, the same safety precautions apply. In addition to *Taenia* spp., humans and canine carnivores may be infected by *Dipylidium caninum* and *Hymenolepis* spp., while six other cestode genera are recorded occasionally in humans. These are described by Lloyd (22) and all can be differentiated from *Taenia* spp. by egg/proglottid morphology. Recently however, *T. taeniaeformis* with morphologically indistinguishable taenid eggs was recorded in a child. In canids, *Echinococcus* spp. eggs cannot be distinguished from *Taenia* spp. eggs, but the presence of the former can be determined by tapeworm size and, more recently, *Echinococcus* species-specific antigen-capture enzyme-linked immunosorbent assay (AG-ELISA) (2). Other worms, *Dipylidium* and *Diplopylidium*, *Mesocoenurus* and *Dipylidium caninum* spp. have morphologically distinct eggs and proglottids (22, 32).

Adult cestodes can be expelled from humans using an anthelmintic followed by a saline purgative and are identified on the basis of scolex and proglottid morphology. A self-detection tool has been developed and tested in Mexico (10); medical staff in health centres are supplied with preserved tapeworm segments in a bottle and a manual of questions to ask patients to try to identify carriers. In animals, arecoline purgation has been useful; again, the recovered tapeworms are identified morphologically. Arecoline is no longer available as an anthelmintic, but can be obtained from chemical supply companies. As it has side-effects, old, infirm and pregnant animals should be excluded from treatment. A dose of 4 mg/kg should result in purgation in under 30 minutes, provided food has been withheld for several hours (i.e. administer to dogs with empty stomachs). Walking and abdominal massage of recalcitrant cases or enema for constipated dogs may avoid the use of a second dose (2 mg/kg), which should be given only sparingly. Fortunately, arecoline purgation is being replaced rapidly by the copro-antigen ELISA for *Echinococcus* spp. and perhaps in the future this will also be the case for *Taenia* spp. Tapeworms can be recovered after anthelmintic treatment, and require appropriate disposal.

Verstey (34) and Loos-Frank (24) have given descriptions of parasitic diagnosis of all the *Taenia* spp. of humans and animals, their hosts and geographical distributions. Keys for identification are given by Khalil et al. (16). Mayta et al. (25) and Loos-Frank (24) give methods for mounting, embedding, sectioning and staining the proglottids. The following staining technique is that of Loos-Frank (24). Worms, after relaxation in water, can be stained directly, although small worms should be fixed in ethanol for a few minutes. Alternatively, worms can be fixed and stored in 70% ethanol containing 10% lactic acid, the scolex and worm being stored separately. The rostellar hooks of scolecites or protoscoleces should be cut off and mounted in Berlese’s fluid (made by dissolving 15 g gum arabic in 20 ml distilled water and adding 10 ml glucose syrup and 5 ml acetic acid, the whole then being saturated with chloral hydrate, up to 100 g). The stain is lactic acid carmine: 0.3 g carmine is dissolved at boiling point in 42 ml lactic acid and 58 ml distilled water, 5 ml of 5% iron chloride solution (FeCl₂.4H₂O) is added after cooling and can be used again to refresh older solutions. Specimens are allowed to sink in the stain in a vial and then are left in the stain for some more minutes to allow the stain to penetrate. Specimens are then washed in 1-day-old tap water until blue in colour. They are then fixed in 50–70% ethanol and dehydrated under the slight pressure of plastic foil keeping the segments flat. Salicylic acid methyl ester is used as clearant.

When segments break from the end of the worm, some eggs are expelled in the intestine and can be found in the faeces. Spontaneous migration of *T. saginata* or *T. asiatica* from the anus is likely to be noticed by the patient (>95%). When the segments migrate, the sticky eggs are deposited in the perianal area and might be detected by application and examination of sticky tape. These signs are far less likely for chains of *T. solium* (3). Segments of all three may be found on the faeces, but are passed intermittently. In dogs, approximately 50% of the segments migrate spontaneously from the anus. These segments, when they fall to the ground, will migrate, shedding eggs. The remainder of the segments are passed in the faeces, but commonly, the segments migrate and void the majority of their eggs in trails on the surface of the faeces and...
surrounding area. Even if a migrating segment sheds all its eggs, it can be identified as a cestode by the many concentric calcareous corporules contained within its tissues. Faeces, after mixing to reduce aggregation, can be examined for eggs. Various techniques are used throughout the world and include ethyl acetate extraction and flotation. For the latter, NaNO₃ or Sheather's sugar solution (500 g sugar, 6.6 ml phenol, 360 ml water), with their higher specific gravities, are superior to saturated NaCl as flotation media for taeniid eggs. Flotation can be carried out in commercially marketed qualitative or quantitative flotation chambers or by centrifugal flotation that includes a modified Wisconsin technique (faeces, diluted in water, are sieved and centrifuged, the pellet is resuspended in sugar or Sheather’s solution and centrifuged at 300 g for 4 minutes). Eggs adhering to the cover-slip can then be detected. Faecal egg examination will be less sensitive for T. solium than the other species. Species cannot be determined by egg morphology, but DNA probes, the polymerase chain reaction (PCR) and PCR restriction fragment length polymorphism (RFLP), have proved useful for differentiation in the laboratory. These have been largely used experimentally to differentiate faecal eggs of T. solium, T. saginata and T. asiatica (11, 12, 13). While equally applicable to differentiation in dogs, the same examinations have not been done.

An AG-ELISA to detect Taenia coproantigen in faeces is no longer available commercially, but can be developed if laboratory facilities are available (2, 3). Information on availability for epidemiological studies or collaborative use can be obtained from Professor P.S. Craig, OIE Reference Expert on Echinococcosis (see Table given in Part 3 of this Terrestrial Manual). This AG-ELISA was developed experimentally by Allan et al. (2) to detect coproantigen in dogs, and so, with appropriate controls, could be used to detect Taenia infection in this species. The technique, however, is only Taenia species specific. The test is a solid-phase, microwell assay with wells coated with polyclonal, rabbit anti-Taenia-specific antibody (TSA). The following is the basic technique:

i) Faecal supernatants are recovered from fresh, frozen or formalinised (5% formalin at 4°C) faecal samples. The sample is vigorously shaken forming a slurry in an equal weight/volume of 0.15 M phosphate buffered saline (PBS) containing 0.3% Tween 30. The supernatant is recovered by centrifugation at 2000 g for 30 minutes.

ii) A soluble aqueous extract of non-gravid proglottids from Taenia are obtained following emulsification in PBS and centrifugation.

iii) Hyperimmune rabbit antiserum is produced against the soluble proglottid extract and the IgG fraction is isolated by passage into and elution of the bound IgG from Protein A-Sepharose CL 4B (Pharmacia). Some of the IgG fraction is conjugated to peroxidase type VI (Sigma). The sera are stored in small quantities frozen at –20°C. Sera may need to be absorbed by packed normal dog faeces in a ratio 2/1 with mixing for 1 hour and recovered by centrifugation.

iv) Flat-bottomed microtitre plates (Dynatech) are coated with rabbit anti-Taenia IgG (protein content 5–25 µg/ml determined by UV spectrophotometry) using 100 µl/well, the antisera are diluted in 0.05 M NaHCO₃/Na₂CO₃ buffer, pH 9.6. Plates are incubated overnight at 4°C, the wells are washed three times with PBS/0.1% Tween, blocked with PBS/0.3% Tween for 1 hour and washed again. 100 µl of faecal supernatant containing 50% fetal calf serum is added and the plates are incubated for 1 hour and then washed three times. 100 µl of the peroxidase-conjugated anti-Taenia IgG (diluted 1/100 or 1/200) is added and the plates are incubated for 1 hour before washing three times. Substrate solution (100 µl of 5-amino-salicylic acid [Sigma] and 0.005% H₂O₂ in 0.1 M phosphate buffer containing 1 mM Na₂EDTA [ethylene diamine tetra-acetic acid] at pH 6.0) is added for 25 minutes and the result is read at 450 nm. Cut-off values are the mean plus 3 standard deviations of values for normal dog faeces.

b) Diagnosis of metacestodes

In live animals, T. solium or T. saginata metacestodes might be palpable in the tongue but, both in the living animal and on post-mortem examination or meat inspection, tongue palpation is of diagnostic value only in pigs or cattle heavily infected with metacestodes; these will also be difficult to differentiate from large sarcocysts.

- Meat inspection – the main diagnostic procedure

Metacestodes are visible first as very small, about 1 mm, cysts, but detection of these requires thin slicing of tissues in the laboratory. Many young cysts are surrounded by a layer or capsule of inflammatory cells (mononuclear cells and eosinophils being prominent histologically). Cysts may later degenerate, but the parasites' abilities to evade the immune response mean that later in infection, as the cyst matures, few inflammatory cells are present in its vicinity and the cysticercus in its intermuscular location is surrounded by a delicate fibrous tissue capsule.

In theory, cysts can be visualised or felt in tissues such as the tongue of heavily infected animals as early as 2 weeks after infection. Cysts are readily visible by 6 weeks and, when mature, are usually oval, about 10 ×
5 mm or larger, with a delicate, fairly translucent, white parasite membrane and host capsule; pale fluid within the cyst and the scolex, visible as a white dot within the cyst, usually invaginates midway along the long axis of the cyst.

At meat inspection many of the cysts detected, often as many as 85–100%, are dead. The rate at which cysts age and die and so degenerate varies with the parasite species and also with the tissue within which the cyst is embedded. Death usually occurs within 9 months of infection of adult cattle with *T. saginata*. However, cysts may remain viable for several years. Cysts of *T. hydatigena* in the peritoneal cavity of sheep and those of *T. solium* also have been described as surviving in sheep and pigs for long periods. *Taenia solium* cysts survive for many years in the brain of humans, and frequently symptoms begin only as the cyst begins to degenerate. In general, cysts tend to die more rapidly in the muscular predilection sites, such as heart. The preferential distribution of parasites in these areas may be because of greater blood circulation to these muscles. Conversely, the higher rate of activity in these muscles may damage the parasites, allowing leakage of fluid and perhaps disrupting the parasite’s ability to evade the immune response. Cysts at different stages of viability and degeneration can be found in the same host.

Degenerating cysts vary in appearance. The host’s fibrous tissue capsule thickens and becomes opaque, but initially the cyst within remains apparently normal. The fluid gradually becomes colloid and inflammatory cells infiltrate. The cyst cavity becomes filled with greenish (eosinophilic) and then yellow, caseous material but initially the cyst within remains apparently normal. The fluid gradually becomes colloid and inflammatory cells infiltrate. The cyst cavity becomes filled with greenish (eosinophilic) and then yellow, caseous material and is very unaesthetic, usually being larger in size and certainly more obvious in meat than the original viable cyst. Later the cyst may calcify. While PCR assays have been used largely for the differentiation of and is very unaesthetic, usually being larger in size and certainly more obvious in meat than the original viable cyst. Later the cyst may calcify. While PCR assays have been used largely for the differentiation of different stages of viability and degeneration can be found in the same host.

After treatment of *T. saginata* and *T. solium* in cattle and pigs with drugs such as albendazole and oxfendazole, the cysts may lose their fluid and collapse. The resultant lesion is much smaller than lesions observed following natural death. However, cysts that have died before treatment of the animal will remain large and visible. Treatment of pigs with oxfendazole (30 mg/kg) 3 months before slaughter has been suggested as a control measure.

Meat inspection procedures vary with the parasite and the host involved, i.e. zoonosis or not, the tissue involved, and regulations within a country. Examinations tend to be more extensive with the zoonotic infections *T. saginata* and *T. solium*.

In general, meat inspection procedures consist of:

i) Visual inspection of the carcass, its cut surfaces and the organs within it. This may reveal *T. saginata*, *T. solium* and *T. ovis* in the muscles, *T. hydatigena* on the liver or mesenteries and omentum, or *T. multiceps* in the brain.

ii) The external and internal masseters and the pterygoid muscles each must be examined and one or two incisions made into each, the cuts being parallel to the bone and right through the muscle.

iii) The freed tongue is examined visually and palpated, particularly for *T. solium*.

iv) The pericardium and heart are examined visually. The heart usually is incised once lengthwise through the left ventricle and interventricular septum so exposing the interior and cut surfaces for examination. Incisions may go from the base to the apex and regulations also may require additional, perhaps four, deep incisions into the left ventricle. Alternately, the heart may be examined externally and then internally after cutting through the interventricular septum and eversion.

v) The muscles of the diaphragm, after removal of the peritoneum, are examined visually and may be incised.

vi) The oesophagus is examined visually.

vii) In African countries in particular, the triceps brachii muscle of cattle is incised deeply some 5 cm above the elbow. Additional cuts into it may be made. The gracilis muscle also may be incised parallel to the pubic symphisis. These cuts are usually also undertaken for *T. solium* in pigs. Such incisions into the legs are made in Africa as it is suspected that more parasites lodge in these muscles in working or
range animals walking long distances due to the exercise and consequent increased blood flow to these muscles. Other countries may also require such incisions into the legs. However, as this devalues the meat, such incisions are made most commonly once one or more cysts have been found at the predilection sites so as to determine the extent of the infection.

Overall, the initial incision into any tissue is the most important, but additional incisions may be required by the regulations or are required if cysts are found on the initial incision(s). Details on meat inspection are supplied by Herenda (15) and by Kyvsgaard & Murrell (17).

Additional or fewer procedures may be required for specific parasites and the judgements on the carcass, viscera, offal and blood will vary dependent on Taenia species and regulations within a country. Judgement on infected carcasses will fall into three main categories: i) approve for human consumption; ii) partially condemn and pass the remainder of the carcass, but in the case of the zoonoses, *T. saginata* and *T. solium*, the carcass, meat and viscera must be treated; and iii) totally condemn heavily infected carcasses or emaciated diseased ones.

**Taenia saginata**: Calves under 6 weeks or <32 kg are not usually examined. Predilection sites are the heart, tongue, masseters and diaphragm, presumably because they receive the greatest circulation. Nonetheless, cysts may be found in any muscle of the body. If one carcass in a lot is found to be infected, all carcasses from the same lot can be held until laboratory confirmation is obtained. If *T. saginata* infection is confirmed, additional incisions are usually made in the carcasses in the lot; all suspicious lesions found in the rest of the lot are considered to be *T. saginata* without laboratory confirmation. Lesions of *T. saginata* may need to be differentiated from Sarcocystis sarcocysts and other lesions. In recent PCR studies in Germany, Switzerland and New Zealand up to 20% of viable, presumed *T. saginata* cysts could not be positively identified; this suggests there may be an unidentified cestode infecting cattle (1). Conversely, aberrant *T. saginata* oncospheres can lead to cerebral oncospheral lesions and thus need to be taken into account for differential diagnosis of neurological disorders (9).

**Judgement**: If a carcass is considered to be heavily infected then the carcass, meat, offal and blood are condemned. The description of a heavy infection varies, but generally it is the detection of cysts at two of the predilection sites plus two sites in the legs. In the case of a lesser infection, the infected parts and surrounding tissues are removed and condemned. Even a single dead cyst requires that the carcass and edible viscera must then be treated and this is justifiable as about 10% of lightly infected carcasses were found on dissection to have both dead and viable parasites within them. Treatment varies with country and facilities available and includes: i) freezing at lower than −10°C for >10 or 14 days, or lower than −7°C for 21 days; ii) boxes of boned meat are frozen at less than −10°C for >20 days; iii) heated to above 60°C throughout; iv) steamed at moderate pressure (0.49 kg/cm²); v) heated at 95–100°C for 30 minutes; or vi) pickled in salt solution for 21 days. Blast freezing needs examination; generally a 30 kg box is reported to require 2 × 24-hour cycles at −30.9°C followed by 72 hours cold storage at −23.3°C for death of oncospheres. Often no treated meat can be exported although in some countries it can be exported in canned form.

**Taenia solium**: The predilection sites are for *T. saginata* although there are reports of higher prevalence in shoulder and thigh. Commonly one or more cuts are required 2.5 cm above the elbow joint. This is said to detect some 13% of infected carcasses that would otherwise have been missed.

**Judgement**: In some countries, any lightly or heavily infected pigs and their viscera and blood are condemned. In areas where infection is common, lightly infected carcasses can be passed for cooking and pickling and occasionally freezing.

**Taenia hydatigena**: The parasite migrating in the liver leaves haemorrhagic tracks that then become green/brown with inflammation and later white due to fibrosis. For the records, these must be differentiated from those of liver flukes, if possible, by identification of the cysticeri or adult flukes. White spot from *Ascans* infection is differentiated as the lesions appear as pale to white, small, isolated foci. Some cysts remain trapped below the liver capsule. These usually are small and degenerate early and then calcify into cauliflower-like lesions. *Taenia hydatigena* cysts usually mature in the omental or mesenteric fat. Those that are retained at the liver surface are usually superficial and subserosal, while *Echinococcus granulosus* hydatid cysts tend to be deeper in the parenchyma. If viable, the former has a long-necked single scolex in a virtually translucent fluid-filled cyst. Fertile hydatid cysts have thicker white outer membranous walls from which brood capsules containing numerous protoscoleces. These appear as a sandy deposit with the cysts. Differentiation can be important in the implementation and monitoring of hydatid disease control measures for which histology may be required. H&E-stained sections will reveal the laminated membrane of very young hydatid cysts as indicated by Lloyd et al. (23). Its presence or absence can be confirmed by periodic acid–Schiff staining when the highly glycosylated proteins in the laminated membrane stain red. *Taenia hydatigena* lesions in cattle and pigs can be similar to tuberculosis. However, the portal and mesenteric
lymph nodes are not involved, the contents of parasite cysts are more easily shelled-out and remainders of hooks and calcareous corpuscles may be seen or Ziehl–Neelsen staining may reveal bacteria.

**Judgement:** Usually only a few cysts or tracks are present and these can be trimmed. Heavily infected livers and omentum are condemned. Rarely, acute infections are seen with large numbers of migrating parasites producing traumatic hepatitis, ascites, oedema, etc., and would result in secondary condemnation of the carcass.

*Taenia multiceps:* The parasites have a predilection site for the brain and spinal cord.

Early migrating parasites can cause reddish haemorrhagic and later grey purulent tracks in the brain, and in heavy infections, the sheep may have a meningoencephalitis. Clinical signs due to the mature cyst relate to pressure atrophy of adjacent nervous tissue and vary according to location in the brain. There may be impaired vision or locomotion if cysts are in the cerebral hemispheres and the sheep gradually may be unable to feed and will become emaciated. Cerebellar cysts may precipitate more acute and severe signs of ataxia or opisthotonus. In heavy infections, parasites migrate and begin development in other tissues, but they die early. These produce small lesions, 1 mm or so in size, that first contain an encapsulated cyst, then eosinophilic, caseous material that later may calcify.

**Judgement:** Initially only the head is condemned or occasional cysts in intermuscular or subcutaneous sites are trimmed. With chronic infection, the animal may have been unable to feed, resulting in condemnation due to emaciation, etc.

*Taenia ovis:* The predilection sites are as for *T. saginata.* Cysts may be confused with large *Sarcocystis gigantea* sarcocysts.

**Judgement:** Commonly detection of up to 2–5 cysts results in trimming and the carcass is passed. This does not prevent the unaesthetic presence of live or degenerate parasites in other tissues. Ultrasound and X-rays are being tested for detection of these. Some authorities may require that the meat be boned, trimmed and frozen or cooked. In heavy infections the carcass is condemned.

In general, meat inspection procedures detect only about 20–50% of the animals that are actually infected. Light infections are easily missed on palpation and meat inspection – in one study, 78% of carcasses infected with >20 cysts were detected compared with those detected following dissection and slicing, while only 31% of those with fewer cysts were detected (35). Meat inspection efficacy will vary with the number and location of incisions (and the skill and experience of the inspector). For example, in Zimbabwe, 58% of cattle were positive in the head only, 20% in the shoulder only and 8% in the heart only, although overall 81% were found to be infected if all three organs were included. Walther & Koske (35) in Kenya also found that the predilection sites were not necessarily infected in 57% of the cattle found positive on dissection. They also confirmed the importance of the shoulder incisions in detection of infection in Africa as 20% of the cattle found to be infected were positive in the shoulder only. Wanzaela et al. (36), also in Kenya, described the insensitivity of meat inspection in detecting cysticerci: only 50% of naturally or artificially infested cattle were identified. Their observations indicated that a number of viable cysticerci may be missed.

In humans, the most common presenting sign in *T. solium* NCC is seizures followed by headache, but a range of signs, such as vomiting, psychoses, etc., are seen depending on the number, location and viability or level of degeneration of the cysticerci (viable, transitional dying, calcification) (4, 21, 26). In humans, clinical evaluation and either computerised tomography (CT) scan (best for calcified cysts) or magnetic resonance imaging (MRI) (detects cysts in both parenchymal and extraparenchymal locations and can follow the progression of the lesion) are used to detect the exact locations and viability of *T. solium* and *T. multiceps* metacestodes. These remain the most efficient means of diagnosis, but access to imaging facilities may not be available in endemic areas. Calcified cysts in tissues are detected by radiography. Serology now is available for NCC.

### 2. Serological tests

The development of an automated sensitive and specific diagnostic test would greatly reduce the costs of damage to the carcass and also the costs of labour. Serological tests for animals have not reached the stage where commercialisation for individual diagnosis or large-scale detection of infected carcasses in slaughter houses is possible. All assays tested – AG-ELISA, antibody ELISA, enzyme-linked immunoelectrotransfer blot (EITB) and tongue inspection – show low sensitivity in rural pigs infected naturally with low levels of *T. solium* (7, 29). This contrasts with their high sensitivity and specificity when applied to commercially reared pigs free from infection and such pigs experimentally infected with *T. solium* (29, 30). This finding is also true for *T. saginata* infections in cattle (28, 33). Thus, only a small percentage (13–22%) of cattle carrying fewer than 30–50 viable cysticerci is detected by AG-ELISA. Conversely, antibody has proven most useful for detecting cysts that are no
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longer viable. Nonetheless, AG-ELISAs do have a use in field-based epidemiological studies for indicating transmission. For example, the detection of viable infections in cattle or pigs could indicate point sources of infection, season of transmission and age of animals at risk. The development of more sensitive and specific assays with recombinant antigens for diagnosis of NCC should improve immunodiagnosis of *T. solium* in pigs.

A number of EITB and ELISA assays for antibodies to *T. solium* in humans are now widely available commercially (i.e. Immunetics, USA; Cypress Diagnostics, USA; Diagnostic Automation, USA; United Biotech, USA, Arup Laboratories, USA; Biopharm, Germany). An AG-ELISA using polyclonal or monoclonal antibody (used to detect antigen in cerebrospinal fluid) has a specificity and sensitivity of up to 86% in selected patients. The specificity of these tests tends to be very high but sensitivity is lower, this is in part related to cyst number. The hierarchy of clinical symptoms, imaging studies and serological tests has been presented by del Brutto et al. (6).

C. REQUIREMENTS FOR VACCINES AND DIAGNOSTIC BIOLOGICALS

Immunological identification of protective antigens and their production by recombinant DNA technology has been uniquely successful in the Taeniidae compared with other eukaryotes, and is described by Lightowlers & Gauci (18, 19, 20). Vaccination with the resultant products has been highly effective. Overall the success was advanced by the fact that a strong protective immunity occurs after natural infection, high levels of protective immunity are induced by antigens in oncospheral extracts, there is good cross-immunity between Taenia species, and immunity is largely antibody mediated as evidenced by passive and maternal transfer of immunity so that antibody could be used to probe for protective antigens. Initially the *T. ovis* vaccine was developed. The *T. solium* 45W antigen was isolated as a recombinant protein from *Escherichia coli*. Potency control is by AG-ELISA and in vivo immunogenicity, and the vaccine has provided protection for a period of at least 1 year in field trials. Two other antigens (To16K and To18K) have been isolated and cloned and each individual *T. ovis* protein is protective. Using the benefits of cross-reaction and probing with the *T. ovis* cDNA, homologues of the *T. ovis* antigens, TSA9 and TSA18, equivalent to To45W and To18, were identified in *T. saginata* and cloned from *T. saginata* oncosphere mRNA. In contrast to the *T. ovis* individual antigens, immunisation with both *T. saginata* antigens was required to produce high level protection. Homologues of the *T. ovis* antigens also were cloned from *T. solium* mRNA and TSO45S and TSOL18 antigens of *T. solium*. Both the *T. ovis* and *T. saginata* vaccines have given >94% and 98% protection in cattle and sheep, respectively. Comparable antigens now have been identified for *T. multiceps*. The *T. ovis* vaccine was registered in 1994 by the New Zealand Animal Remedies Board. However, due to market changes in New Zealand, the vaccine is not available commercially. Costs of large-scale production of antigens, processing conditions and potential variants in expressed antigens have been outlined by Lightowlers & Gauci (20). The *T. solium* antigens, in particular TSOL18, have given high levels of protection experimentally (99%) (18); the antigen is undergoing the steps similar to the *T. ovis* antigens to develop a practical vaccine. Other avenues for vaccine antigens are being explored. Synthetic peptides from the sequences of the recombinant Taeniidae antigens induced antibody but not protection, indicating that the protective epitopes seemed conformational. Reasonable levels of protection were induced experimentally in piglets exposed to natural *T. solium* challenge using synthetic peptides based on protein sequences of the murine parasite *T. crassiceps* (14). The S3Pvac *T. solium* subunit vaccine has had some field efficacy in protecting against natural infection but requires further examination (5, 31). It is possible that cost–benefit analyses concerning the use of the *T. saginata* vaccine could obviate its use in many countries, as cost of the vaccine is very important to the livestock industries. The importance of *T. solium* in humans increases the costs of the disease, but it remains to be seen whether the significance of the disease in endemic countries will be sufficient to push commercial production of the vaccine for use in pigs. Sensitive immunodiagnosis for metacestodes still requires development to access vaccine efficacy.

REFERENCES


* * *
Hendra virus (HeV) and Nipah virus (NiV) emerged in the last decade of the twentieth century as the causes of outbreaks of respiratory and neurological disease that infected a number of animal species. In 1994, HeV caused severe respiratory disease and the death of 13 horses and a horse trainer at a stable in Brisbane, Australia. Between September 1998 and April 1999, after spreading unrecognised as a respiratory or encephalitic infection in Malaysian pigs, NiV appeared in the human population there and was the cause of fatal encephalitis. Over one million pigs were culled to stop spread of the disease. HeV has caused the death of two people while it has been reported that there have been 400 cases of NiV in humans, with approximately 200 deaths, in Malaysia, Singapore, Bangladesh and India. Fruit bats (flying foxes) in the genus Pteropus are natural hosts of both viruses.

HeV infection of horses is characterised progressively by high fevers, facial swelling, severe respiratory difficulty and, terminally, copious frothy nasal discharge. Some horses display neurological signs. The most common post-mortem observations are dilated pulmonary lymphatics, severe pulmonary oedema and congestion. The underlying lesion is generalised degeneration of small blood vessels in a range of organs. Syncytial endothelial cells containing viral antigen are common in capillaries and arterioles. HeV infection of horses is not uniformly fatal and some horses manifesting clinical signs survive infection. Laboratory transmission experiments have shown that HeV is not readily transmitted between horses, a finding consistent with the observation that in the original outbreak, the infection did not spread widely to horses in adjacent properties.

NiV infection of pigs is highly contagious, but it was not initially identified as a new disease because morbidity and mortality were not marked and clinical signs were not significantly different from other known pig diseases. Observations made during the outbreak investigation and during experimental infections confirmed that NiV infection of pigs is characterised by fever with respiratory involvement. In animals showing disease, nervous signs have been frequently reported, but many infections are subclinical. Some infected animals display an unusual barking cough. Abortion is reported in sows. Immunohistochemical lesions are found in either or both the respiratory system (tracheitis and bronchial and interstitial pneumonia) and the brain (meningitis) of infected animals. Syncytial cells containing viral antigen are seen in small blood vessels, lymphatic vessels and the respiratory epithelium.

Both viruses affect companion animals. HeV causes pulmonary disease in cats similar to that observed in horses. Natural infection of dogs with NiV causes a distemper-like syndrome with a high mortality rates; there is serological evidence that some dogs survive infection. Experimentally NiV causes a similar disease to HeV in cats. Syncytial endothelial cells containing viral antigen were demonstrated in both HeV and NiV infections in cats and in NiV infection in dogs.

Infection of humans is from animal contact, usually from an amplifier host rather than directly from the natural, reservoir host: NiV from swine and HeV from horses. However investigations of outbreaks of human NiV in Bangladesh have indicated infection from Pteropid bats. Human-to-human transmission has not been seen with HeV or with NiV in Malaysia and Singapore, but human-to-human transmission is suspected in recent outbreaks of NiV in Bangladesh.

HeV and NiV are closely related members of the family Paramyxoviridae. Differences between them and other family members have led to their classification in a new genus, Henipavirus, in the subfamily Paramyxovirinae. HeV and NiV are biosafety level 4 agents and it is important that
samples from suspect animals be transported to authorised laboratories only under biologically secure conditions according to international regulations.

**Identification of the agent:** Both HeV and NiV may be propagated in a range of cultured cells. Virus isolation from unfixed field samples should be attempted, but only in situations where operator safety can be assured. Identification procedures following virus isolation include immunostaining of infected cells, neutralisation with specific antisera and molecular characterisation. Real-time polymerase chain reaction (PCR) is now available as a diagnostic test.

Viral antigen is present in vascular endothelium, and in the case of NiV in pigs, the respiratory epithelium. A wide range of formalin-fixed tissues can be examined to detect HeV and NiV antigens. Submissions for immunohistochemistry should include samples of brain at various levels including meninges, lung, spleen and kidney. In pregnant animals or in cases of abortion, uterus, placenta and fetal tissues should be included as appropriate. Specimens for virus isolation and molecular detection of virus should be fresh tissues from the same organs, or urine or throat or nasal swabs.

**Serological tests:** Virus neutralisation tests (VNT) and enzyme-linked immunosorbent assay (ELISA) are available. VNT is currently accepted as the reference procedure. The ability of antisera to HeV and NiV to cross-neutralise to a limited degree means that a single VNT using either virus does not provide definitive identification of antibody specificity. Neutralising antibodies to HeV and NiV can be differentiated by the greater capacity to neutralise the homologous compared with the heterologous virus. This may not be a major impediment in outbreak situations where the causative agent is known, but serum samples from suspect cases or from areas of the world other than Australia and Malaysia should be subjected to VNT analyses with both HeV and NiV. The serological relationship between HeV and NiV ensures that ELISAs using HeV or NiV antigen can be used to detect antibodies to both viruses.

**Requirements for vaccines and diagnostic biologicals:** There are no vaccines currently available for either HeV or NiV.

### A. INTRODUCTION

Hendra virus (HeV) and Nipah virus (NiV) occur naturally as viruses of fruit bats commonly known as ‘flying foxes’, members of the genus *Pteropus*. Antibodies to HeV are found in approximately 50% of the four Australian *Pteropus* species (35). Serological surveys of antibodies to NiV show seroprevalences up to 20% in Malaysian pteropid bats (10, 18). Antibodies to NiV or putative closely related viruses have subsequently been detected in pteropid bats in Bangladesh (15), Cambodia (24, 27), Indonesia (29), Madagascar (19) and Thailand (31). HeV has been isolated from Australian flying foxes (12), and NiV from flying foxes from Malaysia and Cambodia (4, 27). NiV RNA has been detected by polymerase chain reaction (PCR) in pteropid bat urine, saliva and blood in Thailand (30, 31).

HeV disease emerged in Brisbane, Australia, in September 1994 in an outbreak of acute respiratory disease that killed 13 horses and a horse trainer (22). The virus was initially called equine morbillivirus, but subsequent genetic analyses indicated that it did not resemble morbilliviruses closely enough to merit inclusion in that genus. There have been other instances of fatal HeV infection of horses in northern Queensland and further instances of infection of people. Two horses developed an acute disease and died almost 1 month before the Brisbane outbreak, but HeV was determined to be the cause of death only after the horse owner, who probably acquired HeV during necropsy of the horses, died 13 months later with HeV-mediated encephalitis (28). A third horse died in January 1999 with no associated human disease (11). Two further equine cases occurred in 2004, one confirmed and the other unconfirmed, the latter identified by an associated human infection (13). In 2006 Australia reported two further cases in horses, one in Southern Queensland and one in northern New South Wales. All outbreaks since 1995 have involved infection of only one horse at pasture without transmission to in-contact animals.

In Malaysia, retrospective studies of archival histological specimens indicate that NiV has caused low mortality in pigs since 1996, but remained unknown until 1999 when it emerged as the causative agent of an outbreak of encephalitis in humans that had commenced in 1998 (3, 23). Unlike respiratory disease caused by HeV in horses, which was frequently fatal but characterised by poor transmissibility (33), respiratory disease caused by NiV in pigs was often subclinical but highly contagious, properties that led to rapid virus dispersal through the Malaysian pig population and forced authorities to choose culling as the primary means to control spread (23). Over one million pigs were destroyed, 106 of 267 infected humans, mostly pig farmers in Malaysia and abattoir workers in Singapore who had direct contact with live pigs, died of encephalitis (3, 26).
New foci of human NiV disease have subsequently been identified in Bangladesh and India. In outbreaks in 2001 and 2003 an animal source of the human infections was not identified (15), but pteropid bats, *Pteropus giganteus*, were present and had antibodies capable of neutralising Nipah virus. Clustering of cases and time–sequence studies indicated that there is human-to-human transmission but at low levels (15). In another outbreak in 2004 in which 27 of 36 infected humans died, epidemiological evidence indicated person-to-person transmission and serological studies identified seropositive fruit bats at the location (1). Drinking fresh date palm sap contaminated by fruit bat saliva, urine or excreta has been identified as one possible route of transmission from the wildlife reservoir to humans (20). As a result of these ongoing outbreaks it is estimated that across Malaysia, Singapore, Bangladesh and India there have now been up to 400 cases of NiV in humans, with approximately 200 deaths.

NiV and HeV are classified taxonomically as paramyxoviruses in the subfamily Paramyxovirinae, and have been grouped in a separate and new genus, the henipaviruses (9).

Diagnosis of disease caused by Henipaviruses is by virus isolation, detection of viral RNA in clinical or post-mortem specimens or demonstration of viral antigen in tissue samples taken at necropsy (8). Detection of specific antibody can also be useful particularly in pigs where NiV infection may go unnoticed. Identification of HeV antibody in horses is less useful because of the high case fatality rate of infection in that species. Human infections of both HeV and NiV have been diagnosed retrospectively by serology. Demonstration of specific antibody to HeV or NiV in either animals or humans is of diagnostic significance because of the rarity of infection and the serious zoonotic implication of transmission of infection.

### B. DIAGNOSTIC TECHNIQUES

1. **Identification of the agent**

   a) **Virus isolation and characterisation**

   HeV and NiV are classified as biosafety level 4 (BSL4) agents, as they are dangerous human pathogens with a high case fatality rate and for which there is no vaccination or effective antiviral treatment. BSL4 is similar to containment group 4 as described in the OIE biosafety guidelines in Chapter 1.1.2 Biosafety and biosecurity in the veterinary microbiology laboratory and animal facilities, provide additional information. However, due to the high risk consequences of human infection in the laboratory, BSL4 requirements surpass the OIE containment level 4 requirements. Virus isolation greatly facilitates identification procedures and definitive diagnosis should be undertaken where operator safety can be guaranteed. Isolation is especially relevant in any new case or outbreak, particularly in countries or geographical areas where infection by HeV or NiV has not been previously documented. Implication of wildlife species as natural hosts of the viruses requires positive serology, PCR or virus isolation from wild-caught animals (7).

   i) **Sampling and submission of samples**

   Diagnostic samples should be submitted to designated laboratories in specially designed containers. The International Air Transport Association (IATA), Dangerous Goods Regulations (DGR) for shipping specimens from a suspected zoonotic disease must be followed (17). The requirements are summarised in Chapter 1.1.1 Collection and shipment of diagnostic specimens.

   The range of tissues yielding virus in natural and experimental cases has been summarised (6). Brain, lung, kidney and spleen should always be submitted. Samples should be transported at 4°C if they can arrive at the laboratory within 48 hours; if shipping time will be over 48 hours, the samples should be sent frozen on dry ice or nitrogen vapours should be used. Samples should not be held at –20°C for long periods.

   ii) **Isolation in cultured cells**

   Virus propagation should be conducted under BSL4 conditions. Strict adherence to this guideline would limit the handling of diagnostic specimens where the presence of HeV or NiV may be suspected but not confirmed to laboratories with BSL4 facilities. Primary virus isolation from suspect samples may of necessity be conducted under BSL3 conditions. However, if this is to be attempted, stringent local guidelines must be developed to ensure operator safety and applied if a ‘paramyxovirus-like’ cytopathic effect (CPE) develops in infected cultures. Such guidelines will emphasise good laboratory practice, the use of class II safety cabinet with appropriate personal protective equipment or a class III cabinet and may require acetone fixation of infected cells, to destroy infectious virus, followed by immunofluorescent detection of Henipavirus antigen. The culture medium from Henipavirus-positive cells should be transferred to a BSL4 laboratory.

   At the recipient laboratory tissues are handled under sterile conditions, and 10% (w/v) suspensions are generated by grinding the tissues in a closed homogenisation system, e.g. stomacher/bag mixer...
using plastic bags or mixer mills using autoclavable steel balls in closed metal cylinders. All processes should be carried out in a Class III cabinet or a Class II cabinet with appropriate personal protective equipment, with the stomacher operated in the cabinet and the centrifuge pots, with aerosol covers, loaded and unloaded in the cabinet. Following clarification of the homogenate by centrifugation at 300 g, the supernatant is added to cultured cell monolayers. Virus isolation is aided by the fact that HeV and NiV grow rapidly to high titre in many cultured cells. African green monkey kidney (Vero) and rabbit kidney (RK-13) cells have been found to be particularly susceptible. HeV also replicates in suckling mouse brain and in embryonated hen’s eggs, and although the former may represent a viable method of primary isolation, there are no data on the relative susceptibility of in-vivo systems such as these compared with the more convenient cell culture systems. A CPE usually develops within 3 days, but two 5-day passages are recommended before judging the attempt unsuccessful. After low multiplicity of infection, the CPE is characterised by formation of syncytia that may, after 24–48 hours, contain over 60 or more nuclei. Syncytia formed by NiV in Vero cell monolayers are significantly larger than those created by HeV in the same time period. Although the distribution of nuclei in NiV-induced syncytia early in infection resembles that induced by HeV, with nuclei aggregated in the middle of the syncytia, nuclei in mature NiV-induced syncytia are distributed around the outside of the giant cell (16).

iii) Methods of identification

• Immunostaining of fixed cells

The speed with which HeV and NiV replicate and the high levels of viral antigen generated in infected cells make immunofluorescence a useful method to rapidly identify the presence of Henipaviruses using either anti-NiV or anti-HeV antiserum. At present the Henipavirus genus consists of HeV and NiV and there are no known antigenically related viruses.

The serological cross-reactivity between HeV and NiV means that polyclonal antiserum to either virus or mono-specific antisera to individual proteins of either virus, will fail to differentiate between HeV and NiV. Monoclonal antibodies (MAbs) are currently being generated and tested to fulfil this function both in primary identification of the virus upon isolation and for use in immunohistochemical examination of tissues from suspect cases.

• Test procedure

Under BSL4, monolayers of Vero or RK-13 cells grown on glass cover-slips or chamber slides are infected with the isolated virus, and the monolayers are examined for the presence of syncytia after incubation for 24–48 hours at 37°C. It is recommended that a range of virus dilutions (undiluted, 1/10, 1/100) be tested because syncytia are more readily observed after infection at low multiplicity. Once visible syncytia are detected, infected cells are fixed by immersion in a vessel filled completely with acetone. The vessel is sealed and surface sterilised prior to removal to a less secure laboratory environment, for example BSL2, where the slides are air-dried. Viral antigen is detected using anti-HeV or anti-NiV antiserum and standard immunofluorescent procedures. A characteristic feature of Henipavirus-induced syncytia is the presence of large polygonal structures containing viral antigen. These are observed most readily with monospecific and MAbs to the nucleocapsid protein N and phosphoprotein P.

• Immunoelectron microscopy

The high titres generated by HeV and NiV in cells in vitro permits their visualisation in the culture medium by negative-contrast electron microscopy without a centrifugal concentration step. Detection of virus– antibody interactions by immunoelectron microscopy provides valuable information on virus structure and antigenic reactivity, even during primary isolation of the virus. Other ultrastructural techniques, such as grid cell culture (13), in which cells are grown, infected and visualised on electron microscope grids, and identification of replicating viruses and inclusion bodies in thin sections of fixed, embedded cell cultures and infected tissues complement the diagnostic effort. The details of these techniques and their application to the detection and analysis of HeV and NiV have been described (16).

b) Virus neutralisation: differentiation of HeV and NiV

Neutralisation tests rely on quantification methods and three procedures are available to titre HeV and NiV. In the traditional plaque and microtitre assay procedures, the titre is calculated as plaque forming units (PFU) or the tissue culture infectious dose capable of causing CPE in 50% of replicate wells (TCID<sub>50</sub>), respectively. In an alternative procedure, the viruses are titrated on Vero cell monolayers in 96-well plates and after 18–24 hours, foci of infection are detected immunologically in acetone-fixed cells using anti-viral antiserum (5). The virus titre is expressed as focus-forming units (FFU)/ml.

Neutralisation assays using these three methods are described below. A virus isolate that reacts with anti-HeV and/or anti-NiV antisera in an immunofluorescence assay is considered to be serologically identical to either HeV or NiV if it displays the same sensitivity to neutralisation by anti-HeV and anti-NiV antisera as
HeV or NIV. Anti-HeV antiserum neutralises HeV at an approximately fourfold greater dilution than that which neutralises NIV to the same extent. Conversely, anti-NIV antiserum neutralises NIV approximately four times more efficiently than HeV (3). Virus quantification procedures should be conducted at BSL4. A new version of the differential neutralisation test has been recently described, which avoids the use of infectious virus by the use of ephrin-B2-bound biospheres (2). Although the test has yet to be formally validated, it appears to have the potential to be a screening tool for use in countries without BSL4 facilities.

i) Plaque reduction

Stock HeV and NIV and the unidentified Henipavirus are diluted in media and replicates of each virus containing approximately 100 PFU in 50–100 μl are mixed with an equal volume of either Eagle's minimal essential media (EMEM) or a range of dilutions of anti-HeV or anti-NIV antiserum in EMEM. The virus–antiserum mixtures are incubated at 37°C for 45 minutes, adsorbed to monolayers of Vero cells at 37°C for 45 minutes and the number of plaques determined by traditional plaque assay procedures after incubation at 37°C for 3 days.

ii) Microtitre neutralisation

Stock HeV and NIV and the unidentified Henipavirus are diluted and replicates of each virus containing approximately 100 TCID50 in 50 μl are added to the test wells of a flat bottom 96-well microtitre plate. The viruses are mixed with an equal volume of either EMEM or a range of dilutions of anti-HeV or anti-NIV antiserum in EMEM. The mixtures are incubated at 37°C for 45 minutes and approximately 2.4 × 10^4 cells are added to each well to a final volume of approximately 200 μl. After 3 days at 37°C, the test is read using an inverted microscope and wells are scored for the degree of CPE observed. Those that contain cells only or cells and antiserum should show no CPE. In contrast, wells containing cells and virus should show syncytia and cell destruction. A positive well is one where all or a proportion of cells in the monolayer form large syncytia typical of henipavirus infection.

iii) Immune plaque assay

Vero cells (2 × 10^4 in 200 μl medium/well) are added to flat-bottom microtitre plates and grown overnight at 37°C. Stock HeV and NIV and the unidentified Henipavirus are diluted and replicates containing about 60 FFU/50 μl are mixed with an equal volume of either EMEM or a range of dilutions of anti-HeV and anti-NIV antiserum diluted in EMEM. Virus–antiserum mixtures are incubated for 45 minutes at 37°C and adsorbed to Vero cell monolayers for 45 minutes at 37°C. Virus–antiserum mixtures are removed, 200 μl EMEM is added to each well and incubation is continued at 37°C. After 18–24 hours the culture medium is discarded and plates are immersed in cold, absolute acetone for 10 minutes and then placed in plastic bags, which are filled with acetone, heat-sealed and surface sterilised with 4% (v/v) lysol during removal from the BSL4 laboratory. Gluteraldehyde can also be used for sterilisation at concentrations as low as 0.1% for 24 hours. It is recommended that each laboratory determine the concentration of gluteraldehyde required for sterilisation within the time frame required. Acetone-fixed plates are air-dried, the wells are blocked with phosphate buffered saline (PBS) containing 0.05% Tween 20 and 2% skim milk powder, and incubated for 30 minutes at 37°C with antiserum to either HeV or NIV or a monospecific antiserum to a virus protein. Anti-viral antibody binding to syncytia can be detected using alkaline phosphatase-conjugated species-specific antibody and the substrate 5-bromo-4-chloro-3-indolyl phosphate and p-nitro blue tetrazolium substrate (NBT/BCIP; Promega, Catalog number S3771). When purple plaques appear against a clear background (10–30 minutes), the substrate is removed and the plates are rinsed with distilled water and air-dried. Plaques are counted using a magnifying glass.

c) Nucleic acid based recognition methods

The complete genomes of both HeV and NIV have been sequenced (32), and PCR-based methods have been used to detect virus and are being validated in a number of laboratories.

A particularly sensitive and useful approach to the detection of henipavirus genome in specimens is real-time PCR. This method has the biosafety advantage of not propagating live infectious virus. Test methods and primers used depend on the technology platform and associated chemistry being used in individual laboratories (21, 30). The virus-specific reagents used in one such assay (21) based on Taqman chemistry are as follows:

<table>
<thead>
<tr>
<th>Primer #</th>
<th>Sequence (5' to 3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>HENDRA-N1433F</td>
</tr>
<tr>
<td>2</td>
<td>HENDRA-N1572R</td>
</tr>
<tr>
<td>TaqMan</td>
<td>HENDRA-N1510T-FAM</td>
</tr>
</tbody>
</table>

For Hendra virus detection:

<table>
<thead>
<tr>
<th>Primer #</th>
<th>Sequence (5' to 3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5'-ATC-TCA-GAT-CCA-GAT-TAG-CTG-CAA-3'</td>
</tr>
<tr>
<td>2</td>
<td>5'-ATC-ATT-TTG-GGC-AGG-TTT-GG-3'</td>
</tr>
<tr>
<td>TaqMan</td>
<td>5'-6FAM-AAC-CGC-CCT-CAG-GCA-GAC-TCA-GGA-TAMRA-3'</td>
</tr>
</tbody>
</table>
For Nipah virus detection:

<table>
<thead>
<tr>
<th>Primer # 1</th>
<th>(Nipah-N1198F) 5'-TCA-GCA-GGA-AGG-CAA-GAG-AGT-AA-3'</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primer # 2</td>
<td>(Nipah-N1297R) 5'-CCCCTTCATCGATATCTTGATCA-3'</td>
</tr>
<tr>
<td>TaqMan probe</td>
<td>(Nipah-1247comp-FAM): 5'-6FAM-CCT-CCA-ATG-ACA-CCT-GCA-G-TAMRA-3'</td>
</tr>
</tbody>
</table>

Laboratories wishing to establish molecular detection methods should refer to published protocols or consult the OIE Reference Laboratory.

d) Henipavirus antigen detection in fixed tissue – immunohistochemistry

Immunohistochemistry is also a useful test in HeV and NiV detection. Performed on formalin-fixed tissues or formalin-fixed cells, it is safe and has allowed retrospective investigations on archival material. As virus replication and the primary pathology occur in the vascular endothelium (14), there is a wide range of tissues in which HeV and NiV antigen can be detected (8). It is thought that HeV antigens may be cleared from lung tissue early in the course of infection and so the sample submitted should include a range of tissues, not just lung. HeV antigen has been detected in the kidney of a horse 21 days post-infection (33) and so this organ should always be submitted. Ideally a submission for immunohistochemistry would include samples of the brain at various levels, lung, mediastinal lymph nodes, spleen and kidney. In pregnant animals the uterus, placenta and fetal tissues should be included.

A range of antisera to HeV and NiV may be used in immunohistochemical investigations of HeV- and NiV-infected tissues, but rabbit antisera to plaque-purified HeV and NiV have been found to be particularly useful. Some MAbs are also available. The Nipah Virus Pathology Working Group has described a detection system (34). A biotin–streptavidin peroxidase-linked detection system has also been used successfully (14). The following detection system is an anti-rabbit/anti-mouse dextran polymer-linked reagent conjugated with alkaline phosphatase.

- Test procedure
  
i) Dewax slides containing formalin-fixed, paraffin-embedded test material and positive and negative control tissue sections by immersion three times in xylene for 1 minute. Hydrate sections through two changes of 98–100% ethanol, one change of 70% ethanol and running tap water to remove residual alcohol.
  
ii) Rinse slides in distilled water, immerse in 0.01 M CaCl₂ (adjusted to pH 7.8 with 0.1 M sodium hydroxide) containing 0.1% (w/v) trypsin (Difco Trypsin 250) for 20 minutes at 37°C and wash in distilled water.
  
iii) Lay slides flat in a humid chamber and rinse with PBS for 5 minutes. Add 200 µl 3% aqueous H₂O₂ to each slide for 20 minutes at room temperature to block endogenous peroxidase. Rinse slides in PBS for 5 minutes.
  
iv) Add 200 µl of an appropriate dilution of rabbit anti-Nipah or anti-Hendra antibody in PBS containing 0.1% (w/v) skim milk powder to test tissue slides and positive and negative control slides. To a duplicate set of test and positive and negative control slides add rabbit antibody to an unrelated pathogen. Cover the slides and incubate at 37°C for 1 hour.
  
v) Rinse slides in PBS for 5 minutes and apply 2–3 drops of Envision™ solution (anti-rabbit Ig conjugated to peroxidase-labelled dextran polymer [DAKO Corporation, 6392 Via Real, Carpinteria, CA 03013]). Incubate at 37°C for 20 minutes.
  
vi) Prepare the substrate by dissolving 2 mg 3-amino-9-ethylcarbazole (AEC) in 200 µl dimethyl formamide (Merck) and add to 10 ml 0.02 M acetate buffer, pH 5.0. Add 5 µl H₂O₂ (30% w/v) and mix. Check the positive control slide for sufficient staining, usually 2–5 minutes, and stop the reaction by rinsing in distilled water. The substrate solution should be made fresh prior to use.
  
vii) Counterstain the slides in haematoxylin for 1–3 minutes, rinse in tap water, add Scott’s solution (0.04 M sodium bicarbonate, 0.3 M magnesium sulphate), and wash well in running tap water. Rinse the slides in distilled water and mount with a cover-slip using aqueous mounting medium.
  
viii) Read the slides for cytoplasmic deposition of the chromogen indicating the presence of viral antigen. Brown/red granular staining will be seen in the cytoplasm of positive cells. The cell nuclei are blue and this facilitates identification of tissue morphology and assists in the location of viral antigen within the tissue.
2. Serological tests

In laboratories doing serological testing, particularly in outbreak situations, several strategies have been adopted to reduce the risk of exposure of laboratory personnel to HeV and NiV. Sera may be gamma-irradiated (6 kiloGreys) or diluted 1/5 in PBS containing 0.5% Tween 20 and 0.5% Triton-X100 and heat-inactivated at 56°C for 30 minutes. The process used will be based on a risk assessment. Specimens for surveillance testing and testing for animal movement certification may be considered a lesser biosafety risk than those for disease investigation. In some circumstances heat inactivation may be adopted as a sufficient precaution. However there is value in having a standardised approach for all samples in managing a test, rather than be maintaining multiple test methods.

a) Virus neutralisation tests

Henipaviruses can be quantified by plaque, microtitre or immune plaque assays and these assays can be modified to detect anti-virus antibody (see above). The virus neutralisation test (VNT) is accepted as the reference standard. In the most commonly used microtitre assay, which is performed under BSL4 conditions, sera are incubated with virus in the wells of 96-well microtitre plates prior to the addition of Vero cells. Sera are screened starting at a 1/2 dilution although this may lead to problems with serum-induced cytotoxicity. Where sample quality is poor or sample volumes are small, as may be the case with flying fox or microbat sera, an initial dilution of 1/5 may be used. Cultures are read at 3 days, and those sera that completely block development of CPE are designated as positive. If cytotoxicity is a problem the immune plaque assay described above has merit because the virus/serum mixtures are removed from the Vero cell monolayers after the adsorption period, thereby limiting their CPE.

b) Enzyme-linked immunosorbent assay

Henipavirus antigens derived from tissue culture for use in the enzyme-linked immunosorbent assay (ELISA) are irradiated with 6 kiloGreys prior to use, a treatment that has negligible effect on antigen titre. In the indirect ELISA developed in response to the initial outbreak at Hendra in 1994, antigen was derived from HeV-infected cells subjected to several cycles of freezing and thawing and treatment with 0.1% (w/v) sodium dodecyl sulphate (P. Selleck, unpublished data). In the national swine surveillance programme in Malaysia in 1999 (6) a similar indirect ELISA format was used in which antigen was derived by non-ionic detergent treatment of NiV-infected cells. Subsequently, to control for high levels of nonspecific binding activity in some porcine antisera, a modified ELISA was developed based on the relative reactivity of sera with NiV antigen and a control antigen derived from uninfected Vero cells. At the Centers for Disease Control (CDC), Atlanta, USA, the approach has been to not only have an indirect ELISA for detection of IgG but also to use a capture ELISA for detection of IgM. For NiV, an ELISA using a recombinant nucleocapsid antigen has also been described (36), which is also configured to detect either IgG or IgM.

The specificity of the indirect NiV ELISA (98.4%) (25) means that in surveillance programmes, the test will yield false positives. This may not be a significant problem in the face of a NiV outbreak where a high proportion of pigs are infected and the purpose of the surveillance is to detect infected farms. However, this level of test specificity creates a problem in the absence of an outbreak or if the number of samples to be tested is limited. If a positive ELISA result was indicative of a bona fide infection, failure to respond may lead to virus spread and human fatalities. In contrast, initiating control measures in response to a false-positive ELISA result would be wasteful of resources (8). The current approach is to test all ELISA reactive sera by VNT, with sera reacting in the VNT considered to be positive. Confirmatory VNT should be done under BSL4 conditions and this may entail sending the samples to an internationally recognised laboratory.

The following procedure for the NiV ELISA has been developed at Australian Animal Health Laboratory (AAHL) for porcine sera and standardised after collaborative studies in the Veterinary Research Institute, Ipoh, Malaysia.

- **Test procedure**

  **Preparation of NiV antigens**

  i) Grow Vero cells until confluent in roller bottles in EMEM containing 10% (v/v) fetal calf serum (FCS). To infect with virus, pour off all but 5 ml of the medium from each roller bottle and, in a BSL4 laboratory, add low passage, plaque-purified NiV to a multiplicity of infection of 0.1 TCID<sub>50</sub>/cell.

  ii) Rotate roller bottles for 30 minutes at 33°C to adsorb virus, add 60 ml EMEM containing 10% FCS to each bottle and roll for a further 48 hours at 33°C. The multiplicity of infection, incubation time and temperature are chosen so that although the majority of cells become infected and are incorporated into syncytia within 48 hours, few cells detach into the culture medium. The culture medium of cells infected under these conditions is an excellent source of virus for further purification.
iii) Wash monolayers of virus-infected cells once with cold 0.01 M PBS and, using a large scraper, scrape cells from each roller bottle into 5–10 ml ice-cold PBS.

iv) Pool scraped cells into 50 ml tubes kept in ice and pellet the cells at 300 g for 5 minutes at 4°C. Pour off PBS and resuspend cells in ice cold TNM (10 mM Tris, 10 mM NaCl, 1.5 mM MgCl₂, pH 7.2), approximately 0.5 ml TNM per roller bottle.

v) Add NP40 (non-ionic detergent, Nonidet P40) to 1% (by addition of 1/10 volume of 10% [v/v] NP-40 in water) and lyse cells using 5–10 strokes of a Dounce homogeniser. This also releases from the cytoskeleton viral antigen that would otherwise be removed by centrifugation (step vi).

vi) Pellet the nuclei at 600 g for 10 minutes at 4°C. The nuclei will not lyse under these conditions and should form a tight white pellet.

vii) Gently remove the supernatant cytoplasmic extract into a clean tube and add ethylene diamine tetra-acetic acid to 1.5 mM. Make up to 10 ml with TNE, aliquot in small amounts, freeze at –80°C and gamma-irradiate with 6 kiloGreys. Store aliquots at –80°C.

Preparation of control, uninfected Vero cell antigen

viii) Grow Vero cells in roller bottles in EMEM containing 10% FCS. When confluent, wash monolayers once with cold PBS and scrape the cells from each roller bottle into 5–10 ml ice-cold PBS. Proceed as described for virus-infected cells in steps iv–vii above.

Preparation of test sera

ix) In a biological class II safety cabinet with appropriate personal protective equipment or a class III cabinet, dilute test serum 1/5 in PBS containing 0.5% (v/v) Triton X-100 and 0.5% (v/v) Tween 20 in the wells of a 96-well microtitre plate. Seal the microtitre plate. Laboratory personnel should wear gowns and gloves and spray both their hands and the sealed microtitre plate with 1% Virkon before removing the microtitre plate from the biosafety cabinet to heat at 56°C for 30 minutes.

x) Mix 22.5 µl heat-inactivated serum with an equal volume of uninfected Vero cell antigen diluted 1/100 in PBS. Mix thoroughly and incubate at 18–22°C for 30 minutes.

xi) Add 405 µl blocking solution (PBS containing 5% chicken serum and 5% skim milk powder) to give a final serum dilution of 1/100 and incubate at 18–22°C for 30 minutes. Aliquots of 100 µl are added to two wells containing NiV antigen and two wells containing uninfected Vero cell control antigen as described in step xiv.

ELISA procedure

xii) Dilute Vero cell control and NiV antigens in PBS to ensure that control and virus antigen wells are coated with a similar concentration of protein. Antigen is usually diluted 1/1000 to 1/4000, but a specific dilution factor must be determined for each batch of antigen. Add 50 µl virus and cell control antigen to the wells of a Nunc Maxisorp 96-well microtitre plate as follows: virus antigen in columns 1, 3, 5, 7, 9 and 11 and cell control antigen in columns 2, 4, 6, 8 10 and 12 (Fig. 1). Incubate at 37°C for 1 hour with shaking. Plates can be also incubated at 4°C overnight.

xiii) Wash ELISA plates three times with PBS containing 0.5% Tween 20 (PBST) (250 µl/well) and block with PBS containing 5% chicken serum and 5% skim milk powder (100 µl/well) for 30 minutes at 37°C on a shaker.

xiv) Wash plates three times with PBST and add 100 µl of inactivated, absorbed sera from step xi to each well as indicated in the format below. Add 100 µl PBS containing 5% chicken serum and 5% skim milk powder to conjugate and substrate control wells. Incubate the plates without shaking for 1 hour at 37°C and wash three times with PBST.

xv) Dilute protein A/G-horseradish peroxidase conjugate (Protein-A/G-Conjugate Supplied by Pierce, through Progen Biosciences Product No. 32490) in PBST containing 1% (w/v) skim milk powder. The dilution factor is approximately 1/50,000. Mix well and add 100 µl protein A-conjugate to all wells except the substrate control wells. Add 100 µl PBST containing 1% skim milk powder to the substrate control wells. Incubate the plates for 1 hour at 37°C without shaking and wash four times with PBST.

xvi) Prepare the substrate (3,3',5,5'-tetramethylbenzidine; TMB; Sigma, catalogue number T 3405) by dissolving one tablet (1 mg) in 10 ml of 0.05 M phosphate citrate buffer, pH 5.0, and add 2 µl of fresh 30% (v/v) H₂O₂. Add 100 µl of the TMB substrate to each well. Incubate for 10 minutes at 18–22°C and stop the test by adding 100 µl 1 M sulphuric acid to each well.

xvii) Read plates after blanking on a substrate control well. The optical density (OD) at 450 nm on NiV antigen and control Vero cell antigen are used to calculate an OD ratio for each serum (OD on NiV antigen/OD on Vero control antigen).
Interpretation of results

xviii) An OD ratio >2.0 with an OD on NiV antigen >0.20 is considered positive.

xix) An OD ratio >2.0 with an OD on NiV antigen <0.20 is considered negative.

xx) Sera displaying an OD ratio between 2.0 and 2.2 should be considered doubtful.

xxi) Doubtful and positive sera should be tested by VNT.

Fig. 1. ELISA plate format and result sheet.

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Nunc Maxisorp 96 well plate; Ni: Nipah virus infected cell antigen; U: uninfected Vero antigen (control antigen); H+: High positive control sera e.g. LAF pig 6 sera; N: Negative control sera e.g. negative pig sera; L+: Low Positive control sera e.g. LAF Pig 6 sera 1:900

C. REQUIREMENTS FOR VACCINES AND DIAGNOSTIC BIOLOGICALS

There are no vaccines currently available for either HeV or NiV.

ACKNOWLEDGEMENTS

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REFERENCES


17. International Air Transport Association (2002). Dangerous Goods Regulations, 43rd edition. International Air Transport Association (IATA), 800 Place Victoria, P.O. Box 113, Montreal, Quebec H4Z 1M1, Canada.


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NB: There is an OIE Reference Laboratory for Hendra and Nipah virus diseases (see Table in Part 3 of this Terrestrial Manual or consult the OIE Web site for the most up-to-date list: www.oie.int).
CHAPTER 2.9.7.

LISTERIA MONOCYTOGENES

SUMMARY

A wide variety of animal species can be infected with Listeria monocytogenes, but clinical listeriosis is mainly a ruminant disease, with occasional sporadic cases in other species. The main clinical manifestations of animal listeriosis are encephalitis, septicaemia and abortion, and the disease is often associated with stored forages, usually silage. Post-mortem findings and histopathology depend on the clinical presentation.

Listeriosis is one of the most important food-borne diseases of humans. The disease manifestations include septicaemia, meningitis (or meningoencephalitis) and encephalitis, usually preceded by influenza-like symptoms including fever. In pregnant women, intrauterine or cervical infections may result in spontaneous abortion or stillbirths. Listeria monocytogenes has also been associated with gastroenteric manifestations with fever. Although the morbidity of listeriosis is relatively low, the mortality of the systemic/encephalitic disease can be very high, with values in the vicinity of 30%. The elderly, pregnant women, newborns and the immunocompromised are considered to be at high risk of contracting the disease.

A number of molecular and cellular determinants of virulence have been identified for this intracellular pathogen, and although there is evidence of polymorphism among different strains of L. monocytogenes for some of these virulence determinants, this heterogeneity cannot be correlated with the ability or inability of the organism to produce disease. Therefore, all L. monocytogenes strains are considered to be potentially pathogenic.

Identification of the agent: A variety of conventional and rapid methods are available for the detection and identification of L. monocytogenes in food samples and specimens from animal listeriosis. Conventional methods remain the ‘gold standard’ with which other methods are compared. They are usually very sensitive. These methods use selective agents and enrichment procedures to reduce the number of contaminating microorganisms and allow multiplication of L. monocytogenes.

Although not required for regulatory purposes, different levels of subtyping L. monocytogenes strains are available, including serotyping, phage typing, multilocus enzyme electrophoresis, DNA restriction enzyme digestion patterns (conventional and pulse-field gel electrophoresis), nucleic acid sequence-based typing and random amplification of polymorphic DNA.

Serological tests: Serological tests for the detection of antibodies have not been traditionally used for the diagnosis of listeriosis. A number of formats have been tried and they have all been found to be largely unreliable, lacking sensitivity and specificity. Experimental serological assays based on the detection of anti-listeriolysin O have been used in some epidemiological investigations and as support for the diagnosis of culture-negative central nervous system infections. Immunohistochemical detection of L. monocytogenes antigens is a useful tool for the diagnosis of the encephalitic form of the disease.

Requirements for vaccines and diagnostic biologicals: It has proven very difficult to develop effective vaccines against L. monocytogenes which, as an intracellular organism, requires effector T cells for an effective immune response. Experimental vaccines in laboratory animals are being explored to confer protection to L. monocytogenes infection by a number of different approaches, including immunisation with plasmid DNA, CD40 signalling along with heat-killed L. monocytogenes, listeriolysin O-deficient mutants inoculated along with liposome-encapsulated listeriolysin O, and immunisation with listerial antigens and IL-12.
A. INTRODUCTION

A wide variety of animal species can be infected by *Listeria monocytogenes*, including mammals, birds, fish and crustaceans, although most of the clinical listeriosis occurs in ruminants; pigs rarely develop disease and birds are generally subclinical carriers of the organism. Most infections in animals are subclinical, but listeriosis can occur either sporadically or in epidemic form. In addition to the economic impact of listeriosis in animals, there is a link between animals and their role as a source of infection for humans primarily from consumption of contaminated animal products. Infection can be as a result of direct contact with infected animals, especially during calving or lambing (68); however, these infections are very rare. The relative importance of the zoonotic transmission of the disease to humans is not clear, and contamination from the food processing environment is apparently more relevant to public health (52).

The clinical manifestations of listeriosis in animals include encephalitis, septicaemia and abortion, especially in sheep, goats and cattle. The septicaemic form is relatively uncommon and generally, but not invariably, occurs in the neonate. It is marked by depression, inappetence, fever and death. The encephalitic form is sometimes referred to as ‘circling disease’ because of a tendency to circle in one direction, and it is the most common manifestation of the disease in ruminants. The signs include depression, anorexia, head pressing or turning of the head to one side and unilateral facial paralysis. Abortion is usually late term (after 7 months in cattle and 12 weeks in sheep) (34, 67). Only one clinical form of listeriosis usually occurs in a particular group of animals. Bovine and ovine ophthalmitis have also been described (66). Rarely mastitis of ruminants has been associated with *L. monocytogenes* infection. Gastro-intestinal infections can occasionally occur in sheep (21). When listeriosis occurs in pigs, the primary manifestation is septicaemia, with encephalitis reported less frequently and abortions rarely. Although birds are usually subclinical carriers, sporadic cases of listeriosis have been reported, most frequently septicaemia and far less commonly meningoencephalitis. Avian listeriosis may be the result of a secondary infection in viral disease conditions and salmonellosis (68).

The post-mortem findings and histopathology, in animal listeriosis, depend on the clinical presentation. In the encephalitic form, the cerebrospinal fluid may be cloudy and the meningeal vessels congested. Gross pathological lesions of the brain are rare. On occasion, the medulla shows areas of softening. However, the histopathology is characteristic of the disease, consisting of foci of inflammatory cells with adjacent perivascular cuffing, predominantly of lymphocytes and histiocytes, plasma cells and occasional neutrophils. The microabscesses in the brain stem often more severally affect one side of the brain. More extensive malacic pathology may occur. The medulla and pons are most commonly involved. In the septicaemic form, multiple foci of necrosis in the liver and, less frequently the spleen, may be noted. Aborted fetuses of ruminants show very little gross lesions, but autolysis may be present if the fetus was retained before being expelled (48, 67).

The evidence indicates that animal listeriosis is predominantly associated with stored forage and with the environment as the main source of contamination. Silage is the most frequent source (28, 69). The intestinal mucosa is the main route of entry, after oral ingestion, in the case of septicaemic/abortive listeriosis. The incubation period can be as short as 1 day. The incubation period for the encephalitic form is usually 2–3 weeks, and the course of the disease is usually short in sheep and goats; 1–4 days (52), although it can be more protracted in cattle.

Although *Listeria monocytogenes* has been recognised as an animal pathogen for many years, its significant role as a food-borne human pathogen became evident only in the 1980s, when documented reports of listeriosis outbreaks, traced to contaminated food, started to appear in the literature (57). Today, *L. monocytogenes* is considered to be one of the most important agents of food-borne disease. Possible explanations for the emergence of human food-borne listeriosis as a major public health concern include major changes in food production, processing and distribution, increased use of refrigeration as a primary preservation means for foods, changes in the eating habits of people, particularly towards convenience and ready-to-eat foods, and an increase in the number of people considered to be at high risk for the disease (elderly, pregnant women, newborns, immunocompromised) (53, 63).

The primary manifestations of listeriosis in humans include septicaemia, meningitis (or meningoencephalitis) and encephalitis, usually preceded by influenza-like symptoms including fever. Gastroenteric manifestations with fever also occur. Although the morbidity of listeriosis is relatively low, the mortality can reach values around 30%. In pregnant women, infection may result in abortion, stillbirth or premature birth (53, 61).

*L. monocytogenes* is a Gram-positive rod and is responsible for almost all infections in humans; although rare cases of infection due to *L. ivanovii* and *L. seeligeri* have been reported. In animals, *L. monocytogenes* is responsible for the majority of infections, but *L. ivanovii* and *L. innocua* infections have also been recorded. *L. ivanovii* has been associated with abortions and has been reported to very occasionally cause meningoencephalitis in sheep.

Although *L. monocytogenes* has definite zoonotic potential, it is also an important environmental contaminant of public health significance.
Subtyping of *L. monocytogenes* strains by a variety of methods is available for epidemiological investigations, but the fundamental question of whether all strains of *L. monocytogenes* are capable of causing disease remains unanswered (31, 42, 45, 46).

Several molecular virulence determinants have been identified that play a role in the cellular infection by *L. monocytogenes* and the unravelling of their mechanism of action has made of *L. monocytogenes* one of the most exciting models of host-pathogen interaction at the cellular and molecular levels. These virulence determinants include, among others, the internalins, listeriolysin O (LLO), ActA protein, two phospholipases, a metalloprotease, Vip protein, a bile exclusion system (BiIE) and a bile salt hydrolase (17, 22, 25, 29, 60). Although there is polymorphism among different strains of *L. monocytogenes* for some of these virulence determinants, it cannot be correlated with the ability or inability of the organism to produce disease (42).

**B. DIAGNOSTIC TECHNIQUES**

1. **Identification of the agent**

There is a variety of conventional and rapid methods currently available for the detection and identification of *L. monocytogenes* in food samples and specimens from animal listeriosis. Conventional bacteriological methods are important for various reasons: Their use results in a pure culture of the organism, which is useful for regulatory purposes. They remain the 'gold standards' against which other methods are compared and validated. These methods are usually very sensitive and they do not require sophisticated and expensive equipment. Some of the disadvantages of this group of methods include the relatively long period of time that the protocols require for completion, several 'hands-on' manipulations, the requirement for many different chemicals, reagents and media, the possibility of contaminating microorganisms masking the presence of the target ones, including overgrowth, the potential overlook of atypical variants of the target organism and the relative subjectivity involved when interpreting bacterial growth on selective and differential agar plates (1).

The isolation and identification of *L. monocytogenes* from food, environmental samples and animal specimens require the use of selective agents and enrichment procedures that keep the levels of contaminating microorganisms to reasonable numbers and allow multiplication of *L. monocytogenes* to levels that are enough for detection of the organism. In the early days of listerial clinical bacteriology, cold enrichment (32) was regularly used to this end, exploiting the ability of the organism to multiply at refrigeration temperatures, whereas contaminating bacteria would not multiply under these conditions. However, this procedure requires very long incubation times, often months, making it unacceptable for current investigations of food-borne outbreaks and sporadic cases, as well as for the implementation of effective hazard analysis critical control points (HACCP) programmes in food production and processing establishments. A number of selective compounds that allow growth of *L. monocytogenes* at normal incubation temperatures have been incorporated into culture media, thus shortening the time required for selective growth of the organism. Examples of these selective compounds include cycloheximide, colistin, cefotetan, fosfomycin, lithium chloride, nalidixic acid, acriflavine, phenylethanol, ceftazidime, polymyxin B and moxalactam (3, 4, 8, 35, 39, 65).

Bacteriological diagnosis of animal listeriosis has traditionally involved direct plating of specimens on blood agar or other enriched media and concomitant use of the 'cold enrichment' technique, with weekly subculturing for up to 12 weeks (32, 51, 67). Immunohistochemical detection of *L. monocytogenes* antigens in formalin-fixed tissue has proven to be more sensitive than direct plating and cold enrichment bacterial culture for the diagnosis of the encephalitic form of the disease in ruminants (20, 44). The introduction of alternative enrichment procedures and selective agents for the isolation of *L. monocytogenes* from food and environmental samples has opened up the possibility of using some of these techniques for the bacteriological analysis of samples from animal listeriosis.

In spite of advances made in the selective isolation of *L. monocytogenes* from food, there is still room for improvement in a number of areas. No single procedure can be credited with being sensitive enough to detect *L. monocytogenes* from all types of food (23). In addition, sublethally injured *L. monocytogenes* cells can be found in processed food due to freezing, heating, acidification and other types of chemical or physical treatment. These sublethally injured bacteria require special culture conditions for damage repair, before being able to be detected in culture.

a) **Isolation methods**

Conventional methods for the isolation of *L. monocytogenes* from food that have gained acceptance for international regulatory purposes include the United States Food and Drug Administration (FDA) method (35), the Association of Official Analytical Chemists (AOAC) official method (8), the ISO 11290 Standards (39–41), the United States Department of Agriculture (USDA)-Food Safety and Inspection Service (FSIS) method (65) and the French Standards (3, 4).

Depending on the nature of the sample, a particular method might be more suitable than others. The International Organization for Standardization Technical Committee ISO/TC 34, Agricultural Food Products,
Subcommittee SC 9, Microbiology, claims that the ISO Standard 11290, parts 1 and 2 (39–41), can be used for the detection of *L. monocytogenes* in a large variety of food and feed products. Although they recognise that this standard might not be appropriate in every detail in certain instances, they recommend that every effort should be made to apply this horizontal method as far as possible.

The FDA and AOAC methods can be used for milk and dairy products. The USDA-FSIS method is recommended for red meat and poultry (raw or cooked ready-to-eat), eggs, egg products and environmental samples.

The traditional procedure for the isolation of *L. monocytogenes* from animal tissues has been direct plating of specimens on sheep blood agar or other rich culture media and concomitant use of the ‘cold enrichment’ technique, with weekly subculturing for up to 12 weeks (32, 51, 67). Isolation of the organism by direct plating is relatively easy when numbers are large in a normally sterile site, like in the case of the septicæmic form of the disease, but isolation is difficult when the organism is present in low numbers, as in the case of the encephalitic form or when samples are heavily contaminated. A comparison of the efficiency of direct plating, cold enrichment and a slight modification of what became the AOAC method, found the latter to be superior over the other two, for the isolation of *L. monocytogenes* from a variety of animal necropsy material, both in terms of the time required for the isolation and identification of the organism, and the isolation rates (26).

For the enumeration of *L. monocytogenes*, the ISO Standard 11290, part 2 (40) applies, as well as optional protocols mentioned in the FDA and USDA-FSIS methods (35, 65).

In the case of animal listeriosis, the samples should be chosen according to the clinical presentation of the disease: material from lesions in the liver, kidneys and/or spleen, in the case of the septicæmic form; spinal fluid, pons and medulla in the case of the encephalitic form; and placenta (cotyledons), fetal abomasal contents and/or uterine discharges in the case of abortion. Refrigeration temperatures (4°C) must be used for handling, storing and shipping specimens. If the sample is already frozen, it should be kept frozen until analysis.

The protocol recommended for isolation of *L. monocytogenes* from animal necropsy material is described below as originally published (26). It is possible that this protocol could be improved by incorporating the recent updates to the AOAC method and new developments for identification and confirmation of *Listeria* isolates, but there is no published evidence to support this hypothesis yet.

- **Isolation procedure from animal necropsy material**
  
i) Inoculate 10–25 g or ml of sample (depending on the amount of sample available) into 225 ml *Listeria* enrichment broth. When dealing with samples from animal listeriosis, the size of the sample for inoculation maybe limited and less than the recommended for food samples (25 g or ml). If that is the case, as much sample material as possible (aiming at 10–25 g or ml) should be inoculated (26). (*Listeria* enrichment broth base: Oxoid tryptone soya broth, 30 g; Difco yeast extract, 6 g; water, 1 litre; selective agents: Acriflavine, 2.3 mg; nalidixic acid, 9.2 mg; cycloheximide, 11.5 mg; add selective agents to 225 ml of the broth base).
  
   ii) Incubate broth at 30°C for 48 hours.
  
   iii) Spread 0.1 ml of the enrichment broth culture onto Oxford agar plates.
  
   iv) Incubate plates at 37°C. Examine bacterial growth after 24 and 48 hours.
  
   v) Test five colonies (or all when fewer available) with typical appearance of *L. monocytogenes* for cell shape, Gram reaction, haemolytic activity on blood agar (defibrinated horse blood), tumbling motility at 20°C, fermentation of glucose (+), rhamnose (+) and xylose (−), hydrolysis of esculin and production of catalase.

All culture media prepared should be subjected to quality control and must support growth of the suspect organism from a small inoculum. The reference strain should be cultured in parallel with the suspicious samples to ensure that the tests are working correctly.

Food samples intended for analysis must be representative of the food, including the outer surface and the interior. The conventional culture methods include an enrichment procedure based on the use of liquid culture media containing selective agents. The nature of the media and the selective agents vary with the method. Both the FDA (35) and the ISO methods include a pre-enrichment step that is intended for the recovery of sublethally injured *L. monocytogenes* cells, whereas in the USDA-FSIS (65) and the AOAC methods (8) the samples are processed directly into enrichment broth. In the case of the FDA method, the pre-enrichment is carried out at 30°C for 4 hours in trypticase-yeast broth containing yeast extract (TSB YE) without selective agents. The ISO protocol uses a ‘primary enrichment’ for 24 hours at 30°C in the presence of selective agents, but at half the concentration (half Fraser broth).
Samples are enriched for 24–72 hours at 30°C, 35°C or 37°C, depending on the method. The FDA method uses TSB YE containing acriflavine, nalidixic acid and cycloheximide. The USDA-FSIS method uses two enrichment steps: The ‘primary’ enrichment is done in University of Vermont medium (UVM), containing nalidixic acid and acriflavine; the ‘secondary’ enrichment is carried out in Fraser broth, containing nalidixic acid, lithium chloride and acriflavine. The ISO standard indicates Fraser broth for the ‘secondary’ enrichment, containing the selective agents at full concentration, whereas the ‘primary’ enrichment is carried out in ‘half Fraser broth’, as indicated above. The AOAC method calls for selective enrichment in tryptone soy broth containing acriflavine, nalidixic acid and cycloheximide (‘selective enrichment medium’).

After selective enrichment, cultures are then plated on to selective/differential agar plates for isolation of presumptive colonies of \textit{L. monocytogenes}. All methods, except that of the ISO standard, use Oxford agar or a modification, MOX agar (USDA-FSIS). Oxford agar contains lithium chloride, cycloheximide, colistin, acriflavine, cefotetan and fosfomycin as selective agents, and typical colonies of \textit{Listeria} spp are small, black and surrounded by a black halo. In addition to Oxford agar, the FDA includes lithium chloride/phenylethanol/moxalactam (LPM) or PALCAM agar, which contains lithium chloride, polymyxin B, acriflavine and ceftazidime. The MOX agar, used in the USDA-FSIS method, contains lithium chloride, colistin and moxalactam. The two selective plating media used in the ISO standard method are: agar \textit{Listeria} according to Ottaviani and Agosti (ALOA), which contains lithium chloride, nalidixic acid, ceftazidime, polymyxin B and amphotericin B (or cycloheximide), and any other selective medium, of each laboratory’s choice, such as Oxford or PALCALM. Typical colonies of \textit{L. monocytogenes} in ALOA agar are green-blue, surrounded by an opaque halo (41).

Bio-Rad Laboratories have developed RAPID’L.Mono, a selective chromogenic medium for direct detection and enumeration of \textit{L. monocytogenes}, based on selective isolation, chromogenic detection of phosphatidylinositol-specific phospholipase C (PI-PLC) and xylose use. \textit{Listeria monocytogenes} develops blue colonies (PI-PLC positive) without yellow halo (xylose negative); \textit{L. ivanovii} produces greenish-blue colonies (PI-PLC positive) with yellow halo (xylose positive). Other \textit{Listeria} spp. colonies are white (PI-PLC negative).

\textbf{b) Conventional identification methods}

Typical \textit{Listeria} spp. colonies, on the above selective/differential agar plates, are then selected for further identification to the species level, using a battery of tests. The tests include the Gram-staining reaction, catalase, motility (both in a wet mount observed under phase-contrast microscopy and by inoculation into motility test media), haemolysis and carbohydrate use. The Christie–Atkins–Munch–Peterson (CAMP) test is a very useful tool to help identify the species of a \textit{Listeria} spp. isolate. It is used in the ISO and AOAC protocols and it is considered to be optional in the FDA and USDA-FSIS methods. The test is simple to perform and easy to read. It consists of streaking a \textit{ß}-haemolytic \textit{Staphylococcus aureus} (ATCC strain 49444 or 25923, NCTC strain 7428 or 1803) and \textit{Rhodococcus equi} (ATCC strain 6939, NCTC strain 1621) in single straight lines in parallel, on a sheep blood agar plate or a double-layered agar plate with a very thin blood agar overlay. The streaks should have enough separation to allow test and control \textit{Listeria} strains to be streaked perpendicularly, in between the two indicator organisms, without quite touching them (separated by 1–2 mm). After incubation for 24–48 hours at 35–37°C (12–18 hours if using the thin blood agar overlay), a positive reaction consists of an enhanced zone of \textit{ß}-haemolysis, at the intersection of the test/control and indicator strains. \textit{Listeria monocytogenes} is positive with the \textit{S. aureus} streak and negative with \textit{R. equi}, whereas the test with \textit{L ivanovii} gives the reverse reactions (51).

\begin{table}[h]
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\begin{tabular}{|l|c|c|c|c|}
\hline
Species & Haemolysis & Production of acid & CAMP test \\
& & & \textit{S. aureus} & \textit{R. equi} \\
& & Rhamnose & Xylose & \\
\hline
\textit{L. monocytogenes} & + & + & – & + & – \\
\hline
\textit{L. innocua} & – & – & – & – & – \\
\hline
\textit{L. ivanovii} & + & – & + & – & + \\
\hline
\textit{L. seeligeri} & (+) & – & + & (+) & – \\
\hline
\textit{L. welshimeri} & – & – & + & – & – \\
\hline
\hline
\hline
\end{tabular}
\caption{Differentiation of \textit{Listeria} species}
\end{table}

V: variable; (+): weak reaction; +: >90% positive reactions; –: no reaction.

Serology, lysogenic typing and the immunocompromised mouse pathogenicity assay are considered to be optional methods.
c) **Rapid identification methods**

The following protocols include conventional and nonconventional commercially available tests, e.g. Vitek, API, MICRO-ID, enzyme-linked immunosorbent assay (ELISA) kits and nucleic acid assay kits, to help in the identification of *L. monocytogenes*. Polymerase chain reaction (PCR), targeting the *hly* gene, has been found to be a sensitive and rapid technique for confirmation of the identification of suspect *L. monocytogenes* isolated on selective/differential agar plates (30). In addition to RAPID'L.Mono and ALOA agars, described above, other chromogenic media have been developed to differentiate *L. monocytogenes* colonies from those of other *Listeriae*: Oxoid chromogenic *Listeria* agar (OCLA), and CHROMagar *Listeria*, developed by CHROMagar Microbiology.

i) **MICRO-ID Listeria**

MICRO-ID *Listeria* is a commercially available system (Organon Teknika Corp., 100 Akzo Ave., Durham, NC 27704, USA) that has been validated by the AOAC (method 992.18) (5) for the presumptive identification of *Listeria* species isolated from food and environmental samples. It provides an alternative to conventional biochemical testing of *Listeria* spp. isolates by the FDA and USDA-FSIS methods. It is based on the principle that the test inoculum contains preformed enzymes that can be detected after 24 hours at 37°C. Differentiation of *Listeria* species is based on an octal code derived after adding the numerical values for each group of three tests and on the reactions obtained from the CAMP test and haemolysis characteristics, which are assayed separately.

ii) **Vitek Automicrobic System**

The Vitek Automicrobic System (bioMérieux Vitek, Inc., 595 Anglum Dr., Hazelwood, MO, USA) is an automated microbial identification system that can be used for the presumptive identification of food-borne *Listeria* species and for screening of non-*Listeria* isolates. It has been validated by the AOAC as method 992.19 (6). The system uses an incubator chamber with an optical reader, a filler/sealer unit for test kit inoculation and a computer. Gram-positive (GPI) and Gram-negative (GNI+) identification cards each contain 30 biochemical tests. Changes are analysed by the computer, which then assigns the test organism a genus and/or species. The identification of the *Listeria* species requires the use of the GPI card and two reactions on the GNI+ card. However, for identification of some *Listeriae*, the analyst must perform the CAMP, haemolysis and/or nitrate reduction tests as described under the FDA method.

Organisms placed in the ‘LM’ category are identified as *L. monocytogenes* or *L. innocua*; in the ‘LI’ category, as *L. ivanovii* or *L. seeligeri*; in the ‘LW’ category, as *L. welshimeri*; and in the ‘LG’ category, as *L. grayi* or *L. murrayi* (a subspecies of *L. grayi*). Organisms in the ‘O’ category are classified as non-*Listeria* species. Further tests should be conducted to identify the species within each category according to the FDA method.

Other commercially available methods for the identification of *Listeria* species include the API LISTERIA (bioMérieux), the MICROBACT 12L (Microgen), the MicroLog System (Biolog), the Sherlock Microbial Identification System (MIS) (Microbial ID; based on fatty acid patterns) and the Walk/Away System (MicroScan).

iii) **Rapid immunological detection methods**

A number of immunological methods have been developed to identify *L. monocytogenes* in foods and the following commercially available methods have been validated by one or more recognised formal validation systems (24, 58).

- **Colorimetric monoclonal enzyme-linked immunosorbent assay (Listeria-Tek)**

  The Listeria-Tek is AOAC official method 994.03 (9) and it is intended for the detection of *Listeria* spp. in dairy products, seafood and meats. Because the monoclonal antibodies (MAbs) used in the test may cross react with other *Listeria* spp., the test is not confirmatory for *L. monocytogenes*.

  The kit is commercially available from Organon Teknika Corp., 100 Akzo Ave, Durham, NC 27704, USA.

  Enrichment cultures found positive by this method are streaked on selective media and suspect colonies are biochemically identified as *L. monocytogenes* according to the FDA method. A positive result is only valid when the positive and negative controls give acceptable absorbance readings.

- **Colorimetric polyclonal enzyme immunoassay screening method (TECRA® Listeria Visual Immunooassay [TLVIA])**

  The TLVIA is AOAC official method 995.22 (10) and it is intended for the detection of *Listeria* spp. in dairy foods, seafood, poultry, meats (except raw ground meat), and leafy vegetables. An optimised version, with enrichment protocols for additional foods and with omission of the toxic antifungal agent cycloheximide, is
AOAC official method 2002.09 (14), which is a screening procedure for the detection of *Listeria* spp. in raw meats, fresh produce/vegetables, processed meats, seafood, dairy foods cultured/noncultured, fruit and fruit juices.

The commercial kit is available from TECRA International Pty Ltd, P.O. Box 788, Willoughby, NSW, Australia.

Enrichment cultures that are positive must be inoculated on to selective media and suspect colonies identified according to the criteria specified under the FDA and USDA methods.

- **Assurance® polyclonal enzyme immunoassay method**

  The Assurance® *Listeria* Enzyme Immunoassay is AOAC official method 996.14 (11) and it can be used for the detection of *Listeria* spp., including *L. monocytogenes*, in dairy foods, red meats, pork, poultry products, fruits, nutmeats, seafood, pasta, vegetables, cheese, bone meal, chocolate, environmental surfaces, and eggs.

  Readings above the cut-off value are considered presumptive positive and the enriched cultures are then confirmed by culture and identification procedures as described under the FDA method.

  The commercial kit is available from BioControl Systems, Inc., 12822 SE 32nd St., Bellevue, WA 98005, USA.

- **Visual Immunoprecipitate assay (VIP™)**

  The VIP™ assay is AOAC official method 997.03 (12). It can be used for the detection of *L. monocytogenes* and other *Listeria* spp. in dairy foods, red meats, pork, poultry and poultry products, seafood, fruits, vegetables, nutmeats, pasta, chocolate, eggs, environmental surfaces and bone meal.

  The test is performed with an enriched culture of the test samples. Presumptive positive tests must be confirmed by culture and identification procedures as described under the FDA method.

  The VIP units are commercially available from BioControl Systems, Inc., 12822 SE 32nd St., Bellevue, WA 98005, USA.

- **VIDAS LIS assay screening method**

  This enzyme-linked immunofluorescent assay (ELFA) is AOAC official method 999.06 (13). It has also been validated by the Association Française de Normalisation (AFNOR) and by the European Microbiological Methods Assessment Scheme (EMMAS) (15). It is used for screening dairy products, vegetables, seafood, raw meats and poultry, as well as processed meats and poultry, for *Listeria* spp. antigens.

  This immunoassay is performed in the automated VIDAS® instrument. The computer compares this value with a standard and a positive or negative report is generated. Positive results must be confirmed by standard culture methods as described under the FDA method. An enrichment modification to this protocol, using demi Fraser and Fraser broths (AOAC official method 2004.06), was evaluated in a multilaboratory study and no significant difference was found in its performance when compared to that of AOAC official method 999.06 (59).

  The VIDAS *Listeria monocytogenes* 2 (LMO 2) method includes specific MAbs to capture *L. monocytogenes* antigens and it has been validated by AFNOR, including its use with environmental samples.

  The VIDAS system is available from bioMérieux, Inc., 595 Anglum Rd., Hazelwood, MO 63042, USA.

Other commercially available immunological methods that have been validated by formal systems include the VIDAS *Listeria Species Xpress* (bioMérieux), validated by AFNOR; the Transia Plate *Listeria ELISA* (Transia, Difffchamb Ltd), validated by AFNOR; the EIAFOSS *Listeria* automated ELISA (Foss Electric), validated by the AOAC Research Institute; the immunochromatographic method REVEAL for *Listeria* (Neogen Corporation), validated by the AOAC Research Institute; the immunochromatographic method Clearview *Listeria* Rapid Test (Oxoid), validated by AFNOR, EMMAS and the AOAC Research Institute and the immunomagnetic separation-based Listertest (Vicam), validated by the AOAC Research Institute (15).

Other commercially available immunologically based methods include the Transia Plate *Listeria monocytogenes ELISA* (Transia, Difffchamb Ltd), the immunomagnetic separation-based Dynabeads anti-*Listeria* (Dynal Ltd), the *Listeria UniQue™ ELISA* (TECRA), the Microscreen *Listeria latex agglutination test* (Microgen BioProducts Ltd), and the *Listeria Rapid Test EIA* (Oxoid).
Chapter 2.9.7. – Listeria monocytogenes

d) **Nucleic acid recognition methods**

A number of methods based on nucleic acid recognition have been developed to identify *L. monocytogenes* in foods. A few of them have been validated by one or more recognised formal validation systems and are commercially available (49). Novel target sequences for diagnostic purposes include the metalloprotease gene (LightCycler foodproof *Listeria monocytogenes* Detection Kit, Roche Applied Science), the *prfA* gene (54) and the *ssrA* gene (50) in a real-time PCR format.

**i) GENE-TRAK *Listeria* assay**

The GENE-TRAK *Listeria* assay is a colorimetric DNA hybridisation method for the detection of *Listeria* sequences, which has been validated by the AOAC as method 993.09 (7) for use with dairy products, meats and seafood. This assay has also been validated by AFNOR. Due to the possibility of encountering false-positive reactions, positive samples must be confirmed by standard cultural methods.

Test portions found positive by this DNA hybridisation assay must be confirmed by streaking a phosphate buffered saline growth suspension on a *Listeria* selective plate and continuing with biochemical identification of presumptive *Listeria* isolates as described under the FDA method.

The GENE-TRAK *Listeria* assay is commercially available from GENE-TRAK™ Systems, 94 South Street, Hopkinton, MA 01748, USA.

**ii) BAX® System**

The USDA-FSIS has adopted the PCR-based BAX® System (Qualicon) (64) as their screening method for *L. monocytogenes* in enriched meat and poultry samples. It reduces the report out time for true negative samples by 24 hours and reduces false-positive results, with a detection limit better that 1 cfu/g in a 25 g sample. All samples that are identified as presumptive positive for *L. monocytogenes* are then subject to cultural confirmation by the conventional method.

**iii) GENE-TRAK test for *Listeria monocytogenes***

The GENE-TRAK test for *L. monocytogenes* (GENE-TRAK™ Systems) is a hybridisation probe-based method validated by AFNOR (15).

**iv) Gen-Probe (AccuProbe®) *Listeria monocytogenes* confirmatory test**

The Gen-Probe (AccuProbe®) *Listeria monocytogenes* Confirmatory Test (Gen-Probe) is another hybridisation probe-based method validated by AFNOR (15).

**v) AD713 method**

The FDA uses a *L. monocytogenes*: a combination of invasion-associated protein and haemolysin (hly) gene probes – AD713 method (33). This method combines the detection of the *L. monocytogenes* haemolysin (also called listeriolsyn O) gene by use of the oligonucleotide probe AD13, and the detection of the invasion-associated protein gene by a synthetic probe, AD07. Both probes are used in combination (designated AD713) to avoid false-negative results because of ‘silent’ mutations in the gene (nucleotide changes that affect DNA probe binding but do not change the gene function). Positive samples must be confirmed with conventional procedures as described under the FDA method.

Other commercially available methods based on nucleic acid recognition, include the Foodproof® *Listeria monocytogenes* PCR assay (Biotecn Diagnostics) and the PROBELIA™ (L. monocytogenes) PCR assay (Sanofi Diagnostics). The application of the real-time PCR as a quantitative detection method, specific for *L. monocytogenes*, has also been developed (18, 36) and it shows good potential for routine analytical use. The IQ-Check *Listeria monocytogenes* kit (for all food products and environmental samples), developed by Bio-Rad, and the GeneDisc (for all food, except milk products) developed by GeneSystems, France, are based on real-time PCR detection and have been validated by AFNOR (2).
Table 2. Some conventional and antibody-based commercial systems for rapid Listeria screening and confirmation

<table>
<thead>
<tr>
<th>Test</th>
<th>ID level</th>
<th>Principle</th>
<th>Approx. test time$^2$</th>
<th>Company</th>
<th>Main use</th>
</tr>
</thead>
<tbody>
<tr>
<td>MICRO-ID Listeria</td>
<td>L. monocytogenes/innocua complex</td>
<td>Enzyme reaction</td>
<td>24 hours</td>
<td>Organon Teknika</td>
<td>Confirmation</td>
</tr>
<tr>
<td>Vitek System</td>
<td>L. monocytogenes/innocua complex</td>
<td>Biochemical tests</td>
<td>24 hours</td>
<td>bioMérieux</td>
<td>Confirmation</td>
</tr>
<tr>
<td>API Listeria</td>
<td>L. monocytogenes</td>
<td>Biochemical tests</td>
<td>24 hours</td>
<td>bioMérieux</td>
<td>Confirmation</td>
</tr>
<tr>
<td>MicroLog System</td>
<td>L. monocytogenes</td>
<td>Carbon source substrates</td>
<td>4 or 24 hours</td>
<td>Biolog</td>
<td>Confirmation</td>
</tr>
<tr>
<td>MICROBACT 12L</td>
<td>L. monocytogenes</td>
<td>Carbohydrate use and micro haemolysis test</td>
<td>4–6 or 24 hours</td>
<td>Microgen</td>
<td>Confirmation</td>
</tr>
<tr>
<td>Sherlock Microbial Identification System (MIS)</td>
<td>L. monocytogenes/innocua complex</td>
<td>Fatty acid patterns</td>
<td>90 minutes</td>
<td>Microbial ID</td>
<td>Confirmation</td>
</tr>
<tr>
<td>Microscreen</td>
<td>Listeria spp.</td>
<td>Latex agglutination</td>
<td>1 minute</td>
<td>Microgen BioProducts</td>
<td>Confirmation</td>
</tr>
<tr>
<td>VIP Listeria</td>
<td>Listeria spp.</td>
<td>Immuno-chromatography</td>
<td>2 minutes (post-enrichment)</td>
<td>BioControl Systems</td>
<td>Screening</td>
</tr>
<tr>
<td>Dynabeads anti-Listeria</td>
<td>Listeria spp.</td>
<td>Immunomagnetic separation</td>
<td>48–72 hours</td>
<td>Dynal</td>
<td>Screening</td>
</tr>
<tr>
<td>REVEAL for Listeria</td>
<td>Listeria spp.</td>
<td>Immuno-chromatography</td>
<td>43 hours</td>
<td>Neogen</td>
<td>Screening</td>
</tr>
<tr>
<td>Cleanview Listeria (Oxoid Listeria Rapid Test)</td>
<td>Listeria spp.</td>
<td>Immuno-chromatography</td>
<td>43 hours</td>
<td>Oxoid</td>
<td>Screening</td>
</tr>
<tr>
<td>Listertest</td>
<td>Listeria spp.</td>
<td>Immunomagnetic separation</td>
<td>24–48 hours</td>
<td>Vicam</td>
<td>Screening</td>
</tr>
</tbody>
</table>

$^1$: Adapted and expanded from ref. 15
$^2$: When used for confirmation, the test time indicated is after enrichment and agar isolation

Table 3. Some ELISA commercial systems for rapid Listeria screening

<table>
<thead>
<tr>
<th>Test</th>
<th>ID level</th>
<th>Principle</th>
<th>Approx. test time$^2$</th>
<th>Company</th>
<th>Main use</th>
</tr>
</thead>
<tbody>
<tr>
<td>Listeria Tek</td>
<td>Listeria spp.</td>
<td>ELISA</td>
<td>50 hours</td>
<td>Organon Teknika</td>
<td>Screening</td>
</tr>
<tr>
<td>TECRA Listeria Visual Immunoassay (TLVIA)</td>
<td>Listeria spp.</td>
<td>ELISA</td>
<td>50 hours</td>
<td>TECRA</td>
<td>Screening</td>
</tr>
<tr>
<td>Assurance Listeria EIA</td>
<td>Listeria spp.</td>
<td>ELISA</td>
<td>50 hours</td>
<td>BioControl Systems</td>
<td>Screening</td>
</tr>
<tr>
<td>VIDAS Listeria (LIS)</td>
<td>Listeria spp.</td>
<td>ELISA</td>
<td>50 hours</td>
<td>bioMérieux</td>
<td>Screening</td>
</tr>
<tr>
<td>VIDAS Listeria monocytogenes (LMO)</td>
<td>L. monocytogenes</td>
<td>ELISA</td>
<td>50 hours</td>
<td>bioMérieux</td>
<td>Screening</td>
</tr>
<tr>
<td>Transia Plate Listeria</td>
<td>Listeria spp.</td>
<td>ELISA</td>
<td>50 hours</td>
<td>Diffchamb</td>
<td>Screening</td>
</tr>
<tr>
<td>Transia Plate Listeria</td>
<td>L. monocytogenes</td>
<td>ELISA</td>
<td>50 hours</td>
<td>Diffchamb</td>
<td>Screening</td>
</tr>
<tr>
<td>EIAFOSS Listeria</td>
<td>Listeria spp.</td>
<td>Automated ELISA</td>
<td>48 hours</td>
<td>Foss Electric</td>
<td>Screening</td>
</tr>
</tbody>
</table>

$^1$: Adapted and expanded from ref. 15
Table 4. Some molecular commercial systems for rapid Listeria screening and confirmation

<table>
<thead>
<tr>
<th>Test</th>
<th>ID level</th>
<th>Principle</th>
<th>Approx. test time</th>
<th>Company</th>
<th>Main use</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gen-Probe (AccuProbe)</td>
<td><em>L. monocytogenes</em></td>
<td>Nucleic Acid Hybridization Probe</td>
<td>30 minutes</td>
<td>Gen Probe</td>
<td>Confirmation</td>
</tr>
<tr>
<td>VIT Listeria Kit</td>
<td><em>L. monocytogenes</em>/<em>Listeria spp.</em></td>
<td>Nucleic acid Hybridisation probe</td>
<td>3 hours</td>
<td>Vermicon</td>
<td>Confirmation</td>
</tr>
<tr>
<td>Foodproof Listeria monocytogenes</td>
<td><em>L. monocytogenes</em></td>
<td>PCR</td>
<td>48 hours</td>
<td>Biatecon Diagnostics</td>
<td>Screening</td>
</tr>
<tr>
<td>Gene Trak Listeria Assay</td>
<td><em>Listeria spp.</em></td>
<td>Nucleic acid Hybridisation probe</td>
<td>50 hours</td>
<td>Gene Trak</td>
<td>Screening</td>
</tr>
<tr>
<td>Gene Trak test for <em>L. monocytogenes</em></td>
<td><em>L. monocytogenes</em></td>
<td>Nucleic acid Hybridisation probe</td>
<td>50 hours</td>
<td>Gene Trak</td>
<td>Screening</td>
</tr>
<tr>
<td>BAX for screening <em>L. monocytogenes</em></td>
<td><em>L. monocytogenes</em></td>
<td>PCR</td>
<td>48 hours</td>
<td>Qualicon</td>
<td>Screening</td>
</tr>
<tr>
<td>BAX for screening <em>Listeria Genus</em></td>
<td><em>Listeria spp.</em></td>
<td>PCR</td>
<td>48 hours</td>
<td>Qualicon</td>
<td>Screening</td>
</tr>
<tr>
<td>PROBELIA</td>
<td><em>L. monocytogenes</em></td>
<td>PCR</td>
<td>48 hours</td>
<td>Sanofi Diagnostics Pasteur</td>
<td>Screening</td>
</tr>
<tr>
<td>LightCycler foodproof Listeria monocytogenes Detection Kit</td>
<td><em>L. monocytogenes</em></td>
<td>Real Time PCR</td>
<td>65 minutes$^3$</td>
<td>Roche Applied Science</td>
<td>Screening</td>
</tr>
<tr>
<td>LightCycler foodproof Listeria Detection Kit</td>
<td><em>Listeria spp.</em></td>
<td>Real Time PCR</td>
<td>75 minutes$^3$</td>
<td>Roche Applied Science</td>
<td>Screening</td>
</tr>
</tbody>
</table>

1: Adapted and expanded from ref. 15
2: When used for confirmation, the test time indicated is after enrichment and agar isolation
3: After enrichment culture

e) Subtyping methods

Regulatory identification of *L. monocytogenes* does not require any specific subtyping of the isolates. However, subtyping schemes can be useful in outbreak investigations, environmental tracking and public health surveillance.

*Listeria monocytogenes* can be subtyped by a number of different approaches including serotyping, phage typing, multilocus enzyme electrophoresis (MEE), DNA restriction enzyme analysis (either using high-frequency cutting enzymes and conventional gel electrophoresis to separate fragments, or using rare-cutting enzymes and pulse-field gel electrophoresis [PFGE] to separate fragments), nucleic acid sequencing-based typing, microarray analysis, amplified intergenic locus polymorphism (AILP), and random amplification of polymorphic DNA (RAPD).

Because of the requirement for specific reagents, stringent quality assurance procedures and some sophisticated equipment, it is recommended that subtyping of *L. monocytogenes* isolates be referred to the appropriate reference centre. A list of international centres and subtyping methods can be found in ref. 16 (pp. 258–259)

i) Serotyping

Strains of *Listeria* can be assigned to 13 different serotypes, based on their combination of somatic (O) and flagellar (H) antigens. Although all of them are considered to be potentially pathogenic, most (>95%) human clinical isolates belong to three serotypes 1/2a, 1/2b, and 4b. Compared with other subtyping methods, serotyping has poor discriminatory power, but can provide valuable information to facilitate the ruling out of isolates that are not part of an outbreak. Isolates from foods and from environmental sources are frequently nontypable with standard typing antisera.

ii) Phage typing

Bacteriophage typing is a technique with very good discriminatory power that can be used to subtype a large number of isolates. However, the current available phage sets are unable to type a high proportion of
REP elements are present in as a very promising subtyping methodology for this microorganism. This approach has been reported for a handful of other microorganisms and it is known as multi locus sequence typing (MLST) (62). Direct amplification and nucleotide sequencing (19, 56), as well as an alternative approach that targets the large fragments of selected metabolic enzymes in starch gels, and can be applied to subtype-related bacterial strains. However, MEE is only moderately discriminatory when used in epidemiological investigations involving L. monocytogenes. Some strains may lack certain enzyme activities and therefore the technique can get complicated. Because of its very nature, the interlaboratory results are highly variable (31).

iii) Multilocus enzyme electrophoresis

This technique takes advantage of nucleotide sequence differences, which result in distinct electrophoretic mobilities of selected metabolic enzymes. This approach has been found to be a highly discriminating and reproducible method. PFGE is particularly useful for subtyping L. monocytogenes, mainly through the use of the restriction endonuclease EcoRI. However, the technique was found to be less discriminating than phage typing, REA or MEE. Qualicon has designed an automated ribotyping system, the RiboPrinter, which generates, analyses and stores riboprint patterns of bacteria, including Listeria.

When restriction endonuclease enzymes that cut infrequently are used to digest unsheared chromosomal DNA, such as Apal, Smal, NotI and Ascl, very large fragments are obtained. Because of their size, these large fragments do not separate when run under conventional agarose gel electrophoresis. However, by periodically changing the orientation of the electric field across the gel, through pulses, the large fragments can ‘crawl’ through the agarose matrix and are separated according to size differences. This technique is known as pulsed-field gel electrophoresis (PFGE) and has revolutionised the precise separation of DNA fragments larger than 40 kilobases. PFGE has been applied to the subtyping of L. monocytogenes and has been found to be a highly discriminating and reproducible method. PFGE is particularly useful for subtyping serotype 4b isolates, which are not satisfactorily subtyped by most other subtyping methods. The main disadvantages of PFGE are the time required to complete the procedure (2–3 days), the large quantities of expensive restriction enzymes required, and the need for specialised, expensive equipment (31). The Centers for Disease Control and Prevention (CDC) in the United States of America has established PulseNet, a network of public health and food regulatory laboratories that routinely subtype food-borne pathogenic bacteria by PFGE. PulseNet laboratories use highly standardised protocols and can quickly compare PFGE patterns from different locations via the Internet. Listeria monocytogenes was added to PulseNet in 1999 (63).

v) Nucleic acid sequence-based typing

Although there have been some reports on the sequence analysis of single genes as a means to type L. monocytogenes strains, determination of allelic variation of multiple genes, has been recently introduced as a very promising subtyping methodology for this microorganism. This approach has been reported for a handful of other microorganisms and it is known as multi locus sequence typing (MLST) (62). Direct amplification and nucleotide sequencing (19, 56), as well as an alternative approach that targets the variable genetic changes directly in a DNA array format (55) have both been used with good discrimination between the strains analysed. Because MLST is based on nucleotide sequence, it is highly discriminatory and provides unambiguous results.

Short repetitive sequence elements are widely distributed among bacteria and palindromic units, known as repetitive extragenic palindromes (REP), are the best-characterised family of repetitive bacterial sequences. REP elements are present in L. monocytogenes and a PCR based on the sequence of these elements (rep-
PCR) has been used successfully to subtype strains of the organism. The four major strain clusters identified by this method matched the origin of their isolation (43).

vi) Random amplification of polymorphic DNA

When arbitrarily selected primers are used in the PCR under low stringency conditions, with chromosomal L. monocytogenes DNA as a template, amplicon patterns are generated that are useful for subtyping strains. RAPD is a viable alternative to phage typing and is highly discriminating. However, despite its relative simplicity and discriminating ability, its main drawback is the inconsistent reproducibility of patterns. The low stringency conditions for primer annealing results in polymerisation with various efficiencies, and therefore the quantities of DNA produced may be widely variable among the different amplicons from a given isolate, which makes it difficult to compare and interpret the RAPD patterns. The technique requires a great deal of standardisation and consistency to obtain reliable results (31).

Based on the results of the WHO multicentre L. monocytogenes subtyping study (27), which compared several different subtyping methods using a well defined set of isolates, serotyping, phage typing, REA, PFGE and RAPD were selected for standardisation in Phase II. This effort should eventually result in a selected set of standardised L. monocytogenes subtyping methods (31).

2. Serological tests

Serological tests for the detection of antibodies have not been traditionally used for the diagnosis of listeriosis. They have been largely unreliable, lacking sensitivity and specificity. A number of formats, including ELISA, complement fixation and microagglutination have been largely unsuccessful in the diagnosis of culture-proven human listeriosis, even in the absence of immunosuppression. Considerable cross-reactivity with antigenic determinants of other Gram-positive organisms has been observed. On the other hand, L. monocytogenes is a ubiquitous organism, and regular exposure of animals and humans to this microorganism is very common. Many healthy individuals are intestinal carriers (2–6%) and anti-L. monocytogenes serum antibody prevalence as high as 53% have been reported in humans. Carriage rate for animals is similar to that of humans, with some differences depending on the species and a little higher rate during indoor season, as compared to animals on pasture (37, 38).

The discovery that the L. monocytogenes haemolysin, listeriolysin O (LLO), is a major virulence factor and that it can stimulate an antibody response, has recently renewed interest in the possibility of using serological tests for the diagnosis of listeriosis, particularly in central nervous system patients, with sterile blood and cerebrospinal fluid, and in perinatal listeriosis. An indirect ELISA based on the detection of anti-LLO was used for the diagnosis of experimental listeriosis in sheep (47). However, LLO is antigenically related to a number of cytolsins, including streptolysin O (SLO) from Streptococcus pyogenes, pneumolysin from S. pneumoniae and perfringolysin from Clostridium perfringens. Problems of cross-reactivity of anti-LLO antibodies with these cytolsins, particularly SLO and pneumolysin, have hampered the development of specific reliable serological tests based on the detection of anti-LLO antibodies. In addition, anti-LLO antibodies have been found in a proportion of healthy individuals and patients with other bacterial, fungal or viral infections (27%, all combined), although at lower titres than in patients with listeriosis. Absorption of diagnostic antisera with SLO is only partially effective in eliminating all cross-reactivity. These experimental assays have been used in some epidemiological investigations and as support for the diagnosis of culture-negative central nervous system infections. Recombinant forms of LLO have been explored as alternatives to wild LLO as a diagnostic antigen in Western blot assays. This is currently an evolving field and we will have to wait for the development of reliable, validated serological tests for the diagnosis of listeriosis.

Immunohistochemical detection of L. monocytogenes antigens in formalin-fixed tissue has proven to be more sensitive than direct plating and cold enrichment bacterial culture for the diagnosis of the encephalitic form of the disease in ruminants (20, 44).

C. REQUIREMENTS FOR VACCINES AND DIAGNOSTIC BIOLOGICALS

It has proven very difficult to develop effective vaccines against L. monocytogenes which, as an intracellular organism, requires effector T cells for an effective immune response. Experimental vaccines in laboratory animals are being explored to confer protection to L. monocytogenes infection by a number of different approaches, but these are still far from becoming available for human and farm animal use. These experimental approaches include immunisation with plasmid DNA, CD40 signalling along with heat-killed L. monocytogenes, LLO-deficient mutants inoculated along with liposome-encapsulated LLO, and immunisation with listerial antigens and IL-12.
Genetically modified *L. monocytogenes* is also being considered as an effective vaccine vector for the expression, secretion and intracellular delivery of foreign antigens for the induction of potent immune responses against viral antigens and tumour cells.

However, the most feasible and practical means to reduce the risk of listeriosis in humans is through dietary and food preparation measures that not only decrease the risk of acquiring listeriosis, but also contribute to the prevention of other common food-borne infections such as those caused by *Escherichia coli* O157:H7, *Salmonella* and *Campylobacter*. These preventive measures include thorough cooking of raw food of animal origin, keeping uncooked meats separate from vegetables, cooked foods, and ready-to-eat foods, thoroughly washing raw vegetables before eating, washing hands, knives, and cutting boards after handling uncooked foods, and avoiding unpasteurised milk or products made from it. Immunocompromised persons, pregnant women and other groups at increased risk of listeriosis should avoid foods that have been epidemiologically linked to this disease, e.g. soft cheeses and pâté. These individuals should also avoid other ready-to-eat foods, unless they are heated until steaming hot before being consumed.

The food industry and public health agencies play a pivotal role in the prevention of food-borne listeriosis by developing and implementing effective HACCP programmes to reduce the presence of *L. monocytogenes* at all critical points in the food production and distribution chain (from the farm to the market).

Likewise, the lack of well designed and tested vaccines for animal use, means that control of listeriosis in animals is most feasible by preventing the environmental conditions that favour its presentation. There is a well-established linkage between silage feeding and listeriosis and, as *L. monocytogenes* is widely distributed in nature, with animals and birds acting as carriers, contamination of silage is not uncommon. Emphasis should therefore be placed on reducing the likelihood of the multiplication of the organism, which occurs more frequently at pH values greater than 5, particularly where ineffective fermentation has occurred and where there is concomitant growth of moulds. Every effort should be made to produce silage of good quality, with early cutting of grass, minimal contamination with soil or faeces and ensuring optimal anaerobic fermentation, which will insure that the pH falls below 5.0; at that level, growth of *Listeria* spp. is inhibited. The best silage for feeding should be selected, especially in the case of sheep, discarding material that has obvious signs of contamination with mould. Material a few centimetres from the top, front and sides of an opened bale or bag, should also be discarded. Leftover silage should be removed (48).

REFERENCES


\(^1\) AFAQ: Association française pour le management et l’amélioration de la qualité
\(^2\) AFNOR: Association Française de Normalisation
\(^3\) AOAC: Association of Official Analytical Chemists
Chapter 2.9.7. — Listeria monocytogenes

Chemicals; Contaminants; Drugs, Horwitz W., ed. AOAC INTERNATIONAL, Gaithersburg, MD, USA, 147–150.


* *
MANGE*

CHAPTER 2.9.8.

SUMMARY

Mange is a contagious skin disease, characterised by crusty, pruritic dermatitis and hair/feather loss, and caused by a variety of parasitic mites burrowing in or living on the skin. Some alternative historical names for mange are ‘la gale’ (in French), ‘itch’, ‘scab’, and ‘scabies’ (a term that should be reserved only for mange caused by Sarcoptes scabiei). Specifically, on domestic hosts (i.e. livestock, poultry, companion and laboratory animals), about 50 mite species in 16 families and 26 genera may cause mange. A number of other skin conditions (e.g. dermatitis, wheals, blisters, nodules) may be confused with mange and must be considered in differential diagnoses, including those resulting from allergic reactions to other kinds of mites, various arthropod bites, fungal diseases, or reactions to physical or chemical aspects of plants. Mange diagnosis in domestic animals is based on clinical manifestations and the demonstration of mites or their developmental stages in host skin scrapings.

Identification of the agent: Mange mites are mostly weakly sclerotised, slow-moving, very small (100–900 µm), and live permanently on their hosts. Although the Acari is an extremely diverse and ubiquitous group of arachnid arthropods, all of the major mange mite species fall within only two acariform lineages, the Astigmata and the Prostigmata. Some economically important mange mite genera are Cheyletiella, Chorioptes, Demodex, Knemidokoptes, Notoedres, Otodectes, Psorobia, Psoroptes, and Sarcoptes. Specialised illustrated diagnostic keys, taxonomic descriptions, and reference specimens should be consulted to properly identify the causative agents of mange. Special collecting techniques and compound microscopy usually are necessary for diagnosis. Certain identifying characteristics of each of the mange mite groups are highlighted in the following discussion. Although availability is limited, serodiagnostic tests have been developed for certain mange mites and are useful in some circumstances.

Requirements for vaccines and diagnostic biologicals: Currently, no commercial vaccines against mange are available.

A. INTRODUCTION

Mange is a contagious skin disease, characterised by crusty, pruritic dermatitis and hair/feather loss, and caused by a variety of parasitic mites burrowing in or living on the skin. The French term for mange is ‘la gale’ (28), and in English, it has been called ‘itch’, ‘scab’, or ‘scabies’ (a term that should be reserved specifically for mange caused by Sarcoptes scabiei). Numerous species of mites cause mange in literally hundreds of species of wild and domestic birds and mammals. In fact, approximately 60 mite families have members that live in or on the skin, hair, or feathers of homoeothermic vertebrates and are potential mange mites. Specifically, on domestic hosts (i.e. livestock, poultry, companion and laboratory animals), about 50 mite species in 16 families and 26 genera may cause mange. Humans are host to the readily transmitted S. scabiei, and human scabies occurs most frequently in elderly nursing homes and children’s day-care centres. Some other mange mites may cause transient disease in humans, but infestations seldom persist.

Mites (Acari) are an extremely diverse, abundant, and ubiquitous group of arachnid arthropods with about 50,000 described species. Higher-level acarine classification is still an unsettled construct, but the following is a consensus system encompassing the mange mites. Acari comprises three major evolutionary lineages, Opilioacariformes, Parasitiformes, and Acariformes, but only certain acariform mites cause mange in domestic animals. Two lineages within the Acaariformes contain mange mites – Trombidiiformes and Sarcoptiformes. Trombidiiformes comprises the order Prostigmata and contains many families, five with mange mites. The
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Sarcoptiformes contains two mite orders, the Oribatida and Astigmata, with many families each, but only 11 astigmatan families contain mange mites.

Some other mites may cause less serious dermatitis in animals or humans (39). Certain Parasitiformes (order Gamasida: e.g. Ornithonyssus, Dermanyssus) and Prostigmata (e.g. Trombicula [and other chiggers], Pymotes) transiently bite a host while feeding, leaving itchy welts and wheals behind. Stored-products, animal-nest, and house-dust mites (e.g. Acarus, Glycyphagus, Dermatophagoides) may cause contact dermatitis (e.g. baker’s itch, grocer’s itch) but no persistent infestation. Certain free-living bird-nest mites (Hypoderatidae) have a parasitic nymphal stage (hypopus) that characteristically lives subcutaneously in the bird host (e.g. Hypodectes propus in domestic pigeons), causing skin irregularities. Pediculosis or certain fungal diseases, such as ringworm, can cause crusty dermatitis, and even the physical (e.g. awns, urticarial hairs) or chemical (e.g. urushiol) aspects of some plants may cause host skin reactions that could be confused with mange.

B. DIAGNOSTIC TECHNIQUES

Mange diagnosis in domestic animals is based on clinical manifestations and the demonstration of mites or their developmental stages in host skin scrapings (16). It is typified by hair loss, crusty or scaly skin lesions, dermatitis, thickened skin, scurf, and pruritus.

1. Detecting the agent

Hair loss and crusty or scaly skin are the most apparent clinical signs of mange. A number of other diseases must be considered when one is confronted with a possible case of mange, including fungi, insect bites, irritating plants, mechanical abrasion, etc. In most cases, scrapings should be taken from the edge of the lesion, from obviously pruritic locations, and from where there are thick, crusty flakes. Take a skin scraping by holding a scalpel blade or other sharp instrument at a right angle to the skin and scraping off the outer surface of the skin. For those mite species that burrow into the skin, the scraping must be deep enough to cause a small amount of blood to ooze from the scraping site. A drop of oil, such as glycerine, may be placed on the blade to help hold the skin scrapings during the procedure. Skin scrapings should be placed in sealed containers (e.g. clean, empty salve tins; stoppered glass/plastic test tubes; small, sealable plastic bags) and promptly taken or sent to a laboratory for more thorough examination. An even more effective method of collecting mites from the skin surface and hair is by using a vacuum cleaner fitted with an in-line filter (17). The material collected, along with the filter, is then examined as a skin scraping would be. An otoscope can be valuable in revealing the presence of ear mites. A cotton-tipped applicator can be used to swab the ear canal if ear mites are observed or suspected; examine it in the same way as a skin scraping.

Perform an initial examination of the skin scraping under a dissecting microscope. Obviously visible mites, especially those that are alive and moving, may be picked up with a dissecting needle dipped in glycerine or a mounting medium and transferred to a drop of mounting medium on a glass slide. When the desired number of mites has been collected, gently place a cover-slip on the drop of mounting medium, taking care to avoid air bubbles. Hoyer’s medium, Berlesse’s fluid, Vitzhum’s fluid, and Heinze’s modified PVA medium are all acceptable mounting media. If permanent mounts are desired, allow the slides to dry for at least 1 week at room temperature, then ring the cover-slips with nail polish or other sealant to keep them from drying out.

Mites that are embedded in oil and exudate, Demodex for example, may be demonstrated by placing a small amount of skin scraping directly on the slide with some glycerine or immersion oil and pressing a cover-slip on top of it. The slide then can be examined directly with a compound microscope.

Skin scrapings that contain dead mites, large amounts of skin flakes or scabs, or large amounts of hair should be processed further. Place the skin scraping (up to several grams of skin and hair) in a suitably sized beaker, then add sufficient 10% potassium hydroxide to immerse the sample. Cautiously bring the solution to a gentle boil, stirring frequently (a laboratory hot-plate with a magnetic stirrer works well for this), for 5–10 minutes or long enough to digest most of the hair and skin. This step should be performed under a chemical fume hood to limit exposure to caustic fumes. Do not boil for an extended period of time, or the mites may disintegrate. Transfer the digested material to suitable test tubes, and centrifuge at 600 g for 10 minutes. Decant the supernatant. Resuspend the pellet in a small amount of flotation medium (e.g. Sheather’s solution or a mixture of 50% corn syrup and 50% water); then, fill the tube completely with flotation medium, and place a cover-slip on top of the tube, assuring that it makes contact with the flotation medium. Let stand for 1 hour, or centrifuge for 10 minutes. Carefully remove the cover-slip by lifting straight up, so that a drop of fluid remains on the underside of the cover-slip, and place on a glass slide. Any mites in the sample will have floated to the top and will be found in the drop of fluid attached to the cover-slip. Another simpler but satisfactory technique, that is used in many laboratories, is to re-suspend the pellet in a small amount of distilled water, drop onto a large (76 × 51 × 1 mm) glass slide and cover with a 40 × 50 mm cover-slip. This is examined under a dissecting microscope (×40 or ×100) with understage lighting. The slide then may be examined under a compound microscope for the presence of mites.
DNA of *Sarcoptes scabiei* has been successfully amplified and detected by polymerase chain reaction (PCR) from human cutaneous scales (5). This technique holds promise as an additional procedure for detecting specific, hard-to-find mange mites in skin scrapings.

In cases where mites are difficult to find in skin scrapings from small domestic pet animals, they sometimes may be demonstrated by faecal flotation.

2. Identifying the agent

Mange mites are mostly weakly sclerotised, slow-moving, very small (100–900 µm), and live permanently on their hosts. The general life cycle of mange mites is brief (1–5 weeks) and includes four stages: egg, six-legged larva, eight-legged nymph (one or more instars), and eight-legged adult (male and female.) Specialised illustrated diagnostic keys (e.g. 3, 13, 15, 16, 18, 40), taxonomic descriptions, and reference specimens should be consulted to properly identify the causative agents of mange. However, certain diagnostic characteristics of each of the mange mite groups are highlighted in the following discussion.

Mange in domestic animals results from the host’s physiological, immunological and behavioural responses to infestation by mites in any of eleven families of Astigmata or five families of Prostigmata.

a) Astigmata

Astigmatan mange mites are generally small, globose or oval in outline, and thin-skinned. The somatic cuticle often shows a pattern of fine, parallel striations (finger print patterns), with distinctively shaped and placed setae, spines, pegs, or scales, and sometimes, lightly sclerotised plates or shields. Adults usually have eight legs and anterior mouthparts that include paired palps and chelicerae used for cutting and feeding. The legs attach proximally to the body through distinct cuticular epimerae (coxal apodemes) and terminate distally in a variety of setal forms or in a pretarsal empodium that may be shaped like either a claw or a bell-like sucker (caruncle or ambulacrum.) Astigmatan mites do not have true, paired pretarsal claws. Males sometimes bear somatic suckers or other sexual characteristics used in mating, but the form and placement of setae and empodia on the legs is usually sufficient to separate the sexes as well as identify the various mange mite species. Fertilised eggs are simple, soft, and translucent ovoids that are produced by mated females through a usually midventral ovipore.

i) Sarcoptidae

Sarcoptid mites are all obligate, burrowing skin parasites of mammals, with over 100 described species (18). Survival time under moderate conditions for mites off the host is limited to about 10 days or less (1). Because of their activities in the epidermal layers of the skin, mange caused by these mites is generally more severe than that caused by mites dwelling above the surface of the skin. The body outline of sarcoptids is generally rounded, dorsoventrally flattened, and the cuticle is striated. The palps are one-segmented, and the legs are usually short. Three genera contain domestic animal parasites of interest.

*Sarcoptes scabiei*

This mite causes sarcoptic mange (scabies) in humans and other mammals (1). It is among the most common, widespread, and serious types of mange extant. More than 100 known species of infested hosts occur worldwide in at least 10 mammalian orders and 26 families (6). Domestic hosts include camels, cattle, dogs, sheep, goats, horses, swine, llamas, and alpacas. Fain (11) suggests that humans were the original host of *Sarcoptes*, and all other hosts were secondarily infested. Despite some dissention (19), scientific consensus generally has followed Fain (11) in viewing all *Sarcoptes* mites on all hosts as no more than host-adapted variants of a single, variable species. Transmission between individuals within a host species or genus may occur easily by close contact, but taxonomically unrelated hosts are not readily infested or infestations are self-limiting. For example, *S. scabiei var. canis* easily transfers among dogs and can move to foxes, coyotes, and other canids (31), but humans serve as no more than transient hosts for this variant (9). Recent molecular analyses support the conspecificity of all *Sarcoptes* variants (42), and an immune response has been demonstrated in Sarcoptes infected hosts (2).

Mature female *S. scabiei* are approximately 500 µm long, with fingerprint-like striations on the cuticle, short and stubby legs, various characteristic setae and pegs, and with a dorsal patch of tooth-like spines. Males are similar but smaller (about 275 µm), and the tooth-like spines are reduced in size and number. The anus is posterior in both sexes, and the first pair of epimeres is fused in a midventral Y-shape. Long-stalked, unjointed pretarsal suckers occur on legs I and II in both sexes and on legs IV in males. The remaining legs all terminate in long, hair-like setae. In addition, each tarsus bears at its tip one or two highly modified setae in the form of short spurs. Nymphs resemble females but are smaller and lack an ovipore. Larvae are similar but smaller still and have only six legs.
Trixacarus caviae

This mite is a specific parasite of captive and laboratory guinea-pigs, Cavia porcellus, but it never has been found on wild-caught animals (18). Although these mites are a little smaller, the morphology and life cycle are similar to S. scabiei. However, all dorsal setae in T. caviae are long and hair-like, unlike some of those in Sarcoptes, which are short and broad or peg-like; males of Trixacarus also lack pretarsal suckers on the fourth pair of legs, and the pedicels (stalks) of all suckers are a bit shorter than those typical of Sarcoptes mites. This mite may cause a severe mange in host animals, especially in the laboratory setting. A similar mite, T. diversus, rarely occurs on laboratory rats.

Notoedres spp.

Notoedres is a large genus comprising some 40 species, most of which are associated with bats (Chiroptera) (18). Four species are of some concern with respect to notoedric mange in domestic animals. The cat mange mite, N. cati, is a cosmopolitan parasite of domestic cats, but it also infests several wild cats (e.g. bobcat, cheetah, serval, snow leopard), palm civets, coatimundis, mongooses, and domestic rabbits. These are highly contagious mites, and they cause intense mange, especially about the host's head and sometimes spreading to the legs, genital area, or even the tail. Laboratory rats are hosts to N. muris, which burrows into the stratum corneum and causes thickening and cornification of the skin on the pinnae, eyelids, nose, and tail. Additional hosts include other Rattus spp., several other rodents, two marsupials, and a hedgehog (18). The laboratory mouse may be infested by two Notoedres, N. musculi and N. pseudomuris, but the latter primarily occurs in wild populations of this host. Each mite also infests a few other murid rodent species. The mange caused is similar to that caused by N. muris in rats. Notoedres mites are generally similar to Sarcoptes but about half the size, and they lack the mid-dorsal field of tooth-like cuticular spines and peg-like setae, which may be replaced by a slight scale-like pattern in the cuticular striations and short, stout setae. The anus is posterodorsal, the first pair of epimeres is not fused medially, and the tarsi of legs I and II each end in three or four short, spur-like setae, not just two.

Psoroptidae

Psoroptic mites are obligate parasites of mammals. They dwell and feed on the surface of the host's skin. Survival time for some of these mites off the host may be two weeks or more (27). The generally oval-shaped body is dorsoventrally flattened, has a striate cuticle with scattered setae but no spines, and bears longer legs and more prominent mouthparts than those of sarcoptic mites. The anus is posteroventral. Males usually each have a pair of terminal posterior lobes bearing diagnostic setae and a pair of ventral anal anal suckers used in mating. The first pair of epimeres is not fused medially. More than 30 genera of psoroptic mites are known from at least seven mammalian orders, with the greatest number on primates (26). Three genera have veterinary importance for domestic animals.

Psoroptes ovis

For decades following Sweatman (36), conventional practice among acarologists has been to distinguish several species of Psoroptes among the mites that cause psoroptic mange worldwide in wild and domestic ungulates and rabbits, e.g. P. cuniculi in the ears of rabbits and various ungulates, P. equi on the bodies of English equids, P. ovis on the bodies of sheep and other ungulates. Distinctions between the species were based primarily on host and anatomical site infested and on morphology of the males. Recently, several workers have invalidated these criteria and used genetic analysis to show conspecificity of the traditionally different species (4, 29, 41, 43). The earliest published description for Psoroptes mites is that for P. ovis (37), making this the proper designation for all such mange mites on all domestic hosts. Thus, the nomenclatural situation in Psoroptes becomes similar to that in Sarcoptes, with one morphologically and genotypically variable species occurring worldwide, albeit on a smaller spectrum of hosts and with a bit less stringent host specificity among the variants. Psoroptic mange in both sheep and cattle seems to vary in its severity according to the variant of P. ovis present, with the most severe form being a reportable condition caused by an especially virulent genotype and known as 'sheep scab'. This form has been eradicated from the USA, New Zealand, Canada, and Australia, although it still persists in many other parts of the world. Thus, particularly for further eradication efforts against psoroptic sheep mange, genotypic analysis of the involved mites may be an especially valuable tool (12).

Mature female Psoroptes are 550–750 µm long, with a striate cuticle and four long and 16 short dorsal somatic setae (32, 36). A noticeable anterodorsal cuticular plate is present behind the mouthparts, and the midventral ovipore is an inverted U-shape. Males are about one-fourth smaller, and they have an additional, larger posterodorsal cuticular plate, a pair of posteroventral anal suckers, and two terminal posterior lobes, each equipped with four setae of varying lengths and structures. Nymphs and larvae are somewhat similar to adults but progressively smaller, and all Psoroptes are pearly white in colour. In all stages, the anterior two pairs of legs are thicker and more robust than the posterior pairs, which are thinner, and in the male, shortened in the fourth pair. Legs I and II terminate in pretarsal empodial suckers on long, segmented pedicels in both sexes, with similar structures on legs IV of the female and legs III of the males. The
female’s third tarsus ends in two long, whip-like setae, and the male has a single short seta on tarsus IV, plus a long, thin seta accompanying the empodial sucker on tarsus III.

**Chorioptes spp.**

This genus currently comprises five putative species of obligate ectoparasitic mites that may cause chorioptic mange in domestic and wild mammals. Three of the species, collected rarely from wild animals, are poorly known and may not be valid entities, but *C. bovis* and *C. texanus*, primarily from domestic animals, have withstood modern biogenetic scrutiny and are accepted species (8, 44). A number of allegedly host-specific varieties within these species are not separable from one another (35). The two species are morphologically distinguishable only by differences in the terminal posterior lobes and setae of males (35). Chorioptic mange, also called ‘barn itch,’ may be the most common form of mange in cattle and horses. It is a relatively mild condition that usually is more localised and less intensely pruritic than psoroptic or sarcoptic manges. This is probably because *Chorioptes* mites are able to feed and survive on host-produced epidermal debris at the skin surface, without necessarily attacking the living parts of the host’s skin. Infestations tend to concentrate on the lower portions of the host, especially the feet and legs, but may include the udder/scrotum, tailhead, and perineum. In some cases, *C. texanus* infests the host’s ears (35). *Chorioptes bovis* has been known for more than 160 years and occurs widely on cattle, goats, sheep, camelds, and possibly domestic rabbits. *Chorioptes texanus* was not discovered until 1924, and for 50 years, it was recognised only from goats and reindeer in the USA and Canada (35). Since 1975, it has been found on European elk, *Alces alces*, and several times on cattle from Brazil, Germany, Israel, and the USA (10, 30, 44). Based on unpublished observations by the USDA, *C. texanus* may now be the prevalent *Chorioptes* species on cattle in the USA.

Both *Chorioptes* species on domestic animals are nearly identical morphologically in all stages. The circular body is dorsoventrally flattened, with a striate cuticle, and about 400 µm long in the female; males are about one-fourth smaller, and the somewhat similar nymphs and larvae are progressively smaller yet. Dorsally, adults of both sexes have both anterior and posterior cuticular shields and a variety of mostly short, hair-like setae. Ventrally, the female ovipore is a transverse slit with a pair of trailing apodemes. The mouthparts are unremarkable, and the legs are moderately long and robust, except the fourth pair in the male are very short, and the third and fourth pairs in the female are more slender. All legs in both sexes terminate distally in empodial suckers with short, unjointed stalks, except for the female’s third pair, which end in two long, whip-like setae each. The male also has a long, whip-like seta on each third leg and a pair of adanal suckers. The terminal posterior lobes of males bear five setae each. The lobes of *C. bovis* each have a nearly rectangular margin, the seta at the external angle is long and whip-like, and the two spatulate setae are moderately shorter (ca. 115 µm) and broad. The lobes of *C. texanus* are each more angulate, almost bilobed, with a very short hair-like seta at the external angle and two much longer (ca. 215 µm) spatulate setae that seem narrowed basally.

**Otodectes cynotis**

Carnivores are the primary hosts for these highly contagious mites, which mainly infest the host’s ear canals but sometimes spread to the pinnae and even beyond. Clinical signs of otodectic mange (otacarasis, ‘ear canker’) may include rubbing and scratching the ears, vigorous head-shaking, but sometimes spread to the pinnae and even beyond. Clinical signs of otodectic mange are moderately shorter (ca. 115 µm) and broad. The lobes of *Otodectes cynotis* are each more angulate, almost bilobed, with a very short hair-like seta at the external angle and two much longer (ca. 215 µm) spatulate setae that seem narrowed basally.

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**Chorioptes spp.**

This genus currently comprises five putative species of obligate ectoparasitic mites that may cause chorioptic mange in domestic and wild mammals. Three of the species, collected rarely from wild animals, are poorly known and may not be valid entities, but *C. bovis* and *C. texanus*, primarily from domestic animals, have withstood modern biogenetic scrutiny and are accepted species (8, 44). A number of allegedly host-specific varieties within these species are not separable from one another (35). The two species are morphologically distinguishable only by differences in the terminal posterior lobes and setae of males (35). Chorioptic mange, also called ‘barn itch,’ may be the most common form of mange in cattle and horses. It is a relatively mild condition that usually is more localised and less intensely pruritic than psoroptic or sarcoptic manges. This is probably because *Chorioptes* mites are able to feed and survive on host-produced epidermal debris at the skin surface, without necessarily attacking the living parts of the host’s skin. Infestations tend to concentrate on the lower portions of the host, especially the feet and legs, but may include the udder/scrotum, tailhead, and perineum. In some cases, *C. texanus* infests the host’s ears (35). *Chorioptes bovis* has been known for more than 160 years and occurs widely on cattle, goats, sheep, camelds, and possibly domestic rabbits. *Chorioptes texanus* was not discovered until 1924, and for 50 years, it was recognised only from goats and reindeer in the USA and Canada (35). Since 1975, it has been found on European elk, *Alces alces*, and several times on cattle from Brazil, Germany, Israel, and the USA (10, 30, 44). Based on unpublished observations by the USDA, *C. texanus* may now be the prevalent *Chorioptes* species on cattle in the USA.

Both *Chorioptes* species on domestic animals are nearly identical morphologically in all stages. The circular body is dorsoventrally flattened, with a striate cuticle, and about 400 µm long in the female; males are about one-fourth smaller, and the somewhat similar nymphs and larvae are progressively smaller yet. Dorsally, adults of both sexes have both anterior and posterior cuticular shields and a variety of mostly short, hair-like setae. Ventrally, the female ovipore is a transverse slit with a pair of trailing apodemes. The mouthparts are unremarkable, and the legs are moderately long and robust, except the fourth pair in the male are very short, and the third and fourth pairs in the female are more slender. All legs in both sexes terminate distally in empodial suckers with short, unjointed stalks, except for the female’s third pair, which end in two long, whip-like setae each. The male also has a long, whip-like seta on each third leg and a pair of adanal suckers. The terminal posterior lobes of males bear five setae each. The lobes of *C. bovis* each have a nearly rectangular margin, the seta at the external angle is long and whip-like, and the two spatulate setae are moderately shorter (ca. 115 µm) and broad. The lobes of *C. texanus* are each more angulate, almost bilobed, with a very short hair-like seta at the external angle and two much longer (ca. 215 µm) spatulate setae that seem narrowed basally.

**Otodectes cynotis**

Carnivores are the primary hosts for these highly contagious mites, which mainly infest the host’s ear canals but sometimes spread to the pinnae and even beyond. Clinical signs of otodectic mange (otacarasis, ‘ear canker’) may include rubbing and scratching the ears, vigorous head-shaking, depression, excessive drainage, and haematoma of the ear. Worldwide, *Otodectes* is probably the most frequent mange mite infesting carnivores, both wild and domestic. In addition to companion animals (e.g. dogs, cats, ferrets), these mites also affect various farm-raised furbearers (e.g. foxes, mink) and occasionally may stray to humans. As with other mange mites, past workers have treated *Otodectes* mites from different localities or different hosts as separate varieties, or even different species, but recent molecular and phenotypic studies conclude that the genus is monobasic (21).

*Otodectes* mites have a typical psoroptid morphology and life history mirroring those of *P. ovis*. The female body is about 435 µm long and oval-shaped; the male length is about 325 µm. The female ovipore is a transverse slit with trailing genital apodemes, and bilaterally, the epimeres of the first pair of legs are joined to those of legs II. The terminal posterior somatic lobes of the male are only weakly produced, but adanal suckers are present. Each lobe bears five hair-like setae of varying lengths. All of the legs are moderately long and robust, except for the fourth pair, which is much reduced, especially in the female. Empodial suckers with very short, simple pedicels occur distally on all legs except for the posterior two pairs in females, which each end in a pair of long setae. The third tarsus of the male also bears a pair of long, whip-like setae in addition to its ambulacrum.

### iii) Knemidokoptidae

This acarine family comprises seven genera and about two dozen species of mites that inhabit the same microhabitats in birds that *Sarcoptidae* occupy in mammals (26). As a result, possibly due to convergence, the morphology of the two groups is similar. The body is generally globose, with cuticular striations that are sometimes modified into patches of scale-, furrow-, or tooth-like structures. The mouthparts and legs are usually short and stubby. Pretarsal suckers may be present, incomplete, or absent on all legs, and the tarsi
may terminate in one or two chitinous spurs. Somatic setae are generally few, unmodified, and quite short. Knemidokoptids have a distinctive anterior dorsal shield marked by a pair of strongly sclerotised, longitudinal, paramedial apodemes running to the base of the mouthparts. Males (but not females) also may have a median posterior dorsal shield, and their first pair of epimeres is fused into a midventral Y-shape. The first epimeres in females (and immatures) may be free or joined by a transverse apodeme into a V- or U-shape. The ovipore is a transverse slit or a three-valved, inverted Y-shape, and the anus is terminal or posterodorsal. Males may or may not have anald suckers. Most species occur, sometimes worldwide, on various wild birds in which they may cause clinical knemidokoptic mange; however, species in three genera are of concern for domesticated and cage birds.

Knemidokoptes mutans commonly burrows in the epidermal layers of the skin on the feet and legs of chickens, turkeys, and pheasants, causing a crusty mange known as ‘scaly leg.’ If untreated, lameness, distortion, or loss of digits may result. The first epimeres of female Knemidokoptes are free; legs I and II each have two terminal spurs, but no ambulacrum occurs on any leg; the ovipore is transverse; the anus is dorsal; and the body has a mid-dorsal patch of cuticular scales. Females are 350–450 µm, and males are less than 240 µm long. As in other knemidokoptids, legs of males are longer than those of females, and all of them terminate in a small, long-stalked sucker. A second, similar species, K. pilae, infests the face, cere, and legs of budgerigars, leading to a condition known as ‘scaly face.’ These mites are slightly smaller than K. mutans, and both species probably occur worldwide on their respective hosts.

Picinemidocoptes laevis infests columboid birds, including the domestic pigeon, sometimes leading to clinical mange. In females, the first epimeres are fused in a U-shape; each leg has an empodial stalk only, and legs I and II end in one spur each; the ovipore is transverse; the anus is terminal; and the dorsal cuticular striae are unbroken by scales.

Neocnemidocoptes gallinae may infest the skin of the back, head, neck, abdomen, and upper legs of chickens, geese, and pheasants, causing intense pruritus. Feathers in these areas may fall out, break, or be plucked by the host, leading to a condition known as ‘depluming itch.’ Affected skin, especially on the neck, may become scaly, thickened, and wrinkly. Although depluming itch is less common worldwide than scaly leg, it may be more damaging and even fatal. Female mites are 340–440 µm long, but males subend about 210 µm. The first epimeres of female Neocnemidocoptes are free; the larsi each end an empodial stalk only, and one spur terminates each of the anterior two pairs of legs; the ovipore is transverse; the anus is dorsal; and the dorsal cuticular cuticle is transversely striate but without scales. Two other, smaller Neocnemidocoptes, N. columbicola and N. columbigallinae, infest columbiform birds in limited circumstances and possibly might cause pathology in domestic pigeons.

iv) Miscellaneous families

Eight remaining astigmatan fur and feather mite families contain a variety of mange mites that are generally of minor significance due to their limited host ranges or relatively mild clinical effects on their hosts.

Three families of mammal parasites are worthy of note. Atopomelidae comprises nearly 50 genera of fur mites with known hosts in five mammalian orders, mostly marsupials in the Southern Hemisphere. The body plan is variable, but most are soft, slightly elongate, flattened or cylindrical, and the legs usually have some flattened segments for grasping the host’s hairs to the mite’s ventral surface, which often is ridged in the coxal areas of legs I and II. Chirodiscoides caviae probably occurs worldwide on guinea pigs, but it has been reported commonly only in Asia and Europe, where it sometimes causes severe pruritis and alopecia to laboratory animals. Listrophoridae is another family of fur mites comprising about 20 genera found on four mammalian orders, mostly rodents and mostly in the Northern Hemisphere. These are somewhat soft, elongate, cylindrical mites with various cuticular striae, spines, and punctate shields, including a sclerotised tegmen dorsally covering the mouthparts. They cling to the host hair-shaft bases by means of a pair of ridged flaps projecting ventrally from the area between the first pair of legs. Lepoacarus gibbus is a common listrophorid that sometimes causes mange in domestic and laboratory rabbits, and Lynxacarus radovskyi lives on several wild felines and the domestic cat, where mild, scurfy mange sometimes results. Myocoptidae is a nearly cosmopolitan family containing six genera of skin-feeding, hair-clasping mites that occur on rodents, insectivores, and marsupials. Myocoptids are generally oval-shaped and dorsoventrally flattened. The cuticle may be extensively striate, scale-covered, or denticulate in females, whereas male cuticles are generally less ornate and more heavily sclerotised. Host hairs are grasped by robust, highly modified legs II and IV in females and legs III in males. Myocoptes musculinus is probably the most ubiquitous ectoparasite of laboratory mice. Infestations are usually benign, but stressed or compromised mice may suffer alopecia, erythema, pruritis, and traumatic dermatitis (myocoptic mange.) Another, smaller myocoptid, Trichoecrius romboutsii, occasionally occurs on laboratory mice, along with M. musculinus or other mites, but its role in clinical mange is unclear.

Five families of mites from the skin and feathers of birds deserve mention. These are classified among 36 astigmatan mite families in three superfamilies loosely known as feather mites (13). Thousands of species of feather mites live on or inside the feathers or skin of nearly every kind of bird worldwide in generally commensal relationships. In rare and unexplained circumstances, the commensal status of nearly
any kind of feather mite may transition to that of a parasite, leading to negative consequences for the host. Some entire families of nominal feather mites (e.g. Cytidotidae, Laminiosioptidae) have become true parasites with distinct associated pathologies, even mange (e.g. Knemidokoptidae). A few species in other families are more prone than is usual to cause debilitation or injury to their hosts. In the Analgidae, Megninia cubitalis, M. ortari, M. holoagstra, and M. ginglymura occur on domestic chickens and may cause depluming behavior and economic losses (14). Dermoglyphus elongatus (family Dermoglyphidae) occurs on caged canaries, and Dubininia melopsittaci (family Kolaligidae) occurs on budgerigars, and excessive presence of each mite species may engender depluming and associated skin lesions in the respective hosts. Members of the families Demotionidae and Epidermoptidae generally feed on the skin or in the feather follicles of their bird hosts, placing them very close to being parasitic. Domestic poultry are hosts to Rivoltasia bifurcata and Epidermoptes bilobatus from the two respective families, and each mite has occasionally been associated with pityriasis (epidermoptic mange) in chickens (3).

b) Prostigmata

With nearly 15,000 named species classified into more than 130 families, prostigmatan mites as a group exhibit tremendous morphological and biological diversity, making generalisations about them difficult. However, all of the prostigmatan mite families belong to the superfamly Cheyletoidea, which includes only about 1000 named species in nine families. There are hundreds of undescribed species. The anterior mouthparts of cheyletoiids may be variously modified by palpal segment elaboration or reduction and by basal cheliceral fusion and extension into elongate, needle-like stylets used to pierce the host's tissues for feeding. Some cheyletoiids have paired, elongate, dorsal respiratory peritremes above the mouthparts. The body usually is elongate, sometimes very much so, and usually soft and thin-skinned, but sometimes with sclerotised plates. Adults usually have eight legs that vary in length and morphology according to the habits of the family, but they each usually terminate distally in a pair of pretarsal claws and a linear empodium that often is equipped with numerous sticky hairs. Proximally, the legs may articulate with simple coxal fields or sclerotised somatic apodemes. The ovipore is a longitudinal, puncture surrounding host tissues and feed on predigested cellular fluids. The normally four pairs of legs are usually short, stumpy, composed of three segments each, and terminate distally in paired pretarsal claws, usually with a linear empodium. Coxal fields occupy much of the anteroventral surface of the body where the legs attach. The palps or one pair of legs of some stages of some species may be greatly elongated or otherwise modified, primarily as holdfast organs. The very thin cuticle of the body and appendages is all but devoid of setae, but the opisthosoma is usually transversely striate. Befitting the confines of their narrow follicular or glandular habitats, the immature stages, including the eggs, of Demodex spp. are usually spindle-shaped or elongate oval, sometimes extremely so. Demodex species are very host specific, only rarely inhabiting more than one species of congenic mammal host. However, it is not uncommon for a host species to harbor two to four different species of parasitic Demodex. Transfer between hosts occurs only by very close contact between individuals (most probably mother to neonate), making transmission between animal species or from animals to humans very unlikely. Their very thin cuticles mean that demodecids cannot survive away from their hosts for more than a few hours.

Although Demodex mites frequently infest the skins of 100% of the individuals of their respective host species, their presence is usually without noticeable consequence for the hosts. On occasion, because of stress or other poorly understood factors, resident mite populations explode in numbers that result in a pathological condition known as demodectic mange. Healthy feral animals almost never suffer from demodectic mange, and laboratory or domesticated hosts are the usual victims (25). Clinical signs may range from presence of small skin papules, to large nodules, to extensive hair loss. Although rare, severe or generalised cases may lead to mites invading the host circulatory system, secondary bacterial skin infection, and even death. Among domestic animals, clinical disease (sometimes called 'red mange') is most often seen in dogs (Demodex canis and D. injai), but swine, (D. phylloides), goats (D. caprae), horses (D. caballi), sheep (D. ovis), cats (D. catt and D. gato), cattle (D. bovis, D. tauri, and D. ghanensis), and rabbits (D. cuniculi) occasionally develop demodectic mange. Humans are normal hosts for two species of Demodex (D. folliculorum and D. brevis).

ii) Psorergatidae

Worldwide, fewer than 100 species of these small parasitic mite species are described in three genera (15) (treated as subgenera of Psorergates by some authors). Known hosts are in eight mammalian orders,
mostly rodents and bats. Adult psorergatids are about 100–200 µm long, generally circular in outline, and dorsoventrally flattened. The cuticle is very thin, finely striate, and a large, punctate, lightly sclerotised shield covers most of the dorsum. The short anterior mouthparts have stylet-like chelicerae and two-segmented palps, each of which ends in a stout, claw-like seta. There are no dorsal peritremes. The four pairs of moderately long legs are radially attached ventrally, have five segments each, and terminate distally in paired pretarsal claws but no empodium. The femur of each leg often bears a sturdy, retrorse spur ventrally. Psorergatids have relatively few setae, including a few on the mouthparts, five or six pairs on the dorsal shield, one small ventral pair, one or two long pairs on posteroventral body lobes, and less than 10 on each leg. The eggs are almost round and large, nearly two-thirds the size of the mature female. They are deposited in hair follicles or in epidermal pits made by the female. Immature stages are much like adults but smaller, with only six legs for larvae and all legs greatly foreshortened. Transfer from host to host is accomplished directly by motile adult mites, which then move selectively to less-keratinised areas of the host skin, frequently about the head, neck, and the back. There, they invade the hair follicles or burrow body-sized pits into the epidermis, feed by puncturing cell walls with their stylets, and reproduce. Psorergatid mites rarely survive off the host for more than a day.

Psorergatid infestations on healthy wild hosts and most domestic animals are generally low and of little consequence. Sometimes, however, populations of a few species may explode, particularly on sheep and laboratory mice, producing psorergatic mange. Skin damage from activities of adult mites usually is mild and only slightly irritating, but their progeny, from egg nests cut into the dermis, may enlarge these pockets into fluid- or keratin-filled papular lesions that may rupture and cause inflammation and other host immune responses (25). Psorergatid mange mites of concern occur in two genera, Psorobia (with four pairs of marginal setae on the dorsal shield) and Psorergates (with three pairs of such marginal setae). Infestations of Psorobia ovis, the sheep itch mite, are most troubling in older animals and cause the hosts to rub, scratch, and bite at the wool in the most irritated areas, giving the fleece a ragged, tufted appearance. Powdery scurf sometimes may be present, as well. The life cycle of P. ovis takes about five weeks, the condition spreads slowly and inconsistently through a flock, and detection of infestations often is difficult. A similar mite, P. bos, occurs widely on cattle, but it seems to have little pathological effect on hosts. Psorobia cercopithec, from Africa (and a similar undescribed Asian species), occasionally cause mange in colonies of laboratory primates (20). The laboratory mouse is subject to papular lesions on the head and neck and auricular mange caused by Psorergates simplex (40). Incidence of these mites in some mouse colonies may be as high as 80 per cent. Another Psorergates mite, P. muricola, has been found on five different rodent species, including Mus musculus, and Psorergates rattus occurs on Rattus norvegicus; whether either of these mites infests or damages laboratory rodents is unknown.

iii) Cheyletiellidae

This is a relatively small family of parasitic mites (about 100 species) that, until 1970, was part of Cheyletiidae, a larger family of mostly predacious prostigmata. The species are arranged into approximately 15 genera, about two-thirds on mammals and the rest on birds. Although a number of the genera contain species capable of causing limited pathology in their hosts, only a few members of the genus Cheyletiella are of concern as mange mites on domestic animals. Cheyletiella mites are 300–530 µm long, elongate rhomboidal, and distinguished by a strongly striate cuticle with one (females) or two (males) large dorsal shields. A number of moderately long, simple or barbed setae occur in distinctive patterns on the mouthparts, and legs. The anterior mouthparts are large, with short piercing stylets and especially robust, five-segmented palps, each of which terminates in a strong, curved claw-like seta that is lined with weak, ridge-like teeth on the inner margin. Prominent M-shaped peritremes occur on the dorsal surface of the mouthparts. The four pairs of legs are long and strong, and each terminates distally in a linear empodium equipped with a double row of sticky hairs. Although almost all other cheyletiellids also have paired pretarsal claws on each leg, none occurs in Cheyletiella. A small sensory organ (solenidion) occurs on the middle segment ( genu) of each leg I, and its shape is (statistically) distinctive for each species (7). Females lay their eggs singly and attach them to host hairs near the skin using a finely woven mass of threads. Transmission between hosts is primarily by close contact, but phoresy on ectoparasitic insects is a possibility, as well.

For many years, the identities of the various pathological Cheyletiella spp. were confused under the single name C. parasitivorax (33), and these mites were mistakenly thought of as predators on other parasitic mites. However, C. yasguri (on dogs), C. blakei (on cats), and C. parasitivorax (on domestic rabbits) are now separately known to be the cause of mange, and any of the three may sometimes afflict humans in close contact with infested hosts, leading to severe dermatitis, pruritus, and other signs of cheyletiellosis for them, as well. The mites move easily among the host hairs on the keratin layer of the skin, periodically attaching to the surface by means of the palpal claws and puncturing cells of the epidermis with their stylets to engorge on predigested host fluids. The disease is similar in all three domestic hosts and usually is most evident on the back, shoulders, and neck. However, clinical signs are generally mild and not very distinctive or definitive. They may include scuffy hair coat, inflammation, occasional pruritus, alopecia, and almost always, hyperkeratosis. The barely visible, moving mites in the fur of the host and the abundant, powdery white scurf associated with cheyletiellid mange have engendered for it the alternative name ‘walking dandruff.’
iv) **Myobiidae**

Myobiids are small (to 900 µm), soft, elongate rectangular, somewhat dorsi-ventrally flattened fur mites known from five orders of mammals worldwide. More than 400 species of myobiids have been identified, at least half of them from bats. The cuticle is generally transversely striate, without sclerotised shields, and dorsally usually bears 12 to 16 pairs of setae, many of which are expanded, leaf-like, and longitudinally striate. The anterior mouthparts are small, with simple two- or three-segmented palps, cheliceral styliets, and dorsal peritremes. The legs, especially the first pair, are strong and highly modified for grasping host hairs, one or two at a time. They terminate distally in large pretarsal claws but no empodium; sometimes one of the two paired claws on a leg is greatly reduced or absent. The apparatus for clasping hairs are characteristic and consist of various combinations of modified leg segments and setae in the form of spurs, hooks, bosses, ridges, and grooved surfaces. Nymphal and larval myobiids generally resemble their respective adults except for size. Myobiid eggs are usually attached by the females with an adhesive secretion to the bases of the host’s hairs. Larvae may actually enter the hair follicles to feed on host fluids issuing from punctures made with the styliets. Nymphs and adults feed at the surface of the host skin in the same way, sometimes even puncturing capillaries and imbibing blood. The life cycles of myobiids are generally brief (ca. 14 days), and the mites freely move between host individuals. Myobiid infestations on wild mammal hosts are usually low in intensity and of little consequence (25), but on laboratory rodents, they frequently expand greatly and cause intense pruritus and hair loss known as myobic mange.

Both *Myobia musculi* and *Radfordia affinis* occur on the laboratory mouse and its wild progenitor, the house mouse, and each may cause pathology in laboratory animals. The two mites are superficially similar in appearance, but differ in many minute details, the most readily observed of which is the number of pretarsal claws present on the second leg; there are two in *Radfordia* and one in *Myobia*. *Radfordia ensifera* infests the Norway rat and the laboratory rat, sometimes causing mange in the latter. Whereas, both pretarsal claws on leg II in *R. ensifera* are of equal size, the posterior claw in *R. affinis* is smaller than the anterior one.

v) **Syringophilidae**

Over 350 species of these very host-specific quill mites have been discovered on a wide variety of bird hosts worldwide, but only a small proportion have been named and described. The body is elongate (about 500–950 µm) and cylindrical in keeping with the infestation site within the quills of the host. The cuticle is thin, striate, and without sclerotised plates, but a variety of usually long setae arise from its surface, particularly at the posterior end. M-shaped peritremes arise above the mouthparts, which are equipped with styliets and simple, linear palps. The legs are short, stubby, and terminate distally in paired claws and haired empodia. Sclerotised epimeres occur in coxal fields I and II. While residing in the quill shafts, syringophilids puncture the quill walls with their styliets to feed on fluids from the surrounding feather follicle tissues.

Two species of quill mites from domesticated hosts sometimes occur in large numbers and cause serious irritation and severe feather loss that might be confused with knemidokoptic mange; *Syringophilus columbae* parasitises domestic pigeons and *S. bipectinatus* occurs in the quills of chickens. Modern poultry production methods that physically separate chick broods from laying hens have been very successful in breaking the chain of passage for *S. bipectinatus* from one host generation to the next, all but eliminating the depulming problem except in more traditional production settings. A second, recently described (2001) quill mite from chickens, *Picobia polonica*, has not yet been associated with host feather loss.

3. **Serological tests**

Researchers have shown that *Sarcoptes scabiei* and *Psoroptes ovis* infestations cause measurable specific antibody responses in hosts (12, 23); this makes possible serological detection of sarcoptic and psoroptic mange. Enzyme-linked immunosorbent assays (ELISA) that detect antibodies to *Sarcoptes* in pigs and dogs are commercially available in some countries and are used for serodiagnostics of scabies (22) in Sweden and Switzerland to support scabies eradication programs in swine. Research efforts are currently in progress to develop a promising new immunodiagnostic assay for *S. scabiei* using recombinant antigens (38). ELISA techniques for serodiagnostics and management of *Psoroptes* infestations are under development and evaluation, but some issues with sensitivity and specificity remain unresolved. Although the only unequivocal proof of mange is finding and identifying mites in skin scrapings, but in the future, this traditional (direct) method will be augmented by better and better biochemical (indirect) methods.

C. **REQUIREMENTS FOR VACCINES AND DIAGNOSTIC BIOLOGICALS**

There are no commercial vaccines for mange. Experimentally, inoculation with *Psoroptes ovis* antigen has reduced the severity of mange. This introduces the future possibility of controlling the effects of mange without the use of acaricides (24, 34).
REFERENCES


* * *
CHAPTER 2.9.9.

SALMONELLOSIS*

SUMMARY

Salmonellosis is an infectious disease of humans and animals caused by organisms of the two species of Salmonella (Salmonella enterica, and S. bongori). Although primarily intestinal bacteria, salmonellae are widespread in the environment and commonly found in farm effluents, human sewage and in any material subject to faecal contamination. Salmonella organisms are aetiological agents of diarrhoeal and systemic infections in humans, most commonly as secondary contaminants of food originating from animals and the environment, usually as a consequence of subclinical infection in food animals leading to contamination of meat, eggs, and milk or secondary contamination of fruits and vegetables which have been fertilised or irrigated by faecal wastes. Human salmonellosis is one of the most common and economically important zoonotic diseases.

Salmonella organisms may also be found in feedstuffs, causing gastro-intestinal asymptomatic carriage or infectious disease in animals, particularly poultry and pigs. Salmonellosis has been recognised in all countries, but appears to be most prevalent in areas of intensive animal husbandry, especially in pigs and calves and some types of poultry reared in confinement. Reptiles are commonly asymptomatic carriers of Salmonella. Several serovars are host specific (e.g. S. Abortusovis in sheep or S. Typhi in humans) or host adapted (e.g. S. Choleraesuis, S. Dublin).

The disease can affect all species of domestic animals; young animals and pregnant and lactating animals are the most susceptible. Enteric disease is the commonest clinical manifestation, but a wide range of clinical signs, which include acute septicaemia, abortion, arthritis and respiratory disease, may be seen. Many animals, especially pigs and poultry, may also be infected but show no clinical illness. Such animals may be important in relation to the spread of infection between flocks and herds and as sources of food contamination and human infection.

Fowl typhoid and Pullorum disease, poultry diseases caused by Salmonella, are addressed in Chapter 2.3.11 of this Terrestrial Manual.

Identification of the agent: Diagnosis is based on the isolation of the organism either from tissues collected aseptically at necropsy or from faeces, rectal swabs or environmental samples, food products and feedstuffs; prior or current infection of animals by some serovars may also be diagnosed serologically. When infection of the reproductive organs, abortion or conceptus occurs, it is necessary to culture fetal stomach contents, placenta and vaginal swabs and, in the case of poultry, embryonated eggs.

Salmonellae may be isolated using a variety of techniques, which may include pre-enrichment to resuscitate sublethally damaged salmonellae, enrichment media that contain inhibitory substances to suppress competing organisms, and selective plating agars to differentiate salmonellae from other enterobacteria.

Various biochemical, serological and molecular tests can be applied to the pure culture to provide a definitive confirmation of an isolated strain. Salmonellae possess antigens designated somatic (O), flagellar (H) and virulence (Vi), which may be identified by specific typing sera, and the serovar may be determined by reference to the antigenic formulae in the Kauffman–White scheme. Many laboratories may need to send isolates to a reference laboratory to confirm the full serological identity and to determine the phage type and genotype of the strain, where applicable.

Serological tests: Serological tests should be conducted on a statistically representative sample of the population, but are of limited value if vaccination is used. In poultry, the whole blood test is used for rapid diagnosis of S. Pullorum/Gallinarum on the farm, being a relatively reliable diagnostic test under certain circumstances. In the laboratory, the tube agglutination test is the method of choice for export and diagnostic purposes for samples from all species of farm animals. Enzyme-linked immunosorbent assays are available for some serovars and may be used for
serological diagnosis and surveillance, especially in poultry and pigs. Vaccination may compromise the diagnostic value of serological tests.

Requirements for vaccines and diagnostic biologicals: Many inactivated vaccines are used against salmonellosis and some live vaccines are available commercially. Due to the low efficacy of inactivated vaccines, oil or alhydrogel adjuvants are used to improve their immunogenic properties. Field efficacy data are often lacking, although laboratory testing may provide a useful indication. Innocuity tests are performed in laboratory animals and, in the case of inactivated vaccines, sterility tests using bacteriological enrichment media are carried out. Further reassurances, such as environmental impact and stability, are necessary for vaccines produced using genetic manipulation. Competitive exclusion may be used to reduce Salmonella infections in poultry and other animal species.

A. INTRODUCTION

According to the latest nomenclature, which reflects recent advances in taxonomy (42), the genus Salmonella consists of only two major species: S. enterica and S. bongori. A third putative species, S. subterranea, has also been proposed following the isolation of a single unusual environmental strain (24, 27, 50, 54). Salmonella enterica is divided into six subspecies, which are distinguishable by certain biochemical characteristics and some of which correspond to the previous subgenera. These subspecies are:

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<th>Original subgenera</th>
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<td>Subspecies I</td>
<td>subspecies enterica</td>
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<td>Subspecies II</td>
<td>subspecies salamae</td>
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<td>subspecies indica</td>
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</tbody>
</table>

For the serovars of S. bongori, the symbol V was retained to avoid confusion with the serovar name of S. enterica subsp. enterica. Strains of Salmonella are classified into serovars on the basis of extensive diversity of lipopolysaccharide (LPS) antigens (O) and flagellar protein antigens (H) in accordance with the Kauffmann–White scheme; currently approximately 2500 serovars are recognised (42). This number is constantly being increased. The most common serovars that cause infections in humans and food animals belong to subspecies enterica. The serovars of the other subspecies are more likely to be found in poikilothermic (cold-blooded) animals and in the environment, but are occasionally associated with human disease. Some serovars of subspecies arizonae and subspecies diarizonae have been associated with disease in turkeys and sheep and others may be carried by free-living or captive reptiles and amphibians.

Names are retained only for subspecies enterica serovars. These names must no longer be italicised. The first letter is a capital letter. In clinical practice the subspecies name does not need to be indicated as only serovars of subspecies enterica bear a name, e.g. Typhimurium, London or Montevideo are serovars of subspecies enterica. The genus Salmonella followed by the serotype name may be used for routine practice (e.g. Salmonella Typhimurium). Most serovars of the other subspecies are designated by an antigenic formula, including subspecies designated by Roman numerical (e.g. Salmonella IV 48:g.z51).

In this chapter, the abbreviated new conventions are followed, i.e. S. Typhimurium rather than the more complete nomenclature S. enterica, subsp. enterica serovar Typhimurium. There are also regular changes to serotype classifications as new evidence on genetic relatedness becomes available, e.g. S. Pullorum is now classified as S. Gallinarum biovar Pullorum (42).

Salmonellosis is an infectious disease of humans and animals caused by organisms of the two species of Salmonella (Salmonella enterica and S. bongori). Although primarily intestinal bacteria, salmonellae are widespread in the environment and may commonly be found in farm effluents, human sewage and in any material subject to faecal contamination. Salmonellosis has been recognised in all countries, but appears to be most prevalent in areas of intensive animal husbandry, especially of poultry or pigs.

The disease can affect all species of domestic animals; young animals and pregnant animals are the most susceptible. Enteric disease, often presenting as a bloody or profuse watery diarrhoea with pyrexia, is the commonest clinical manifestation, but a wide range of clinical signs, which include acute septicaemia, abortion, arthritis, necrosis of extremities and respiratory disease, may be seen. The signs and lesions are not pathognomonic. Many animals, especially poultry and pigs, may also be infected but show no clinical illness (65). Such animals may be important in relation to the spread of infection between flocks and herds and as causes of
human food poisoning. In the latter case, this can occur when these animals enter the food chain thus producing contaminated food products (64, 65).

The course of infection, the clinical signs, and epidemiological patterns vary according to the serovar and the animal species involved. Some serovars only affect certain hosts, e.g. S. Gallinarum in poultry or S. Choleraesuis in pigs, although most serovars may cause disease in a wide range of animal species (51). Many serovars, including some that are host adapted such as S. Choleraesuis and S. Dublin, have been shown to cause disease in humans, and animal attendants, veterinarians and abattoir workers may be infected directly during the course of their work, as may laboratory personnel.

Disease is usually referred to as salmonellosis, although the term paratyphoid may be used, e.g. swine paratyphoid. In poultry, pullorum disease or bacillary white diarrhoea and fowl typhoid are often used to describe infections caused by S. Pullorum and S. Gallinarum, respectively (51). Fowl typhoid and Pullorum disease are covered in detail in Chapter 2.3.11 of this Terrestrial Manual.

For detailed epidemiological investigations, strain identification is necessary and such investigations have traditionally relied on biochemical and serological methods, phage typing of some serovars, and antibiograms. Genotypic analysis of the organism by use of real time-polymerase chain reaction (RT-PCR) and molecular fingerprinting of DNA has been used to good effect in recent years. Plasmid profile analysis is a quick and relatively easy method to fingerprint strains, and has been used in both human and veterinary medicine to study the spread of *Salmonella*. This technique has limitations as not all strains of *Salmonella* harbour plasmids, and plasmids may be readily acquired or may be of similar size but genetically different. The method has however proved to be useful in outbreak investigations to supplement other methods. Alternative genetic techniques, such as pulsed-field gel electrophoresis, AFLP (amplified fragment length polymorphism), VNTR (variable number tandem repeat), SNP (single nucleotide polymorphism) analysis and automated ribotyping, are increasingly used (4, 32, 53, 56). Genotyping is a rapidly expanding field and many new methods have been developed in recent years. It should be remembered that a single method may not work for all isolates and it may be necessary to evaluate a number of different techniques to find a method or combination of methods that is satisfactory and capable of differentiating clones of a particular serovar or phage type (45, 55). The molecular techniques are often more discriminatory and rapid and are replacing the phenotypic methods, such as serotyping and phage typing, for epidemiological investigations in some laboratories. However, these molecular tools are not necessarily available in all laboratories or standardised to give reproducible results in different laboratories and isolates may need to be forwarded to a suitable Reference Laboratory.

Genetic techniques such as micro-array and multiplex PCR analysis aimed at identifying specific serotypes as well as providing additional information on gene content are being developed (18, 19, 44).

The isolation and subsequent identification of salmonellae depend not only on the quality of the sample but also on the culture medium and growth characteristics of the serovar, particularly those adapted to a host species. A comprehensive review of *Salmonella* infection in domestic animals has recently been published (65).

National schemes have been implemented in many countries to control *Salmonella* infections in animals in order to protect the consumer. In the European Union, the Zoonoses Directive 2003/99/EC requires the monitoring of breeding flocks of more than 250 birds and hatcheries for S. Enteritidis and S. Typhimurium. In further legislation S. Virchow, S. Infantis and S. Hadar will also be subject to special controls (10). Culture of chicken delivery-box liners and dead or culled chickens is carried out on the day of arrival. At 4 weeks of age and 2 weeks prior to laying, pooled faeces of up to 60 samples, depending on the flock size, are cultured. Subsequently, adults are sampled every 2 weeks. At the hatchery, the meconium or dead-in-shells are cultured every 2 weeks, though there are plans to replace this with on-farm monitoring or monitoring of hatch basket liners. New EU legislation controlling *Salmonella* in commercial laying flocks, broilers, turkeys and pigs is also being introduced. Serological monitoring is permitted as an additional measure but can no longer replace bacteriological monitoring in poultry. In Denmark serological monitoring for *Salmonella* is used for pig herds and commercial laying flocks. Several other countries also now have serological monitoring programmes for finishing herds of pigs using ‘meat-juice samples or serum taken at slaughter.

*Salmonella* infections of food animals play an important role in public health and particularly in food safety, as food products of animal origin are considered to be the major source of human *Salmonella* infections (64). Special programmes have been implemented for surveillance of poultry, swine and cattle and include the surveillance of healthy animals that may be subclinical carriers of *Salmonella* organisms. Cross-contamination during food processing is also monitored as contamination by healthy food handlers can occur (65).

Feed contaminated with *Salmonella* has been the most common original source of introduction of new strains of *Salmonella* into livestock production networks, from whence it is further distributed by movement of carrier animals and other routes. In many situations international or national trade in livestock or other animals may be the major threat; feed also may contain less pathogenic ‘environmental’ serovars that may not be a cause of disease or cycles of infection in animals. As feed contamination may be caused by *Salmonella* serovars of relevance to public health, feedstuffs should be investigated for the presence of salmonellae (65). As feed is
milled with ingredients of mixed global origin a wide range of ‘exotic’ Salmonellas may be found in feed. Once established on a holding, spread between animals, environmental contamination and farm pests become more important in perpetuating and disseminating the infection.

Samples of food and feed tested for Salmonella should be truly representative. Proper steps should be taken to prevent contamination during transport or storage (20, 21). Because of the large variety of food and feed products there is no single sampling method appropriate to all products. Therefore different methods specific to the product should be used (11, 22).

The World Health Organization provides information on the development of appropriate measures for the prevention and control of food-borne diseases, including Salmonella infections, of humans. The most common vehicles of infection are eggs and egg products, poultry meat and meat from other food animals, and meat products. Contaminated salad crops and spices have also been involved in numerous outbreaks. Salmonella Enteritidis and S. Typhimurium are the most widespread serovars in many European countries (although Salmonella is rare in livestock production, some EU countries have strict control programmes), while S. Typhimurium is the dominant serovar in North America (64).

A Salmonella control policy for public health purposes should cover all stages from ‘the stable to the table’. It should include the mandatory reporting of all outbreaks of the disease (13), and animal, food and feed testing (65). Feed monitoring includes sampling of compound feed and other feed materials which are fed unprocessed, and ingredients, as well as sampling during feed processing. World-wide epidemiological investigations should be done to monitor Salmonella transmission and support Salmonella control policies.

Health and hygiene controls at slaughter are essential, and special precautions should also be applied when slaughtering potentially infected herds. Decontamination measures should be implemented during processing. Vaccines are increasingly used to reduce Salmonella in poultry and it is essential that these can be distinguished from field infections for monitoring purposes and to ensure that the vaccines do not spread beyond the vaccinated group of animals (60).

Another essential element in the prophylaxis of human salmonellosis is retailer and consumer education, in particular awareness of safe handling and storage of food, kitchen hygiene and proper cooking to limit the risk of infection.

**B. DIAGNOSTIC TECHNIQUES**

1. **Identification of the agent**

The frequency of sampling and the type of samples obtained will depend largely on the objectives of the testing programme (including any statutory requirements), clinical findings, level of detection or precision of prevalence estimates required, cost and availability of sampling resources and laboratory facilities.

Individual samples for bacteriological tests are collected as aseptically as possible and in the case of clinical disease or routine monitoring, samples should be collected before any antibiotic treatment has commenced. Preferably clinical samples are collected during the acute phase of the disease or as soon as possible after death. In the case of flocks of poultry or other avian species, environmental samples, such as naturally pooled faeces, litter and dust or drag or boot swabs from floor surfaces (5, 25), may be the most cost-effective way to identify infected flocks. Precautions should be taken to avoid cross-contamination of samples during collection, transit and at the laboratory. Packages should be kept cool and accompanied by adequate information. For smaller animal species, it may be preferable to submit a representative number of sick or recently dead animals to the laboratory, if that is possible (63). Host-adapted serovars are usually more difficult to isolate from faeces so if these are suspected, infected tissues should be cultured where possible.

Particular attention should be given to the isolation of salmonellae from animals with subclinical infection, as these may only excrete bacteria intermittently and in low numbers. An increased sample size, increased number of samples representing more individuals, combined in some cases with pooling of samples and repeat sampling can provide an increased diagnostic sensitivity. In such situations bacteriological or serological methods should be used to identify infected flocks or herds rather than to identify infected individual animals.

- **Culture**

There are numerous methods for isolation of Salmonella in use world-wide (9, 14, 17, 29, 46, 63). Some of the more common methods are described below. The culture techniques and media that may work best in a particular diagnostic situation depend on a variety of factors, including the type of Salmonella, source and type of
specimens, animal species of origin, experience of the microbiologist, and availability of selective enrichment and selective plating media.

All culture media should be subjected to quality control and must support growth of the suspect organism from a small inoculum. The routine use of a reference strain in parallel with routine samples may lead to cross contamination of samples if careless techniques are used, so a rare serovar with typical growth characteristics that are similar to the highest priority target strains should be used.

The increasing application of external quality assurance programmes has led to greater use of international standard methods, such as ISO 6579:2002; even though this has not been validated for faecal and environmental samples and was intended for foodstuffs and feedingstuffs. In recent years a standard method for detection of Salmonella from primary animal production has been developed and evaluated, and an ISO-method is now nearly adopted (35). The core of the standard method is pre-enrichment in buffered peptone water, enrichment on modified semi-solid Rappaport–Vassiliadis (MSRV) and isolation on xylose-lysine-deoxycholate (XLD) and an additional plate medium of choice.

a) Pre-enrichment media

The number of salmonellae in faeces from asymptomatic animals, environmental samples, animal feed and food is usually low, and it is necessary to use pre-enrichment media, such as buffered peptone water or universal pre-enrichment broth, to assist isolation. This may allow the small numbers of salmonellae, which may otherwise be killed by the toxic effect of enrichment media, to multiply, or it may help to resuscitate salmonellae that have been sublethally damaged, e.g. by freezing, heating, exposure to biocides or desiccation. Pre-enrichment may not be the best method for isolating less vigorous Salmonella strains, such as the host-adapted strains, from faeces because of overgrowth by competing organisms during non-selective pre-enrichment.

b) Enrichment media

Enrichment media are liquid or semi-solid agar media that contain additives that selectively permit salmonellae to grow while inhibiting the growth of other bacteria. Some, however, are also relatively toxic to certain serovars of Salmonella, e.g. selenite inhibits S. Choleraesuis, and brilliant green is toxic to many strains of S. Dublin. Elevated temperatures have also been used to increase the selectivity of enrichment media, e.g. tetrathionate and Rappaport–Vassiliadis at 43°C inhibit temperature-sensitive strains, especially S. Dublin and 41.5°C is now recommended for incubation of Rappaport–Vassiliadis broth (22). Selective motility enrichment may also be used to increase the sensitivity of Salmonella isolation and semi-solid enrichment media, e.g. MSRV or diagnostic semi-solid Salmonella medium (DIASALM), may provide greater sensitivity (59). The formulation of the medium, which may vary between suppliers, or even between batches in some cases, temperature and duration of incubation, and the volume of the samples used to inoculate the medium, may all serve to influence the isolation rate, and these variables should always be taken into account. Examples of selective enrichment media that may be used are selenite tetrathionate, as in Muller–Kauffmann broth, selenite F, selenite cysteine, brilliant green broth and Rappaport–Vassiliadis broths, or semi-solid Rappaport–Vassiliadis medium. In some cases it may be advantageous to use more than one selective broth or to culture by both pre-enrichment and direct selective enrichment/direct plating, although often the benefit does not justify the extra cost. Additions such as Ferrioxamine E may be added to selective media to enhance isolation of Salmonella from iron or nutrient-limited samples such as eggs, water or soil (46) or antibiotics may be added to enhance the isolation of antimicrobial resistant strains.

c) Selective plating media

These are solid, selective agars that permit differential growth to varying degrees. They inhibit growth of bacteria other than Salmonella and give information on some of the principal differential biochemical characteristics – usually nonlactose fermentation and hydrogen sulphide (H2S) production. The results are read after 24 and 48 hours of culture at 37°C. Salmonellae form characteristic colonies on such media that are usually distinguishable from the colonies of other bacteria on the plate, with the possible exceptions of Proteus, Pseudomonas and Citrobacter. Lactose-fermenting salmonellae may occasionally be isolated and H2S production may be variable. Such atypical strains may be more effectively detected when semi-solid selective media are used. DIASALM medium is particularly useful in this respect as presumptive confirmation by slide agglutination testing using polyvalent O, H or specific antisera can be carried out on liquid from the growth zone in the plate. Salmonella Abortusovis is a slow-growing serovar and it is usual to incubate plates for up to 72 hours and to use the nonselective blood agar. Examples of selective media are brilliant green agar, xylose lysine deoxycholate agar, deoxycholate/citrate agar, Rambach agar, and bismuth sulphite agar, although many more will be found in the literature and media catalogues. A wide range of chromogenic agars are now available. Many of these may aid differentiation of suspect colonies, but must be validated for the sample matrices, culture systems and serovar range used as sensitivity can be poor in some circumstances.
• Identification of suspect colonies

Suspect colonies are subcultured onto selective and non-selective agars to ensure that possible contaminants, such as Proteus spp., are absent. If there is an abundant pure growth, suspect colonies may be tested by slide agglutination with polyvalent Salmonella-typing sera (28). In some cases, the suspect colony may not agglutinate or auto-agglutinate and it is necessary to use biochemical tests to confirm the identity. These tests can be performed with peptone water sugars or commercial systems (such as the Analytical Profile Index [API] system), OBIS test, or composite media (such as triple sugar iron agar [TSI]), can be used to screen organisms (12).

The determination of the O factor(s) and the H antigen(s), and in special circumstances the Vi antigen, is performed by direct slide agglutination or tube agglutination using specific antisera. In the case of biphasic organisms, it is necessary to determine both phases, by the use of phase inversion – this involves passage through semi-solid agar containing antiserum to the known phase. Screening is facilitated by the availability of antisera directed against several factors, which can be pursued further by the use of monovalent typing sera. While many laboratories can identify the more common serotypes, it is necessary to use the facilities of a reference laboratory to confirm the identity of an isolate and possibly to obtain information on the phage type, if there is a scheme available, and genetic profile.

Additional biochemical tests may be necessary to identify some serotype variants, e.g. d-tartrate, which can be used to differentiate S. Paratyphi B var. Java from S. Paratyphi B. Isolates should also be tested for their sensitivity to a range of antimicrobial agents as there is increasing concern about the emergence of new multiple resistant strains harbouring transferable resistance genes to cephalosporins and fluoroquinolones (26, 43, 61). Live vaccine strains are also commonly identified by antimicrobial resistance markers, biochemical changes such as auxotrophy or roughness.

• Immunological and nucleic acid recognition methods

Numerous alternative Salmonella detection methods are in use and are commercially available (3, 6, 8, 12, 48, 49, 52, 57, 66). These include electrical conductance/impedance, immunomagnetic separation (IMS), enzyme-linked immunosorbent assay (ELISA), gene probes PCR methods, including nucleic acid sequence based amplification (NASBA) (6) and real time (16, 31, 40) or quantitative PCR (41). Many of these methods have not been validated for faecal and environmental samples and are more suited to analysis of human foodstuffs (39). The rapid methods are usually more expensive than conventional culture, but can be economically viable for screening materials where a low prevalence of contamination is expected or where materials such as feedstuffs are held pending a negative test. An enrichment/IMS method linked with ELISA or PCR can give results within 24 hours. Currently none of the rapid methods has been shown to be suitable for direct detection of Salmonella so non-selective or selective enrichment stages are required (37). Typically this introduces more steps and operator time in the detection procedure. For DNA-based methods, inhibition of the PCR reaction by elements of the test sample matrix, especially in the case of faeces, is problematic and requires suitable DNA extraction techniques (23). Rapid isolation methodologies may also be linked with sophisticated detection systems, such as biosensors (38). There are many variations and developments in rapid methods for Salmonella detection, but none has been shown to satisfactorily replace culture in all circumstances. It is therefore not possible to provide details of all the methods in this chapter or to make recommendations, but the review articles cited above will provide further information. Efforts are currently being made to standardise the use of certain rapid methods internationally (30), but there is a considerable amount of work still to be done.

• Example test procedures for isolation of Salmonella from food, feedstuffs, faecal and environmental samples

i) Add a 10–25 g sample to ×10 volumes of buffered peptone water at ambient temperature. (NB: for many host-adapted serovars and some arizonae serovars, it is preferable to add the sample to selective enrichment medium, such as selenite cysteine broth, and to test tissue samples where possible [including direct plating]; see culture method for S. Pullorum/Gallinarum in Chapter 2.3.11 of this Terrestrial Manual.)

ii) Incubate buffered peptone water for 16–20 hours at 37°C.

iii) Inoculate 20 ml modified semi-solid Rappaport–Vassiliadis or DIASALM in a Petri dish with 0.2 ml incubated buffered peptone water broth.

iv) Inoculate 10 ml tetrationionate broth with 1 ml incubated buffered peptone water broth.

v) Incubate modified semi-solid Rappaport–Vassiliadis or DIASALM at 41.5°C and tetrationionate broths at 37°C (ensure that a reputable brand of tetrationionate suitable for use at 37°C is used).

vi) After 24 and 48 hours of selective enrichment, plate out modified semi-solid Rappaport–Vassiliadis or DIASALM by taking 1 µl loop of material from the edge of the turbid growth zone and streaking over
one plate of Rambach agar or brilliant green agar and one plate of xylose lysine desoxycholate agar plus novobiocin.

vii) Plate out 10 µl of tetrathionate broth on Rambach agar or brilliant green agar and xylose lysine desoxycholate agar plus novobiocin.

viii) Incubate plates at 37°C for 24 hours.

ix) Check up to five suspect colonies (red/pink with reddening of the media on brilliant green agar, crimson with pale borders or orange/colourless on Rambach agar, red with black centre on xylose lysine desoxycholate agar) using poly ‘O’ and poly ‘H’ (phase 1 and phase 2) antiserum or composite biochemical media.

x) Subculture strongly suspect colonies that do not agglutinate with poly H antisera on to nonselective media then repeat testing. If a strong poly ‘O’ and poly ‘H’ agglutination can be obtained, this is sufficient for presumptive confirmation. Such isolates can then be serogrouped. If agglutination results are unclear then carry out further biochemical testing using composite media, such as TSI or use ONPG (o-nitrophenyl-beta-d-galactopyranoside) and urea or commercial biochemical tests such as API ID 32 E.

2. Serological tests

• Serological identification of infected animals, flocks and herds

A number of serological tests have been developed for the diagnosis of Salmonella infections in animals. In poultry, the whole blood test, which uses a stained antigen, and the serum agglutination test (SAT) have been used successfully for over 50 years for the identification of flocks infected with S. Pullorum/Gallinarum. Because S. Enteritidis possesses the same group D somatic antigen as S. Pullorum/Gallinarum, the whole blood test and related tests can be used for the diagnosis of infection but the sensitivity is low. In recent years, other tests, such as the ELISA (2, 58) have been developed for the diagnosis of S. Enteritidis and S. Typhimurium infections in poultry and for other serovars in farm animals. The ELISA has been used effectively to identify serologically S. Dublin carrier cattle and can be applied to bulk milk for screening dairy herds. The mix-ELISA is used in Denmark on serum or tissue fluid released by freezing then thawing muscle samples to detect Salmonella infections in pigs (36). A similar test is used to detect antibodies to S. Enteritidis and S. Typhimurium in egg yolk from commercial laying flocks (13).

Some ELISAs are now in routine use and are available commercially and there is a need for standardisation of their use. The purpose of this section is to consider the serological tests that have been fully evaluated and are in routine use for the diagnosis of salmonellosis in animals. Other tests that are still in the development stage will therefore not be considered. New tests for Salmonella diagnosis are developed frequently so an Internet search is often the best means of obtaining current information.

• Factors affecting serological diagnosis

1. Serological methods should be used to identify infected flocks/herds rather than to identify infected individual animals, although repeated herd tests can be used as an aid to selective culling of chronic carrier animals. Serological tests are normally designed to detect a limited range of Salmonella serovars or serogroups.

2. It is well recognised that some animals with a positive serological response may no longer be infected with Salmonella organisms. Likewise, animals that are actively excreting salmonellae may be serologically negative. Similar considerations may also apply to bacteriological culture methods, and negative faecal culture results may not necessarily indicate that an animal is not infected. However, neither of these situations should be considered as a major problem if enough tests are carried out. Animals that are serologically positive may have ceased to excrete salmonellae although circulating immunoglobulin concentrations may remain high. Other animals on the farm may still be infected. Serologically negative animals may result from a recent infection causing excretion before immunoglobulin production is maximal, or infection with less invasive serotypes. Animals that have been infected recently would, in all probability, eventually be detected serologically by an appropriate monitoring programme throughout the life of the flock/herd.

3. Newborn animals are immunologically immature and do not respond serologically to the somatic LPS antigen until 2–3 weeks of age. They do, however, produce a serological response to the flagellar protein antigens. Cattle may be unresponsive until about 10–12 weeks of age, and sucking pigs may fail to develop an immune response or have an antibody response that reflects maternal immunity. Differential responses involving different antibody classes (IgM, IgA, IgG) can be used in pigs to differentiate recent infection from infection that occurred some time ago, but this is often not useful for herd testing where individuals are usually at different stages of infection. Most tests are based on IgG and raised antibody levels typically appear 1–3 weeks after infection and last 2–3 months.
Chickens may also acquire anti-

Salmonella antibodies passively from their parents via the yolk sac; this may indicate an infected parent flock. Mammals can acquire maternally derived antibodies via the colostrum.

4. Immunisation has been used for many years to control certain Salmonella infections in farm animals, and if diagnostic serology is to be used, it is necessary to differentiate the vaccine response from that of actual infection. Many live vaccines given orally do not provide a significant serum antibody response in the majority of animals but there may be occasional exceptions. Injectable killed vaccines used for control of S. Enteritidis in chickens may produce a very prolonged antibody response. It would be advantageous if a marked live vaccine could be produced which could be differentiated from field challenge by serological testing.

5. The effect of antibiotic therapy on the serological response remains unclear. Some workers found reduced titres following therapy whereas others found no effect. Serology, however, may be a more useful diagnostic technique for salmonellosis than culture if antimicrobial therapy has been used.

6. Approximately 2500 different Salmonella serovars exist. Depending on the antigen and test used, serological cross-reactions between different serovars may occur, e.g. S. Typhimurium, S. Pullorum and S. Enteritidis. In some cases cross-reactions may also occur as a result of exposure to organisms other than Salmonella.

7. In poultry, egg yolk may be tested for immunoglobulins to Salmonella, and eggs may provide a method to screen flocks. This approach is used for monitoring commercial laying flocks in Denmark. In cattle, milk may be tested for anti-Salmonella antibodies to screen dairy herds.

8. The use of filter-paper discs for serum collection obviates the necessity to separate serum. The discs also provide long-term storage and reduce transport costs to the laboratory. The sensitivity of the test may be slightly reduced compared with tests carried out on fresh serum.

a) The whole blood test

The whole blood test provides a rapid test for fowl typhoid and pullorum disease that can be used on the farm. The sensitivity of the whole blood test is low and in inexperienced hands false-positive and false-negative results may be recorded.

For a detailed description of the whole blood test, see Chapter 2.3.11 Fowl typhoid and Pullorum disease.

b) Rapid slide agglutination test

Serum (0.02 ml) is mixed with polyvalent crystal-violet-stained antigen (0.02 ml). The tile is rocked gently for 2 minutes, after which the test is read. The test components are stored at 4°C and must have reached room temperature before being used.

Test sera should be free from contamination and haemolysis. It may be helpful to centrifuge serum samples that have been stored for any period of time.

If nonspecific false-positive reactions are suspected, positive/suspicious sera may be retested after heat-inactivation at 56°C for 30 minutes.

c) Serum agglutination test

The SAT is relatively insensitive, and many older animals have low levels of agglutinins in their sera caused by enterobacteria other than Salmonella. Single samples are of little diagnostic value except for initial screening on a herd basis. Paired samples are needed as the minimum requirement for confirmation of active infection. The test is relatively inexpensive; the antigens can be readily prepared and expensive equipment is not necessary. The SAT can be adapted to the microtitre format and can be readily used to determine somatic and flagellar titres. It is advisable to use standard sera and other confirmatory methods for quality control of the purity and immunogenicity of SAT antigen preparation(s) that are not dependant on sera produced from those antigens.

• Preparation of somatic antigen

i) Plate out the Salmonella culture from the appropriate stock culture onto a blood agar base (BAB) plate, or other suitable medium, for single colony growth. Incubate overnight at 37°C (±2°C).

ii) Select a smooth colony and carry out a slide agglutination test to ensure that the required somatic antigen is present.

iii) Using a sterile loop, inoculate a nutrient agar slope in a universal container from the selected colony.
iv) Incubate the culture for 8–12 hours at 37°C (±2°C).

v) Using a Pasteur pipette, wash off the culture, preferably inside a safety cabinet, with approximately 2 ml of absolute alcohol, and transfer into a sterile universal container.

vi) Leave the antigen for 4–6 hours at room temperature to enable the alcohol to kill the bacteria and detach flagellae.

vii) Spin the universal container in a bench-top centrifuge for 5 minutes at 1000 g. Pour off the liquid and add enough phenol saline to make the antigen up to an opacity equivalent to Brown’s tube No. 2 (approximately 10⁸ colony-forming units/ml) or other appropriate standard.

viii) Carry out standard titration with known serum to ensure that the antigen is positive for the required factor.

ix) Store in a refrigerator at 4°C until required.

• Preparation of flagellar antigens

i) Plate out the appropriate Salmonella stock culture on to a BAB plate, or other appropriate medium. Incubate overnight at 37°C (±2°C).

ii) Passage in semi-solid agar (about 0.3%) in a Craigie’s tube, or other suitable container, to induce optimum expression of the appropriate flagellar antigen. If the serovar is biphasic, H antiserum corresponding to the phase to be suppressed is added to the agar.

iii) Use slide agglutination to check that the Salmonella is in the required phase. If this is correct, inoculate a loop of culture into 20 ml of nutrient broth. Incubate for 12–18 hours at 37°C (±2°C) for optimum growth. (If the phase is incorrect, repassage through semi-solid agar.)

iv) Pipette 250 µl of 40% formaldehyde into the antigen suspension (use gloves and preferably work in a safety cabinet), and leave overnight.

v) Test the antigen by SAT using the appropriate typing serum.

• Test procedure

i) It is easiest to screen the sera at a dilution of 1/20; 0.25 ml of antigen is added to 0.25 ml of serum prediluted to 1/10 in normal saline.

ii) The tests are incubated in a water bath at 50°C for 24 hours in the case of somatic antigens and for 4 hours for the flagellar antigens. The dilution and time of incubation will vary depending on the antiserum that is used.

iii) Sera that give a positive reaction are then diluted from 1/20 to 1/320 and retested with the appropriate antigen.

d) Enzyme-linked immunosorbent assays for Salmonella Enteritidis

Two main basic systems are available for detection of IgG (IgY) specific for S. Enteritidis: the indirect ELISA (2) and the competitive ‘sandwich type’ ELISA (58).

The indirect ELISA involves the use of a detecting antigen coated on to the wells of a microtitre plate. After the application of a blocking reagent to reduce nonspecific binding, test samples are applied to the wells. Specifically bound antibody in the sample is detected by an antibody/enzyme conjugate. A variety of antigens, including LPS, flagella, SEF14 fimbriae, outer membrane proteins and cruder whole cell antigen preparations have been used.

The competitive sandwich ELISA employs a specific reagent – a monoclonal antibody (MAb) or polyclonal antibody – for coating antigen to wells. This is then followed by a pure or crude antigen preparation. Test samples are applied followed by conjugated antibody, which will not bind to the antigen if the sample contained specific antibodies. The assay time can be shortened by adding both test sample and conjugate together. MAbs have been prepared for LPS, flagella and SEF14 for S. Enteritidis.

There are advantages and disadvantages to both systems. The indirect assay is simpler and reagents are available for all Salmonella serotypes of chickens, turkeys, ducks and mammalian hosts. The competitive ELISA can be applied to all animal species and in general shows higher specificity. However, reagents are not available commercially for all serotypes. There are also some affinity problems and it may be less sensitive than the indirect assays. In the field, both systems have produced false-positive reactions and in some cases screening with an indirect LPS ELISA may be followed by confirmation with a flagellar competitive ELISA.
Both types of assay may be used with serum, egg yolk or reconstituted dried blood eluted from filter paper discs. A mix-ELISA (or meat-juice ELISA), is used in Denmark and other countries to detect Salmonella infections in pigs (36). This ELISA contains the 'O' LPS antigens 1, 4, 5, 6, 7 and 12, from S. Typhimurium and S. Choleraesuis, which enables it to detect serologically 95% of the Salmonella serogroups found in pigs in most European countries. Group D antigens have also been added to some ELISA kits. Serum is used to screen breeding and multiplying herds, whereas for pigs in the abattoir, the assay is performed on the tissue fluid ('meat-juice') that is liberated when a frozen 10 g muscle sample is thawed.

With some ELISAs differentiation can be made between infections produced by Salmonella serotypes from different serogroups. Some cross-reaction occurs between groups B and D and other invasive serovars. There is, however, usually a greater antibody response when LPS from the homologous serotype is used in the ELISA. The optimal method for choosing a 'cut-off' absorbance value, above which sera are designated as having come from an S.-Enteritidis-infected flock, without producing an unacceptable level of false-positive tests, has not yet been decided on and agreed upon internationally.

ELISAs are readily adapted to automation and hence to large-scale testing programmes. A major problem is that expensive equipment is necessary and many of the reagents are also expensive. Several commercial ELISA kits for S. Enteritidis, S. Typhimurium and Group B/C mix-ELISAs are available. Ideally these should be validated by international ring trials before adoption for surveillance purposes.

An example of a validated ELISA is the one developed at the OIE Reference Laboratory at VLA Weybridge (see Table in Part 3 of this Terrestrial Manual for address). The requirements are given below.

**Equipment**

Falcon microtest III PVC plates; appropriate pipettes and measuring cylinders; ultrawash microtest plate washer; ELISA plate reader; test filter of 405–410 nm and reference filter of 630 nm.

**Antigen**

i) Phenol-extracted S. Enteritidis LPS is available commercially (Sigma Cat. No. L6011). This is reconstituted in 1 ml deionised water and stored at –20°C in 100-µl aliquots in phosphate buffered saline (PBS), pH 7.2, at a concentration of 2.5 mg/ml. For use, the antigen should be thawed in coating buffer at the appropriate concentration.

ii) The LPS antigen can also be prepared by the technique of Westphal & Luderitz (62) and standardised as to its carbohydrate concentration by the method of Gerhardt (15), and adjusted to 1000 µg/ml.

**Serum and conjugate diluent**

Add bovine serum albumin (BSA) (2 g) and Tween 20 (0.05 ml) to PBS (100 ml). (Alternatively, powdered milk [1 g] can replace the BSA.) Store at 4°C and make fresh solutions every week.

Coating buffer: add sodium carbonate (1.59 g) and sodium bicarbonate (2.93 g) to deionised water (1 litre) and adjust to pH 9.6. (Alternatively, dissolve one tablet of 0.05 M Sigma carbonate/bicarbonate buffer [Cat. No. C-3041] in deionised water [100 ml].) Store at 4°C and renew every 2 weeks.

Substrate buffer: make a 10% (v/v) solution of diethanolamine in deionised water. The diethanolamine should be prewarmed to 37°C before dispensing, and the pH of the solution should be adjusted to pH 9.8 with 1 M hydrochloric acid. Store at 4°C and renew every 2 weeks.

Enzyme conjugate: goat anti-chicken immunoglobulin conjugated to alkaline phosphatase (e.g. ICN Immunobiologicals, alternative supply Sigma: A9171) or other species anti-chicken globulin. Store at 4°C diluted in diluent at the appropriate concentration and renew every week.

Enzyme substrate: dissolve one tablet of p-nitrophenyl phosphate disodium (5 mg) in substrate buffer (5 ml) no earlier than 30 minutes before dispensing, and store in the dark.

**Standards**

i) Positive control antiserum prepared by intramuscular inoculation of four 1-week-old specific pathogen free (SPF) chickens with an inoculum containing $10^6$ S. Enteritidis. The serum is subsequently obtained 3–4 weeks later when antibody titres are maximal.

ii) Negative control serum A from four 1-week-old SPF birds.

iii) Negative control serum B from 58 1-week-old breeders known to be free from Salmonella infections. Pool the sera and store in 100 µl volumes at –20°C.
C. REQUIREMENTS FOR VACCINES AND DIAGNOSTIC BIOLOGICALS

Many inactivated vaccines are used against salmonellosis caused by different serovars in various animal species, including a combined S. Enteritidis and S. Typhimurium vaccine for use in poultry. Inactivation is usually achieved by either heating or the use of formalin and an adjuvant, such as alhydrogel, is usually used. Live vaccines have also been used in a number of countries; these include the semi-rough strains, such as 9R for fowl typhoid and HWS51 for S. Dublin infections (33). Other attenuated vaccines include auxotrophic and ‘metabolic drift’ mutants, which are used to prevent Salmonella infections in farm animals in Germany and for S. Enteritidis and S. Typhimurium in poultry in the United Kingdom. Mutant vaccines attenuated rationally by molecular biological gene-deletion techniques have been developed for poultry and other species; these include aroA mutants and strains with mutations in the genes encoding adenylate cyclase (cya) and the cyclic adenosine monophosphate receptor protein (crp) (7), which is available in the United States of America. In Europe genetically modified organisms are not normally permitted for use as vaccines.

Guidelines for the production of veterinary vaccines are given in Chapter 1.1.8 Principles of veterinary vaccine production. The guidelines given here and in Chapter 1.1.8 are intended to be general in nature and may be supplemented by national and regional requirements.

1. Seed management

a) Characteristics of the seed

For killed or living vaccines, the bacterial strains should be an organism as closely related to currently circulating field strains as possible. It should be carefully chosen from cases of severe clinical disease, and virulence and antigen production should be assessed. It is best to evaluate a panel of potential strains in this way before testing the final selection. The final vaccinal strain should be identified by historical records and characterised by stable phenotypic and/or genetic markers. Living vaccinal strains should be marked by stable characters allowing distinction from wild strains. Markers, such as resistance to antimicrobials, for example rifampicin, may be used. Attenuation of virulence should be stable and preferably obtained by two independent defined mutations. The stability of live vaccine strains can be verified by regular checks using sensitive molecular fingerprinting and micro-array techniques.

b) Method of culture

The seed culture is propagated and maintained using suitable media, of which many have been described (in textbooks) for growth of Salmonella. The media used must not contain serum or animal tissues. Culture may be on solid medium, in Roux flasks, or in liquid medium, in which case large-scale fermentation equipment may be used. Iron limitation or low temperature incubation on a minimal media may enhance LPS antigen production by the vaccine strain.

c) Validation as a vaccine

i) Purity

The vaccine strain must be checked as follows:

• Staining of a smear of bacterial suspension on a glass slide using Gram stain.
• Homogeneity of culture on nonselective media.
• Metabolic requirements as indicated by biochemical tests.
• Detection of markers, and phage type.
• Agglutination with specific antiserum.

ii) Safety

The LD<sub>50</sub> (50% lethal dose) or ID<sub>50</sub> (50% infectious dose) may be determined in mice. Ten times the field dose of live vaccine or twice the dose for killed vaccines must be given to the target species at the recommended age and by the recommended route. The animals are observed for absence of adverse reactions. Stability and non-reversion to virulence after serial passages in susceptible species should be shown for live vaccines. It is also necessary to consider repeat vaccination. Live vaccine should be shown not to persist for long in vaccinated animals or be transmitted to milk or eggs that may be consumed, and the method of application should not present a hazard to operators.

iii) Efficacy

Laboratory experiments and field trials should be used to show that the vaccine is effective. The laboratory experiments consist of vaccination–challenge tests in the target species at the recommended dose and age. The efficacy data can also be used as the basis for a batch potency test.
Field trials are more difficult to undertake with respect to testing efficacy because of difficulties with standardising the challenge and providing appropriate controls.

iv) Environmental aspects

Live vaccine strains should be tested for their ability to persist in the environment and infect non-target species such as rodents and wild birds that are likely to be exposed. Prolonged survival of some live vaccines in faeces and litter may present an unacceptable environmental hazard when the material is removed from the animal houses. Live vaccines should not be used in poultry during lay.

2. Method of manufacture

Vaccine must be made in suitable clean rooms to which only approved personnel have access. Care must be taken to avoid cross-contamination between areas where live organisms are processed and other areas. Contamination from operators and/or the environment must be avoided and vaccine preparation should take place in a separate area from diagnostic culture work. Operators must not work with vaccine whilst ill and must not be subject to immunosuppressive conditions or medications. Personnel must be provided with protective clothing in production areas and in animal rooms.

Seed-lot cultures are prepared from the primary seed-lot, and the number of passages is dependent on the validation of the process. The vaccine may be prepared by inoculation of a suitable medium, such as nutrient broth, with a fresh culture and incubation on a shaker at 37°C for 24 hours, with or without aeration. The organisms are harvested by sedimentation or centrifugation. Alternatively, the organisms may be grown on and harvested from a solid medium, such as nutrient agar. In the case of live vaccines, the suspension is diluted in PBS, pH 7.0, and may be freeze-dried.

The time of inactivation of dead vaccines should be at least 33% more than that taken to reduce the viable number to an undetectable level. The inactivation process must be applied to the whole volume of the vaccine cell harvest.

Preservatives, excipient for lyophilisation, stabiliser for multidose containers or other substances added to or combined with a vaccinal preparation must have no deleterious effect on the immunising potency of the product.

3. In-process control

The following points require attention:

- Visual control of the suspension, homogeneity by Gram stain, culture on nonselective medium.
- Slide agglutination with specific antisera.
- Titration of bacteria by turbidimetry and/or plate count.
- Test of effective inactivation (dead vaccine) by plating on nonselective medium or use of a medium that gives optimum chance of recovery e.g. production medium with neutralisation of the inactivating compound.
- Titration of viable bacteria (living vaccine) before and after lyophilisation.

4. Batch control

a) Sterility

Tests for sterility and freedom from contamination of biological materials may be found in Chapter 1.1.9.

b) Safety

A laboratory test in mice that has previously shown a correlation with safety in the target species may be used to determine the LD₅₀ and/or ID₅₀. Each batch should be tested in the target species at the recommended age and route, using at least twice the field dose for killed vaccines and ten times the dose for live vaccines.

c) Potency

Potency is tested using vaccination–challenge assay in mice and/or other species, including (if practicable) the target species and immunological response in target species.
d) **Duration of immunity**

The duration of immunity is likely to vary considerably between products, vaccination regimes and individual vaccinated animals. Immunity to *Salmonella* is normally serogroup specific. Consultation among colleagues suggests that most killed vaccines will provide protection for 6 months, while some live vaccines given by injection may elicit stronger immunity, which may persist for 1 year or more. It should be remembered, however, that a strong challenge such as that associated with continuously occupied farms or infected rodents may overwhelm vaccinal immunity and commercial live vaccines may be attenuated to reduce environmental survival in a way that reduces the immune response. There may also be problems with ensuring effective oral administration with live vaccines or accuracy of injection with killed and live injectable vaccines.

e) **Stability**

Information is lacking on the stability of killed vaccines. Stability is affected by storage conditions and by the presence of contaminating microorganisms growing in the product. The stability is assessed by potency tests repeated at appropriate time intervals. The stability of live vaccines can be assessed by performing counts of the number of viable organisms repeated at appropriate time intervals.

f) **Preservatives**

Chemicals with antimicrobial activity, such as thiomersal, phenol or crystal violet, are often included as preservatives in killed bacterial vaccines.

g) **Precautions**

Certain killed vaccines may occasionally cause abortion in pregnant animals because of their LPS content, and likewise live vaccines should be used with caution in pregnant animals. It is often necessary, however, to vaccinate pregnant animals to provide maternal immunity for their offspring. It may be useful to include endotoxin assay in the safety test programme so that the levels can be compared with those shown to be safe in the double-dose tests. Vaccines may also cause swelling at the site of injection.

5. **Tests on the final product**

a) **Safety**

Killed vaccines are assessed in a double-dose test, and live vaccines are assessed in a test using ten times the dose, in the target species.

b) **Potency**

If possible, the potency test should relate to the efficacy of the vaccine in the target species, and suitable criteria should be applied for passing batches. It may be possible to assess killed vaccines by the O-H antibody response produced, although it should be remembered that serum antibodies are only part of the host’s protective mechanism against *Salmonella*. Alternatively, the potency of the vaccine may be assessed by its effect on the LD$_{50}$ in mice.

D. **COMPETITIVE EXCLUSION**

Susceptibility to *Salmonella* infection in poultry can be substantially reduced by spray or oral treatment prior to exposure (ideally in the hatchery) with an anaerobic culture of caecal microflora that inhibits colonisation by *Salmonella* by occupying intestinal colonisation sites, producing acids and other inhibitory substances and stimulating local and mucosal generalised immune responses. This treatment is widely used in some countries but only used as an aid to decontaminating persistently infected farms in others. It is also useful to minimise perturbation of intestinal flora following antimicrobial treatment or stress and to potentiate the effect of vaccines given subsequently. As with any treatment that reduces the prevalence or numbers of *Salmonella* organisms excreted by infected groups of animals, there may be some interference with monitoring programmes and the sampling and test sensitivity may have to be adjusted to compensate for this (1, 34, 47).

REFERENCES


Chapter 2.9.9. — Salmonellosis


64. WORLD HEALTH ORGANIZATION (WHO) REGIONAL OFFICE FOR EUROPE (2000). WHO Surveillance Programme for Control of Foodborne Infections and Intoxications in Europe. Seventh Report on Surveillance of Foodborne Disease in Europe 1993–1998. Institute for Health Protection of Consumers and Veterinary Medicine BGVV. FAO/WHO Collaborating Centre for Training and Research in Food Hygiene and Zoonoses, P.O. Box 33 00 13, 14191 Berlin, Germany.


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NB: There are OIE Reference Laboratories for Salmonellosis (see Table in Part 3 of this Terrestrial Manual or consult the OIE Web site for the most up-to-date list: www.oie.int).
CHAPTER 2.9.10.

TOXOPLASMOSIS

SUMMARY

Definition and description of disease: Toxoplasmosis is a zoonotic infection of animals caused by the protozoan parasite Toxoplasma gondii. It has the capacity to infect all warm-blooded animals and, while infection does not cause clinical illness in the majority of animal species, in some it causes acute life-threatening disease and in others, particularly sheep and goats, it may manifest itself as a disease of pregnancy by multiplying in the placenta and fetus. In these latter animals it can result in the abortion or the birth of weak lambs/kids, which may be accompanied by a mummified fetus. Characteristically, in these cases, the placental intercotyledonary membranes are normal, but white foci of necrosis, approximately 2–3 mm in diameter, may be visible in the cotyledons. Microscopically, these foci appear as areas of coagulative necrosis that are relatively free of inflammation. Inflammation, when present, is nonsuppurative. Toxoplasma tachyzoites are seen only rarely in association with these foci, usually at the periphery of the lesion. Examination of the brain may reveal focal microgliosis. The lesions often have a small central focus of necrosis that might be mineralised. Focal leukomalacia in cerebral white matter, due to anoxia arising from placental pathology, is often present. Focal microgliosis is more specific, as leukomalacia reflects placental damage but may occur in other pathological conditions where the placenta is compromised, including, though rarely, ovine chlamydiosis. Infection in pigs may cause severe fetal losses in pregnant sows, but more usually is mild and unnoticed. Acute fatal infections affect New World monkeys, marsupials and certain other animals.

Identification of the agent: Toxoplasma gondii is an obligate intracellular parasite that has a sexual cycle in felidae and a two-stage asexual cycle in all warm-blooded animals. It predominantly comprises three clonal lineages (I, II and III). In the acute phase of infection, tachyzoites multiply in cells to cause varying degrees of tissue destruction and, in fatal cases, tachyzoites may be demonstrated in ascitic fluid or in lung impression smears. With the onset of an immune response, tachyzoites are transformed into bradyzoites that multiply slowly in cells to produce tissue cysts. In aborting sheep, goats and pigs, T. gondii is often difficult to find in tissue sections, but is more likely to be seen in sections of brain and placenta. Its identity can be confirmed by immunohistochemistry, while the polymerase chain reaction may be used to identify parasite DNA in tissues. Isolation of T. gondii from samples is expensive and slow but, if required, is best achieved by inoculation of mice with tissue homogenate derived from fetal brain or placenta. The sexual life-cycle of the parasite takes place exclusively in epithelial cells of the feline intestine and can result in the excretion of large numbers of oocysts in the faeces. Oocysts may remain viable in the environment for many months.

Serological tests: The dye test is the longest established serological method, and in many ways represents the ‘gold standard’, at least in humans. The dye test uses live, virulent Toxoplasma tachyzoites, a complement-like ‘accessory-factor’ and test serum. When specific antibody acts on the tachyzoites, the latter do not stain uniformly with alkaline methylene blue. The test has proven unreliable in some species. In addition, as live Toxoplasma is used, the test carries a potential risk of human infection as well as being expensive to conduct. The indirect fluorescent antibody (IFA) test is safer, gives titres comparable with the dye test and can be used to differentiate IgM and IgG antibodies. The direct agglutination test and the latex agglutination test are both relatively rapid and neither requires complex laboratory facilities. The enzyme-linked immunosorbent assay requires more sophisticated laboratory equipment but can handle large numbers of samples and does not rely on human interpretation for the result.
Toxoplasma gondii is a zoonotic, obligate intracellular protozoan parasite that has the capacity to infect all warm-blooded animals and, while infection does not cause clinical illness in the majority of animal species, in some it causes acute life-threatening disease and in others, particularly sheep and goats, but also pigs, it manifests itself as a disease of pregnancy by multiplying in the placenta and fetus. Acute, potentially fatal, infections are recorded in New World monkeys (8), marsupials (6) and certain other animals (see below). In these cases clinical signs may include lymphadenopathy, hepatomegaly, interstitial pneumonia and nervous signs. At post-mortem examination lymph nodes, spleen and liver may be enlarged and the latter may have pale foci. In sheep, goats and pigs a primary infection established during pregnancy may result in apparent infertility or in stillbirths and abortion, according to the stage of pregnancy at which infection was initiated. In a typical case of abortion, a ewe or doe infected in mid-gestation produces a stillborn lamb/kid a few days earlier than the predicted end of pregnancy. The aborted fetus is often accompanied by either a weak sibling or a ‘mummified’ fetus (5). The ewe/doe remains clinically normal. In such cases, placental cotyledons are typically speckled with white foci around 2–3 mm in diameter while the intercotyledonary membranes appear normal. Infection in early pregnancy, when the fetus has only a rudimentary immune system, results in fetal death and resorption. In this case the mother may present as barren, which in turn can mimic a flock/herd infertility problem. Mothers that become infected in late pregnancy would be expected to produce infected clinically normal offspring. Following an infection, either during or outside of pregnancy, the parasite would not be expected to cause abortion in any subsequent pregnancy. While recent research has questioned this conclusion (13, 38) the majority of current thinking tends towards the view that recrudescence of a persistent infection during pregnancy leading to repeat abortions is not normally a significant occurrence (28). Infection in pigs may cause severe fetal losses in pregnant sows but under modern intensive farming conditions, when contamination of the housing and feed by T. gondii oocysts is minimal or absent, infection would generally be expected to be at a very low incidence and cause only mild or unnoticed signs of infection (24). However when pigs are maintained outdoors under extensive systems, they are much more likely to encounter oocysts, and so infection would be expected to be more common (21).

Toxoplasma gondii is an obligate intracellular parasite that has a two-stage asexual cycle in warm-blooded animals and a sexual cycle in felidae. The parasite comprises three clonal lineages (I, II and III) in the main, with type II and III being associated with disease in animals while type II is the predominant form identified in human disease (17, 20). In the asexual cycle, the two developmental stages are the rapidly multiplying tachyzoite and the slowly multiplying bradyzoite. In acute infection, tachyzoites actively penetrate host cells where they multiply causing the cell to rupture and release organisms locally and into the bloodstream. As the host develops immunity, the parasite retains its overall size and shape but transforms into the bradyzoite stage and multiplies more slowly within tissue-cysts to establish a persistent infection. These microscopic tissue cysts are present most frequently in brain and skeletal muscle and represent the quiescent stage of the parasite within the host. Viable tissue cysts within muscle (meat) are a significant source of human infection. In animals that succumb to acute infection tachyzoites may be demonstrated in ascitic fluid or in lung impression smears as well as in tissue sections of the liver and other affected organs.

The sexual cycle occurs in enteroepithelial cells of the feline definitive host and results in the production of Toxoplasma oocysts. Following a primary infection of a cat, oocysts may be shed in the faeces for several days. The oocysts sporulate in the environment over the next 1–5 days (depending on aeration, humidity and temperature), at which time they become infective. They are very resistant to environmental conditions and may remain infective for a year or more. Sporulated oocysts are 11 × 13 µm in diameter and each contains four sporozoites in each of two sporocysts (11). When a susceptible animal ingests sporulated oocysts the sporozoites are released to penetrate the intestinal lining, become tachyzoites and establish an infection.

In sheep, goats, pigs, horses and humans, tissue cysts may remain for the rest of the life of the individual (11). Toxoplasma does not usually cause clinical illness in cattle, cameldids or deer but, as noted, can cause fatal disease in New World monkeys, marsupials and certain other animals including hares (Lepus europaeus: L. timidus) (16), the Pallas cat (2), the arctic fox (32), some birds (12) and sea mammals (15). It is suggested that these and other similarly affected animals have had minimal exposure to T. gondii in their natural habitat through the ages, making them particularly vulnerable to the parasite.

Abortion in sheep and goats due to T. gondii must be differentiated from that caused by other infectious agents, including infections with Chlamyphila abortus (see Chapter 2.7.7 Enzootic abortion of ewes), Coxiella burnetii.
Chapter 2.9.10. – Toxoplasmosis

(see Chapter 2.1.12 Q fever), *Brucella melitensis* (see Chapter 2.7.2 Caprine and ovine brucellosis [excluding *Brucella ovis*]), *Campylobacter fetus fetus* (see Chapter 2.4.5 Bovine genital campylobacteriosis), *Salmonella* spp. (see Chapter 2.9.9), border disease (see Chapter 2.7.1), and the viruses that cause bluetongue, Wesselsbron's disease and Akabane disease. In pigs, *Brucella suis* (see Chapter 2.8.5) may also cause fetal death, mummification and abortion.

- **Human health risks**

*Toxoplasma gondii* readily infects human beings and while infection is relatively common (approximately 30% of the population depending on age and environment), clinical illness is relatively uncommon. Those particularly at risk of developing clinical illness include pregnant women, as the parasite can pose a serious threat to the unborn child if the mother becomes infected for the first time while pregnant, and individuals who are immunosuppressed, such as tissue transplant patients, AIDS patients, patients with certain types of cancer and those undergoing certain forms of cancer therapy. These individuals are at risk of developing acute lethal infection if left untreated. The very young and very old may also be more susceptible. On occasions, people with no apparent immune deficiency may develop an illness characterised by general malaise, fever and lymphadenopathy. The most likely sources of human infection are ingestion of raw or lightly cooked meat containing live *T. gondii* tissue cysts, ingestion of raw or lightly cooked vegetables contaminated with oocysts or exposure to oocysts derived from cat faeces, such as may be encountered in gardens and children’s sand pits. Toxoplasmosis is now also recognised to be a water-borne zoonosis (10). This method of transmission occurs where water treatment is ineffective or non-existent and there is a sizeable local felid population that contaminates surface water with oocysts (1, 10). Linked to this there is now also an appreciation that sea mammals are becoming infected by waters from contaminated land and from untreated urban sewage (15).

**B. DIAGNOSTIC TECHNIQUES**

1. **Identification of the agent**

a) **Isolation**

Isolation of *T. gondii* from aborted ovine and caprine fetuses and fetal membranes is best made by inoculation of laboratory mice. The best tissues for inoculation are fetal brain and placental cotyledons, and optimum results are obtained with fresh samples free from contamination. Samples must not be frozen at any stage, as this kills the parasite;

i) With aseptic precautions, remove 2–5 g of placental cotyledon or brain tissue from the aborted fetus.

ii) Homogenise the tissue in an equal volume of 0.3 M sterile phosphate buffered saline (PBS), pH 7.4, with added antibiotics (100 International Units [IU]/ml penicillin and 745 IU/ml streptomycin) in a ‘stomacher’ (Seward Laboratory, London) or other suitable macerating equipment. Brain tissue may be effectively homogenised by passing it through a 16-gauge needle ten times by means of a syringe.

iii) Inoculate each of three *Toxoplasma*-free mice intraperitoneally with 0.5 ml of the homogenate.

iv) Kill the mice 6–8 weeks after inoculation and remove the brains. Blood should also be recovered from the mice at this stage and the serum separated and stored at −20°C. Brains from mice that die before 6–8 weeks should also be harvested.

v) Homogenise each mouse brain with an equal volume of sterile PBS by passing through a 16-gauge needle ten times by means of a syringe.

vi) Spread one drop (5 µl) of a given suspension on each of five slides.

vii) Dry and stain with Giemsa, dehydrate and mount under a cover-slip.

viii) Examine slides under a microscope. Tissue cysts appear as circular structures measuring 5–50 µm filled with blue-staining, crescent-shaped bradyzoites.

An alternative method for examining the mouse brain is to take a small portion of forebrain (approximately match-head size) squashed flat with a cover-slip. Tissue cysts should be easily detected under the microscope.

If the tissues inoculated are heavily infected with *T. gondii*, mice may die at 1–2 weeks.

Failure to demonstrate tissue cysts does not rule out a positive diagnosis. Serum from the mice may be analysed for the presence of antibodies to *Toxoplasma* (e.g. using an indirect fluorescent antibody [IFA] test); if this analysis is also negative, infection with *Toxoplasma* is unlikely.
b) Tissue sections

In animals that die with acute toxoplasmosis, focal mononuclear inflammation with or without focal necrosis may be seen in a number of tissues, including the liver, heart and lungs. The latter may be oedematous. Lymph nodes may have undergone expansion and there may or may not be focal necrosis with or without haemorrhage. Typically Toxoplasma tachyzoites may be demonstrable in association with necrosis and inflammation.

In cases of abortion and stillbirth in sheep and goats, affected placental cotyledons typically contain large foci of coagulative necrosis that may have become mineralised with time. Any associated inflammation is characteristically slight and nonsuppurative. Well preserved samples of placental cotyledons may show moderate oedema of the mesenchyme of the fetal villi with a diffuse hypercellularity due to the presence of large mononuclear cells. Sometimes small numbers of intracellular and extracellular toxoplasmas are visible, usually on the periphery of a necrotic area or in a villus that is in the early stages of infection. The Toxoplasma tachyzoites appear ovoid, 2–6 µm long, with nuclei that are moderately basophilic and located centrally or towards the posterior end.

In the fetal brain primary and secondary lesions may develop. Microglial foci, typically with a necrotic and sometimes mineralised centre and often associated with a mild focal lymphoid meningoitis, represent a fetal immune response following direct damage by local parasite multiplication. Toxoplasma tissue cysts are only rarely found, usually at the periphery of these lesions. Focal leukomalacia is also common and is considered to be due to fetal anoxia in late gestation caused by advanced lesions in the placentome preventing sufficient oxygen transfer from mother to fetus. Such foci occur most commonly in the cerebral white matter cores, but sometimes also in the cerebellar white matter. Focal leukomalacia on its own suggests placental disease or acute insufficiency but the two types of neuropathological change seen together are characteristic of Toxoplasma infection. Confirmation of the identity of T. gondii-like structures in tissue sections from such cases, as well as from instances of acute toxoplasmosis, may be achieved by immunohistochemistry that labels intact T. gondii or antigenic debris. The method is both convenient and sensitive and is used with fixed tissues (including archive tissues) that may also exhibit a degree of decomposition, where isolation would not be appropriate or possible. The ABC indirect immunoperoxidase method and the peroxidase–antiperoxidase (PAP) technique (34) are equally good.

c) Nucleic acid recognition methods

Several polymerase chain reaction (PCR)-based assays have been developed for the detection of DNA from T. gondii. The main target regions are the B1 repetitive sequence (3), the P30 (SAG1) gene (31) and 18S ribosomal RNA (rRNA) (14). The sensitivity of the PCR is dependent on the copy number of the target sequence (P30: 1 copy; B1: 35 copies; rRNA: 110 repeat units). Customised synthetic DNA oligonucleotides are commercially available (e.g. www.sigma-genosys.co.uk). Recently, the method for amplification of the B1 repetitive sequence has been used to analyse the lens aspirates of congenitally infected human cataract patients (25) and was found to be more sensitive than the conventional method used (enzyme-linked immunosorbent assay [ELISA]). However, although the PCR is extremely sensitive, care should be taken if it is the only test available, as in many situations a more reliable diagnosis will be gained if it is used in combination with other diagnostic data.

Recently, a real-time PCR has been developed to allow simultaneous quantification and amplification of DNA. It is very similar to existing PCR methods and can be carried out on 96-well microtitre plates. After each round of amplification, fluorogenic dyes intercalate with the double-stranded DNA and the results, shown on an amplification plot, allow quantification of the parasite DNA in the sample. Real-time PCR has been used to amplify and quantify DNA from the T. gondii B1 gene (7, 23). This quantification of parasite DNA can be used to determine the number of parasites in tissues and fluids, such as the amniotic fluid of patients suspected of being congenitally infected with T. gondii. (27). The real-time PCR is a highly sensitive and specific method, however it is expensive and requires specialised detection systems and therefore may only be cost-effective in laboratories where analysis of large numbers of samples is carried out.

The following method is a nested form of the PCR, amplifying the B1 repetitive sequence of DNA (36). Parasite DNA can be extracted and purified from several tissues, including placenta, the central nervous system, heart and skeletal muscle.

Contaminating red blood cells in tissues are removed by washing in 10 mM Tris/NH4Cl lysis buffer, pH 7.6, followed by centrifugation at 2000 g for 15 minutes. DNA is then extracted from the resultant pellet and resuspended in 10 mM Tris, pH 8.3, 50 mM KCl, 1.5 mM MgCl2 containing protease K 100 µg/ml and 0.5% Tween 20.

Samples are incubated at 55°C for at least 1 hour, then the protease K is inactivated by boiling. The PCR procedure is performed in 50 µl volumes. Amplification of the B1 gene is performed by modifying the
procedure described in ref. 1. The reaction mixture contains 10 mM Tris, pH 8.3, 2.5 mM MgCl₂, 40 mM KCl, 0.01% gelatin, 0.1 mM dNTPs, 0.2 µM of each primer (oligonucleotide primers are those described in ref. 1), two sense primers P1 and P2 and two antisense primers P3 and P4) and 2.5 units of Taq polymerase.

Primary amplification is performed with primers 1 and 4 to give a 193 bp product over 25 cycles of 93°C for 1 minute, 50°C for 1.5 minutes and 72°C for 3 minutes. The amplified product is then diluted 1/20 in distilled water to reduce amplification of non-specific products.

Secondary amplification using nested primers 2 and 3 and the same reaction conditions, is carried out over 15 cycles to give a 94 bp product. The final product is then visualised on 1% agarose gels. Southern blotting, using a labelled probe, can be used to confirm the identity of the B1 PCR products and to distinguish them from non-specific products.

d) Oocyst detection in drinking water

Toxoplasma gondii oocysts have been detected in drinking water using the method for the detection of Cryptosporidium oocysts (18). The method relies on the collection of a large-volume sample of water and passing it through a cartridge filter. Identification of Toxoplasma oocysts was by means of inoculation of rodents.

2. Serological tests

There are several serological tests available for the detection of T. gondii antibodies. In one type of test the observer judges the given colour of tachyzoites under a microscope, such as with the dye test (DT) and IFA test. Another depends on the principle of agglutination of Toxoplasma tachyzoites, red blood cells or latex particles, as with the direct agglutination test (DAT) and indirect haemagglutination test (IHA) and latex agglutination (LA) test, respectively. With the ELISA, the degree of colour change defines the quantity of specific antibody in a given solution. The DT, IFA test, DAT and ELISA are outlined below and the IFA test is given in more detail.

The DT (29) is the so-called ‘gold standard’ serological test for Toxoplasma antibody in humans. Live Toxoplasma tachyzoites are incubated with a complement-like accessory factor and the test serum at 37°C for 1 hour before methylene blue is added. Specific antibody induces membrane permeability in the parasite so that the cytoplasm is able to leak out and the tachyzoite does not incorporate the dye and so appears colourless. Tachyzoites not exposed to specific antibody (i.e. a negative serum sample) take up the dye and appear blue. The DT is both specific and sensitive in humans, but may be unreliable in other species. In addition, it is potentially hazardous as live parasite is used. It is expensive and requires a high degree of technical expertise. It should be noted that on animal welfare grounds, tachyzoites should be grown in tissue culture rather than in mouse peritoneum whenever possible.

The IFA test (26) is a simple and widely used method. Whole, killed Toxoplasma tachyzoites are incubated with diluted test serum, the appropriate fluorescent antispecies serum is added, and the result is then viewed with a fluorescence microscope. Fluorescent-labelled antibodies are available commercially for a variety of animal species, the method is relatively inexpensive and kits are commercially available. However, the method requires a fluorescence microscope and the results are read by eye, so individual variation may occur. It may be difficult to find some species-specific conjugates and there is a risk of possible cross-reactivity with rheumatoid factor and anti-nuclear antibodies.

The DAT (9) is both sensitive and specific. Formalinised Toxoplasma tachyzoites are added to U-shaped well microtitre plates and dilutions of test sera are then applied. Positive samples will produce agglutination that can be graded, while negative samples will produce a ‘button’ of precipitated tachyzoites at the bottom of the well. The test is simple and easy to perform although relatively large amounts of antigen are required. Kits are commercially available. The method of growth and harvesting of parasites is given below. A commercially available latex agglutination test (LAT) is also available. The DAT and LAT are not species specific and are suitable for use in all species.

The original ELISA (35) uses a soluble antigen preparation made from Toxoplasma RH strain tachyzoites (as described below) and layered into wells in a microtitre plate. Test sera (e.g. ovine in origin) are added, followed by an anti-species enzyme-labelled conjugate such as horseradish peroxidase-labelled anti-ovine-IgG. Any attached conjugate causes a colour change in the substrate that is directly related to the amount of bound antibody, and which can be read with a spectrophotometer at the absorbance specific to the substrate used. The assay is simple, can readily test a large number of samples and is easy to perform with the chosen anti-species conjugate. Defined anti-species conjugates, substrates and whole kits are commercially available. However, the assay does require a spectrophotometer. The ELISA is well suited to laboratories required to analyse large numbers of samples.
Recently, a kinetics ELISA (KELA) has been developed (37). The KELA system measures the rate of reaction between bound enzyme and the substrate solution that leads to development of colour. Three optical densities (OD) are read at 45-second intervals (using the KELA data management program) and the results are reported in terms of slopes. The correlation between the ELISA and the KELA is very high, and therefore, the two tests are very good diagnostic tools, differing only in their convenience of application.

To improve the specificity of the conventional ELISA, assays that use recombinant antigens (19) and affinity purified *Toxoplasma*-specific antigens (22) have been developed for use in sheep (30, 33) but these tests are not yet routinely used.

With the conventional ELISA the detection of *Toxoplasma*-specific IgG and IgM antibodies allows a degree of discrimination between acute and chronic toxoplasmosis. More recently avidity assays have been developed. As the immune response matures, after infection is established, so antibodies of increasing avidity (functional affinity) for the antigen develop. This avidity can be measured and used to indicate active or recent *T. gondii* infection. An assay for the detection of avidity of IgG for the P30 antigen of *T. gondii* in sheep has been developed (30). This test is a good diagnostic tool for discriminating relatively recent from more established infections.

### Preparation of antisera and antigens

Antisera to *T. gondii* and conjugated antisera for use in the IFA test and ELISA, to allow screening of a variety of animal species, may be obtained commercially. International standards for animal sera are not available.

Below are protocols for the preparation of tachyzoite antigen for use in the IFA test and ELISA. Tachyzoites may be grown in mice or in tissue culture and retained as whole parasites for the IFA test, or prepared as soluble antigen for the ELISA.

#### Production of *Toxoplasma* tachyzoites in mice

i) Inject each of six *Toxoplasma*-free mice intraperitoneally using a 1-ml syringe and a 23-gauge needle, with 0.2 ml of 1 × 10⁷/ml *T. gondii* tachyzoites of the RH strain, collected fresh from a previous mouse passage or from tissue culture. (For optimum recovery of tachyzoites having minimal host mononuclear cells, mice should be more than 6–8 weeks of age and weigh approximately 22–25 g.)

ii) Kill the mice 3 days later by CO₂ inhalation (avoid cervical dislocation as this may cause contamination of peritoneal fluid with red blood cells).

iii) Pin the mouse out on its back on a clean cork mat. Reflect the abdominal skin with aseptic precautions, remove any peritoneal fluid by means of a 21-gauge needle attached to a 1 ml syringe and gently eject the harvested exudate into an equal volume of PBS.

The optimum time to collect tachyzoites is 72 hours after initial inoculation, when there will be sufficient organisms but before there is significant contamination by host cells. It is also important not to delay harvesting peritoneal fluid much past 3 days as the mice will die. (If tachyzoites for mouse inoculation are taken from a frozen stabilate, it may be necessary to harvest mice 4 or 5 days after the initial inoculation and passage the parasite once more through mice before using it as an antigen in the above tests.)

iv) Centrifuge the fluid at 500 g for 5 minutes, aspirate supernatant and resuspend in Hanks’ balanced salt solution (HBSS). Alternate between PBS and HBSS washes by centrifugation.

v) Calculate the concentration of tachyzoites and contaminating host cells with an improved Neubauer counting chamber (Count numbers of tachyzoites at 1/1000 dilution and cellular contamination at 1/10).

vi) Carry out further washes (step iv above) as required to reduce cellular contamination to <0.5% host mononuclear cells and <0.25% for red blood cells.

vii) Resuspend the tachyzoites in PBS to give a final concentration of 1 × 10⁷/ml.

viii) Tachyzoites may be maintained by continual passage in this manner without the addition of penicillin/streptomycin by observing strict aseptic procedures.

#### Preparation of aliquots of a frozen stabilate of *T. gondii* tachyzoites

i) Centrifuge tachyzoites in mice or tissue culture as described.

ii) Centrifuge at 500 g for 5 minutes and resuspend in Iscove’s modified Dulbecco’s medium (IMDM) (Gibco BRL, Paisley, UK) approximately three times.
iii) Add the following solutions to give these concentrations: 10% dimethyl sulphoxide; 20% normal horse serum (free from antibody to T. gondii); 70% resuspended tachyzoites to give a final concentration of 1 × 10^8 tachyzoites/ml.

iv) Allow the preparation to stand on the bench for 1 hour.

v) Dispense into 1-ml aliquots using screw-topped tubes appropriate for liquid nitrogen storage.

vi) Put the tubes into a small container, wrap in thick insulating material and place in –70°C freezer to allow the tachyzoites to freeze gradually.

vii) The next day transfer to liquid nitrogen, keeping well insulated while transferring.

viii) This stabilate may then be used for mouse inoculation or tissue culture growth of the parasite. When removing from storage thaw the sample rapidly in warm water.

**Production of Toxoplasma tachyzoites in tissue culture**

i) Toxoplasma gondii can be grown and maintained in tissue culture by twice-weekly passage in African green monkey kidney (Vero) cells.

ii) Cells and parasite are grown in IMDM supplemented with 50 IU/ml penicillin, 50 µg/ml streptomycin and 2% fetal bovine serum.

iii) Tachyzoites are harvested from tissue culture flasks by scraping the cell monolayer using a sterile cell scraper.

iv) Using 25 cm² vented tissue culture flasks that have each been seeded with 1 × 10^5 Vero cells, add tachyzoites at the rate of two tachyzoites per monolayer cell and incubate at 37°C in a 5% CO₂ humidified chamber. Harvest after 3–4 days.

**Preparation of whole tachyzoites for use in the IFA test**

i) Produce 4 × 10^7/ml suspension of RH strain T. gondii tachyzoites in PBS.

ii) Add formaldehyde (40%) to give a final concentration of 0.2% (v/v).

iii) Incubate at 4°C overnight and divide into aliquots in suitable sealed tubes and store frozen until required.

**Production of soluble antigen for ELISA**

i) Produce a suspension of RH strain T. gondii tachyzoites in PBS.

ii) Centrifuge at 2000 g for 15 minutes, retain the pellet and resuspend it in nine times its volume of distilled water.

iii) Rupture the tachyzoites by freezing and thawing three times.

iv) The antigen preparation is then sonicated for 20 seconds at 4°C at an amplitude of 20 microns.

v) Remove any cellular debris by centrifugation at 10,000 g for 30 minutes at 4°C.

vi) Retain the supernatant and store at –20°C until required. (Protein estimation might be expected to give a value of between 2 and 4 µg/ml.)

**Protocol for the IFA test**

The following is a protocol for carrying out an IFA test for anti-Toxoplasma IgG antibodies in sheep serum. It only requires minor modifications for testing different species or for measuring IgM antibody.

i) Clean the required number of tissue culture 15-well multitest slides (Flow laboratories) and allow to dry.

ii) Layer 5 µl of a whole tachyzoite preparation on to each well and allow to dry.

iii) Fix in methanol for 10 minutes.

iv) Wash twice for 10 minutes each time in 0.3 M PBS, pH 7.4.

v) Add 5 µl of the given test sheep serum (diluted in PBS) to each well. (Prepare serial dilutions of the test sera, e.g. 1/16, 1/32, etc. up to 1/1024.) Ensure that positive and negative control sera are included in each test as well as a ‘PBS-only’ sample. Incubate for 30 minutes at room temperature.
vi) Wash twice for 10 minutes each time in PBS.

vii) Add 5 µl of an appropriate dilution of rabbit-anti-sheep IgG conjugated to fluorescein isothiocyanate, diluted in 0.2% filtered Evan's blue dye in PBS, to each well and incubate for 30 minutes at room temperature.

viii) Wash three times for 10 minutes each time in PBS.

ix) Mount the slides under cover-slips with buffered glycerol (nine parts PBS one part glycerol) or Citifluor (Citifluor Ltd, London).

x) Examine using a fluorescence microscope, fitted with ×20 and ×40 objective lenses.

With a negative test serum result the whole parasites will appear red due to the autofluorescence of the Evan's blue dye. They may also present with a green fluorescent cap at the parasite pole (nonspecific polar fluorescence). With a positive test serum the parasites will fluoresce red and at least 80% of them within a given well will be surrounded by an unbroken band of green fluorescence. In an adult sheep/goat a positive titre could be defined as ≥1/64 and a negative titre as ≤1/32. For lamb/kid and fetal sera, respective titres could be defined as ≥1/32 and ≤1/16.

An example slide set-up is shown below:

```
Sample 1
1/16 → 1/32 → 1/64 → 1/128 → 1/256

1/512 → 1/1024 PBS only → 1/1024 ← 1/512
↑

1/1256 ← 1/128 ← 1/64 ← 1/32 ← 1/16
```

Sample 2

C. REQUIREMENTS FOR VACCINES AND DIAGNOSTIC BIOLOGICALS

The only available vaccine is a commercially produced live preparation for sheep (Toxovax, Intervet BV, The Netherlands; Toxovax, AgVax, Ag Research, New Zealand), currently licensed for use in the UK, Ireland, France, Portugal and Spain and New Zealand. It consists of tissue culture grown S48 T. gondii tachyzoites attenuated by over 3000 passages in mice. The vaccine stimulates effective protective immunity for at least 18 months following a single subcutaneous injection, but as it is unable to produce tissue cysts, sheep are not left with a persistent vaccinal infection. The vaccine has a short shelf life and is a potential risk to immunosuppressed and pregnant female operatives.

The vaccine should be stored and used strictly according to the manufacturer's instruction, thus it should not be frozen at any time, should be maintained cool (around 4°C) and not exposed to direct sunlight. The diluent provided should be added to the concentrated suspension of tachyzoites immediately prior to use.

REFERENCES


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CHAPTER 2.9.11.

VEROCYTOTOXIGENIC ESCHERICHIA COLI

SUMMARY

Escherichia coli are normal inhabitants of the gastrointestinal tract of animals and humans. Some strains have become highly adapted to cause diarrhoea and a range of extra-intestinal diseases. Since 1977, it has been recognised that some diarrhoeagenic strains of E. coli produce toxins that have an irreversible cytopathic effect on cultured Vero cells. Such verocytotoxigenic E. coli (VTEC) belong to over 100 different serotypes. Escherichia coli O157:H7 is the predominant and most virulent serotype in a pathogenic subset of VTEC, designated enterohaemorrhagic E. coli (EHEC). This designation is based on their capacity to cause haemorrhagic colitis and haemolytic uraemic syndrome in humans, their ability to produce verocytotoxins, their ability to cause attaching and effacing lesions on epithelial cells, and their possession of a characteristic large plasmid. In the past two decades, VTEC O157:H7 has risen in importance world-wide as a public health problem. Other non-O157 serogroups, including O26, O91, O103, O104, O111, O113, O117, O118, O121, O128 and O145, have been associated with occasional outbreaks of human disease, and others may be associated with sporadic cases. Ruminants represent the main natural host of VTEC and are generally healthy carriers of the organisms. VTEC have also been isolated from pigs, cats, dogs, chickens and wild birds. Cattle are considered to be the main reservoir of E. coli O157:H7 infection for humans. Despite its pathogenicity for humans, infection in animals with E. coli O157:H7 is invariably asymptomatic. By contrast, the EHEC serogroups, O26, O111 and O103 may be pathogenic for both humans and animals. The presence of VTEC in animal faeces provides the potential for these organisms to enter the food chain by faecal contamination of milk products, contamination of meat with intestinal contents during the slaughter process or contamination of fruit and vegetables by contact with infected manure. VTEC are also transmitted through contaminated water and by direct contact with infected people or animals.

Identification of the agent: Diagnostic procedures for VTEC have been developed, primarily for E. coli O157:H7, and seek to overcome the problems of isolating low numbers of target organisms from complex matrices such as animal faeces, food and clinical specimens. Identification of E. coli O157:H7 in subclinical animal carriers depends on enrichment of faeces samples in liquid media, usually buffered peptone water with or without the addition of vancomycin, cefsulodin and cefixime, for 6 hours at 37°C followed by immunomagnetic separation using commercially available paramagnetic particles or beads coated with anti-O157 lipopolysaccharide antibody. Beads with bound bacteria are plated on to selective agar, commonly 1% sorbitol MacConkey agar containing cefixime and potassium tellurite, and incubated for 18 hours at 37°C. Non-sorbitol-fermenting colonies are confirmed biochemically as E. coli and by serum or latex agglutination as possessing the O157 somatic antigen and/or H7 flagellar antigen. Potential virulence for humans is confirmed by the demonstration of verocytotoxin production by Vero cell assay, enzyme-linked immunosorbent assay (ELISA) or agglutination tests or the demonstration of genes encoding verocytotoxin by polymerase chain reaction. Detection of non-O157 VTEC relies on direct analysis of colonies on nonselective plates by, for example, immunoblotting or DNA probing for verocytotoxin production. Numerous immunological and nucleic acid-based recognition tests have been described to provide a more rapid presumptive diagnosis of VTEC and many are available commercially. Phage typing and pulsed field gel electrophoresis are widely used by reference laboratories for subtyping VTEC O157 for epidemiological purposes.

Serological tests: Serological tests are not used routinely in animals to diagnose VTEC infection, but it has been shown that cattle infected with VTEC produce serum antibodies to the O157 lipopolysaccharide that can be detected by ELISA.
Requirements for vaccines and diagnostic biologicals: No vaccines are currently available for controlling VTEC infections in animals or humans, but a variety of experimental vaccines are being developed.

A. INTRODUCTION

Escherichia coli are normal inhabitants of the gastrointestinal tract of animals and humans of which only some strains have become highly adapted to cause diarrhoea and a range of extra-intestinal diseases. Escherichia coli are routinely characterised by serological identification of somatic O, flagellar H and capsular K antigens. However, while some serotypes correlate closely with certain clinical syndromes, differentiation of pathogenic strains from the normal flora depends on the identification of virulence characteristics. Since 1977, it has been recognised that some diarrhoeagenic strains of E. coli produce toxins that have an irreversible cytopathic effect on cultured Vero cells (19). Such verocytotoxigenic E. coli (VTEC) have been shown to belong to over 100 different serotypes (16, 33). They are also described as Shiga toxin-producing E. coli (STEC) due to the similarity demonstrated between verocytotoxins (VT) and Shiga toxins (Stx) of Shigella dysenteriae (28). In the past two decades, VTEC O157:H7 has increased in importance world-wide as a public health problem. Escherichia coli O157:H7 is the predominant and most virulent serotype in a pathogenic subset of VTEC, designated enterohemorrhagic E. coli (EHEC). This designation is based on their capacity to cause haemorrhagic colitis and haemolytic uremic syndrome in humans, their ability to produce VT, their ability to cause attaching and effacing lesions on epithelial cells, and their possession of a characteristic large plasmid (27). Other non-O157 serotypes, including O26:H11, O104:H21, O111:H– and O145:H–, have been associated with occasional outbreaks of human disease, and others still with sporadic cases (16).

Ruminants represent the main natural host of VTEC and are generally healthy carriers of the organisms. VTEC have also been isolated from pigs, cats, dogs, chickens and wild birds; these species can be transiently colonised by the organisms (4, 16). Surveys have shown that O157 strains normally represent a minority of the VTECs that colonise the intestinal tract of animals. The presence of VTEC in animal faeces provides the potential for these organisms to enter the food chain via faecal contamination of milk, contamination of meat with intestinal contents during slaughter or contamination of fruit and vegetables by contact with contaminated manure. VTEC are also transmitted through water and by direct contact with infected people, animals or animal waste. Contaminated water, used for irrigating or for washing vegetables, can also be source of infection for humans or animals. Cattle are considered to be the main reservoir of E. coli O157:H7 infection for humans, although the organism has been isolated from a variety of farmed animals, horses, dogs, rabbits, birds and flies. Despite its ability to cause severe disease in humans (29), infection in animals with E. coli O157:H7 is invariably subclinical. Some non-O157 serotypes, however, are pathogenic for animals and humans and include O26:H11; O103:H2; O111:H– and O145:H– (3, 16).

VTEC are also associated with oedema disease in piglets with four serotypes responsible for the majority of outbreaks world-wide, namely O45:K+, O138:K81, O139:K82 and O141:K–. The main virulence factors are a fimbrial adhesin, F18, involved in colonisation and the VT2e toxin, which is responsible for clinical signs. A high degree of genetic relatedness between O101 strains harbouring stx2e genes of human and porcine origin has been demonstrated. The role of pigs as subclinical carriers of STEC in the epidemiology of human disease needs further research.

Because E. coli O157:H7 has become the predominant zoonotic VTEC, diagnostic methods have been developed to detect selectively this serotype in human clinical cases (33) and in food sources (34). For the latter, a validated International Standard detection method is available (EN ISO 16654:2001). In this chapter, however, emphasis will be given to the isolation and identification of O157 and other VTEC from carrier animals (11).

B. DIAGNOSTIC TECHNIQUES

1. Identification of the agent

   • Samples

In most cases, samples taken from animals for VTEC isolation will be faeces collected for surveillance purposes or as part of an epidemiological trace-back exercise following an outbreak of disease in humans. Samples may be taken from the rectum or from freshly voided faeces on the farm or from intestinal contents after slaughter. A variety of VTEC are present in healthy animals and not all are thought to be pathogenic for humans. Escherichia coli O157:H7, which is the most significant VTEC in human disease, is carried subclinically in animals. Cattle are thought to be the most important reservoir of this serotype. In an infected herd, only a proportion of the animals will be detectably infected, the organism is usually present in carriers in low numbers and is shed intermittently in
faeces. Shedding is influenced by the age of the animals, diet, stress, population density, geographical location and season (25). Some animals are thought to contribute disproportionately to transmission of infection and have been termed “super-shedders” (24). Isolation rates may be improved by taking faeces samples in preference to rectal swabs, by increasing the sample size, by increasing the number of individuals sampled and by repeat sampling. Use of recto-anal mucosal swabs is reported to improve detection of colonised as distinct from transiently infected cattle (30). Precautions should be taken to avoid cross-contamination of samples in transit and at the laboratory. Samples should be kept cool and cultured as soon as possible after collection.

- **Safety**

Care should be exercised when handling VTEC-positive samples as the infective dose capable of causing severe human infection may be low (possibly 100 organisms for VTEC O157:H7) and laboratory-acquired infections have been reported (see Chapter 1.1.2 Biosafety and biosecurity in the veterinary microbiology laboratory and animal facilities).

- **Isolation**

**a) Liquid enrichment media**

Clinical samples are routinely plated directly on to solid media for isolation of _E. coli_, but the number of target VTEC organisms in faeces from healthy carriers is usually low and enrichment in liquid media improves recovery. Commonly used enrichment media are buffered peptone water either unsupplemented (which gives good recovery) or supplemented with 8 mg/litre vancomycin, 10 mg/litre cefsulodin and 0.05 mg/litre cefixime (BPW-VCC) to suppress the growth of Gram-positive organisms, _Aeromonas_ spp. and _Proteus_ spp.; modified trypticase–soy broth (mTSB) supplemented with 20 mg/litre novobiocin or 10 mg/litre acriflavin to reduce the growth of Gram-positive organisms; or modified _E. coli_ broth with 20 mg/litre novobiocin (mEC+n). EHEC _E. coli_ grow poorly at 44°C. The optimal incubation for bovine faeces to minimise overgrowth by other organisms is 6 hours at 37°C. For meat samples, enrichment for 6 hours at 41–42°C is used and for water and dairy products, 24 hours at 41–42°C. Nonselective pre-enrichment is necessary for the effective recovery of low levels of stressed _E. coli_ O157. Enrichment broths should be pre-warmed to prevent cold-shocking the organisms and slowing their initial growth; 24 hours’ incubation may increase recovery if the organisms are stressed.

**b) Immunomagnetic separation**

Immunomagnetic separation (IMS) has been used as a selective concentration technique to improve isolation of _E. coli_ O157:H7 where numbers of the organism are low (10). Commercially available paramagnetic particles or beads coated with anti- _lipopolysaccharide_ (LPS) antibody are mixed with an aliquot of incubated broth. Beads with bound bacteria are separated from the supernatant by a magnetic field and after washing are plated on to selective agar and incubated for 18 hours at 37°C to isolate suspect colonies. The technique is serogroup specific. Commercial systems are available for manual or automated separation (9). Recovery may be affected by the bead-to-organism ratio (optimum is 3:1), the enrichment broth used and the problem of nonspecific adsorption of _E. coli_ to the magnetic beads (which can be reduced by the use of a low ionic strength solution in the IMS procedure and careful washing). These factors should be taken into account when trying to maximise the sensitivity of the technique for detecting target _E. coli_.

**c) Selective culture for _Escherichia coli_ O157**

There are no biochemical characteristics that distinguish the majority of VTEC from other _E. coli_, however, the inability of most strains of _E. coli_ O157:H7 to ferment D-sorbitol rapidly and their lack of beta-glucuronidase activity can be exploited in the isolation and identification of these organisms. However, the less common sorbitol fermenting and beta-glucuronidase positive _E. coli_ O157:H– variants (nonmotile due to lack of expression of the H7 antigen), will not be identified by isolation in such selective media chosen for these biochemical characteristics (18). MacConkey agar containing 1% D-sorbitol instead of lactose (SMAC) is a useful and inexpensive medium on which non-sorbitol-fermenting _E. coli_ grow as small, round greyish-white colonies. Selectivity is improved by the addition of 0.5% rhamnose, and addition of 0.05 mg/litre cefixime (CR-SMAC) inhibits overgrowth by _Proteus_ spp. While fewer presumptive colonies require testing on this medium, rhamnose is an expensive supplement. An alternative modification is the addition of 2.5 mg/litre potassium tellurite in addition to cefixime (CT-SMAC), which has a greater inhibitory effect against non-O157 _E. coli_ and other non-sorbitol fermenters, such as _Aeromonas_, _Plesiomonas_, _Morganella_ and _Providencia_, than against _E. coli_ O157 (28). This is currently the most commonly used medium for isolating _E. coli_ O157.

Media containing fluorogenic or chromogenic glucuronides are used to distinguish non-beta-glucuronidase-producing _E. coli_ O157:H7 from beta-glucuronidase-producing _E. coli_. Hydrolysis of 4-methylumbelliferyl-beta-D-glucuronide (MUG) by beta-glucuronidase activity produces a fluorescent compound visible under UV light. The addition of 0.1 g/litre 5-bromo-4-chloro-3-indoxyl-beta-D-glucuronide (BCIG) to SMAC
differentiates white colonies of *E. coli* O157:H7 from green-blue colonies of sorbitol negative, beta-glucuronidase positive organisms. Commercially available chromogenic and fluorogenic media may be found by reference to media catalogues. While advances have been made in improving the selectivity of media for *E. coli* O157:H7, isolation rates, particularly of stressed organisms, may be adversely affected by the additives used. To mitigate against these effects, addition of recovery agents such as 1% sodium pyruvate to tryptone–soy agar or delaying exposure of stressed cells to selective agents can aid recovery of the organism (8).

Sorbitol-fermenting (SF) *E. coli* O157:H– have been isolated from patients with diarrhoea and HUS but the epidemiology of this infection is poorly understood and only rarely has the organism been isolated from animals, including cattle (22). The majority of SF *E. coli* O157:H– isolates are susceptible to tellurite and cannot be identified on CT-SMAC. Microbiological analysis for this organism is laborious and entails plating IMS-separated organisms onto SMAC and testing individual SF colonies by latex agglutination for the O157 antigen. Alternatively, colony sweeps are tested by polymerase chain reaction (PCR) for the presence of vt2, *eae*, *rfb*O157 and *sfpA* (see below). Well-spaced colonies from growth positive by PCR are then tested by colony hybridisation with probes for vt2, *eae* and *sfpA* or colony immunoblot using specific antibody (18, 22).

d) **Isolation of other VTEC**

Non-O157 VTEC grow well on media that permit the growth of *E. coli*, such as blood agar or MacConkey agar, and the majority can only be differentiated from other *E. coli* by their ability to produce VT. The large number of different VTEC serotypes precludes the use of O-antisera for the routine screening and presumptive identification of colonies on these media. IMS can be used for selective concentration of serogroups O26, O103, O111 and O145 from a pre-enriched sample, as for the O157 strains. These serogroups are the non-O157 VTECs most commonly associated with human disease, and commercially produced beads are currently available.

The inability of O26 strains to ferment rhamnose has led to the recent development of media that may prove to be useful in differentiating O26 *E. coli* from other enteric organisms. The first is rhamnose-MacConkey agar (RMAC) in which the lactose in the MacConkey medium is replaced by 10 g/litre rhamnose. Addition of 2.5 mg/litre potassium tellurite and 0.05 mg/litre cefixime (CT-RMAC) is said to improve specificity. The second is a chromogenic rhamnose agar incorporating 10 g/litre rhamnose and 0.02 g/litre phenol red in ES coliiform agar (an indicator medium for beta-galactosidase activity) to which is added 0.5 mg/litre potassium tellurite and 0.05 mg/litre cefixime. On this medium, O26 colonies are reported to be dark blue to black, other *E. coli* serotypes are green, and enterobacteria other than *E. coli* are green, yellow or colourless.

One potentially useful virulence marker for VTEC is enterohaemolsin production, which causes haemolysis of washed sheep erythrocytes after overnight incubation on blood agar supplemented with calcium. This characteristic is shared by 90% of VT-producing *E. coli* isolated from human infections. However, the finding that a proportion of disease-producing VTEC can be negative for enterohaemolysin production reduces the value of enterohaemolysin agar as a screen.

In most cases, therefore, isolation of VTEC relies on direct analysis of colonies on plates by immunoblotting or DNA probing for VT production to identify colonies for further characterisation. Colonies are first replicated so that positive colonies can be isolated after replicates have been tested. Colonies may be blotted on to suitable membranes (nitrocellulose or nylon) from which replicates are made or picked off into 96-well microtitre plates containing broth for replication before transferring aliquots to appropriate filters. Colonies are then analysed using nucleic acid probes or antibodies to identify any VTEC (33). Hull *et al.* (14) developed a mitomycin immunoblot assay for detecting VTEC in faeces that was simple enough to use in routine diagnostic laboratories. Serial dilutions of faeces in broth are inoculated on to MacConkey agar plates and incubated overnight at 37°C. Using standard replica plating techniques, growth from the plate yielding approximately 200 colonies is transferred to two 0.45-µm pore-size nitrocellulose filters laid on Syncase agar with 25 ng mitomycin/ml. This medium induces vegetative growth of bacteriophages carrying the genes for VT and enhances toxin expression. (Alternatively, bacterial or faecal suspensions may be plated directly on to the filters.) The plates are incubated overnight at 37°C. After overnight growth, filters are removed from the plates, immersed in a chloroform bath for 15 minutes, then blocked for 1 hour with 5% non-fat milk in 10 mM Tris, 150 mM NaCl, 0.05% Tween (pH 8) (TNT). The filters are incubated for 1 hour in antisera raised against VT1 or VT2, given three 5-minute washes in TNT, then incubated for 1 hour with alkaline phosphatase-conjugated anti-immunoglobulin G followed by three further 5-minute washes in TNT. Any reaction is visualised by colour development with nitroblue and 5-bromo-4-chloro-3-indolyl-phosphate. VT1, VT2 and VT-negative control *E. coli* are tested in parallel. The use of polyclonal antibodies results in some false positives that are eliminated by using monoclonal antibodies. When the use of DNA probes was compared with the use of the mitomycin immunoblot colony assay, it was shown that the results were comparable. The immunoblot assay has the advantage of being simpler to perform than DNA probing. Mitomycin plates have a long shelf life when stored in the dark at 4°C.
Colony immunoblotting or probing are labour intensive techniques and may be better applied to samples that have been screened and shown to be positive for the presence of VT or VT genes by, for example, enzyme-linked immunosorbent assay (ELISA) or PCR.

- **Identification and characterisation of suspect colonies**

Colonies growing on solid media that are suspected to be VTEC must be confirmed biochemically or genotypically (e.g. by GadA PCR) to be *E. coli*. Somatic O’ and flagellar ‘H’ antigens are identified serologically. Not all VTEC isolated from animals are thought to be pathogenic for people. Some isolates of *E. coli O157*, particularly from pigs, are non-verocytotoxigenic and non-pathogenic for humans. Diagnosis, therefore, must include the demonstration of known virulence factors in the isolates. These include the verocytotoxins VT1 (Stx1) and VT2 (Stx2) and their genes and an outer membrane adhesion protein associated with attaching and effacing lesions, intimin, which is encoded by the *eae* gene (21). For VTEC O157 strains, subtyping methods are available in reference laboratories for epidemiological investigations.

a) **Biochemical tests**

VTEC are biochemically similar to other *E. coli*. VTEC O157:H7 strains differ in failing to ferment sorbitol, failing to produce beta-glucuronidase and fermenting raffinose and dulcitol. *Escherichia coli* can be distinguished from *E. hermanii* by lack of growth in the presence of potassium cyanide and failure to ferment cellobiose. *Escherichia hermanii* is positive for both tests. Ninety-eight per cent of *E. hermanii* strains have a characteristic yellow pigment on nutrient agar that is not seen in VTEC. *Escherichia coli* may be confirmed by demonstration of the use of tryptophan and beta-galactosidase activity (see below) or by commercially available biochemical test strips.

b) **Serological tests**

Commercial latex kits are available for O157, O26, O91, O103, O111, O128, O145 and H7. Tests should be carried out according to the manufacturer’s instructions and should incorporate positive and negative control organisms and control latex. Presumptive diagnosis may also be made using slide or tube agglutination tests with anti-O LPS antiserum (antisera to 181 O- antigens are available). O157 antiserum has been shown to cross-react with other organisms including *E. hermanii* (frequently found in foods), *Salmonella* O group N, *Yersinia enterocolitica* serotype O9 and *Citrobacter freundii*, indicating the need to confirm putative VTEC colonies as *E. coli*. Isolates can be tested for the presence of flagellar antigen (antisera have been raised to 56 H antigens), but this may require passage through motility medium. Some pathogens are nonmotile.

c) **Verocytotoxin production in Vero cell assay (16)**

The Vero cell assay remains a standard method for the confirmation of VT production (see below). Vero cells have a high concentration of globotriaosylceramide (Gb₃) and globotetraosylceramide (Gb₄) toxin-binding receptors in their plasma membranes and will normally detect all variants of VT. The test can be used on faecal suspensions, culture filtrates or live cultures. In mixed faecal cultures, the sensitivity of the assay is increased by treating the suspension with polymyxin B or mitomycin to release cell-associated toxin. While the test is sensitive, it is not available in most routine diagnostic laboratories. It is labour intensive and results can take 3–4 days after the cell culture is inoculated. Where tissue culture facilities are not available, other methods may be used for detecting VT production, including ELISA or agglutination and PCR can detect the vt genes. All of these methods are now available as commercial kits.

d) **Subtyping of *Escherichia coli* O157 for epidemiological studies**

A variety of methods is available in reference laboratories to help discriminate between strains of *E. coli* O157:H7 to aid epidemiological investigations of outbreaks of human disease (13, 33). These methods vary in technical complexity and more than one technique is required to provide useful differentiation. Techniques include phage typing, biotyping and antimicrobial sensitivity testing (resistance being uncommon in strains from most countries), plasmid profiling, restriction fragment length polymorphism analysis, ribotyping, pulsed field gel electrophoresis (PFGE) and various PCR-based analyses (random amplification of polymorphic DNA; repetitive DNA element PCR; amplified fragment length polymorphism analysis). Of these, only phage typing and PFGE are widely used. Despite some difficulties with interpretation of profiles, PFGE has emerged as the standard method used by public health reference laboratories for subtyping VTEC O157 due to its high level of discrimination and accuracy and reproducibility. It is used in ‘Pulsenet’, a network of public health laboratories performing a standardised PFGE method that allows comparison of fingerprints held on an electronic database by the Centres for Disease Control and Prevention in the USA (www.cdc.gov/ncidod/dbmd/pulsenet/pulsenet.htm). The European Union’s ‘Enter-net’ system for the surveillance of *Salmonella* and VTEC relies largely on phage typing to subtype *E. coli* O157:H7 strains. Use of subtyping of genes for intimin and VT has proved valuable for epidemiological studies and source attribution (5, 6). Subtyping methods for non-O157 serotypes have
been less well explored, however, similar molecular approaches to those used for VTEC O157 can be taken.

- **Non-culture techniques for detecting VTEC**

Although definitive diagnosis of VTEC relies on the isolation and characterisation of pure cultures, cultural methods for VTEC are time-consuming and labour intensive. This has led to the development of a range of immunological and nucleic acid hybridisation tests for rapid identification of O and H antigens, VT or genes associated with VT production in the sample. As the tests have a detection level above the numbers at which the target organism is normally present in the faeces, an enrichment step (preferably nonselective for isolation of injured or stressed bacteria) is required to increase the numbers prior to testing.

a) **Immunological methods**

Immunooassays to identify O and H antigens and VT may be used to confirm the identity of the organisms once isolated from clinical, food or environmental samples, while others, including dipstick and membrane technologies, microplate assays, colony immunoblotting, immunofluorescence and ELISA, are used as rapid methods for detecting the presence of potential pathogens in samples prior to isolation, thus shortening the time for a presumptive diagnosis. Most assays for somatic and flagellar antigens are designed to detect the O157 LPS and H7 flagellar antigen. Toxin assays have the advantage of detecting all VTEC. Enzyme immunoassays for O157 and VT, visual immunoassays for O157 and agglutination tests for O157, H7 and VT are available commercially as kits (7, 11, 27, 33). Not all have been validated for use with faeces. Specialised reagents in which anti-O157 LPS antibodies are conjugated to fluorescein, peroxidase or phosphatase are also available. Of the enzyme immunoassays, the most commonly used format is a sandwich assay. Antibody is bound to a carrier surface to capture a specific VTEC antigen; following the addition of an appropriate substrate, a second antibody with an enzyme label binds to this antigen and produces a colour reaction. The kits have been validated with specific pre-enrichment protocols and reagents to ensure reproducible results. Some use heat-treated samples thus improving the safety of the test, and some incorporate an automated processing system to screen large numbers of samples. Others are blot ELISAs developed to screen colonies for O157 antigen. The commercial kits have the advantage of being easy to perform in routine laboratories, and tests should be carried out according to the manufacturers' instructions. Kits validated for food and carcass samples or for human clinical samples may lack sensitivity for animal faeces samples. Immunological assays only give a presumptive result, which must be confirmed by isolation and characterisation of the organisms producing the O157 antigen or the toxin. The availability of kits is changing and the OIE Reference Laboratories should be able to provide the latest information on validated diagnostic kits.

b) **Nucleic acid recognition methods**

i) Colony hybridisation assays

Colony hybridisation is a useful means of detecting VTEC in mixed culture for further characterisation. DNA probes and synthetic oligonucleotide probes are available labelled with digoxigenin or biotin and therefore suitable for use in routine diagnostic laboratories. Assays have been described to detect VT genes, the 60 MDa plasmid in *E. coli* O157 and the *eae* gene individually and in combination (27, 29, 33). Hybridisation assays are less sensitive for detecting VTEC in broth cultures or faecal extracts.

ii) PCR for VT genes and other virulence markers

Many PCRs are described in the literature for detection of VT1, VT2 and VT2 variant genes (27, 29, 33), and a number of these toxin-typing PCR methods has recently been compared (36). Demonstration of the genes associated with VT-production does not confirm gene expression and hence production of toxin. PCR can be used on pure or mixed plate or broth cultures, and extracts from food or faeces. It can also be used to detect genes in non-viable organisms. As well as its role in diagnosis, PCR has the potential to be used to screen samples for VTEC in epidemiological studies. Amplification of target genes in bacterial DNA extracts from faeces is less successful than from pure cultures, and careful preparation of the sample is required to improve sensitivity. Faeces contain nonspecific PCR inhibitors and no single method of removing these is ideal. Sensitivity is improved by nonselective enrichment prior to testing, but remains lower than using IMS or the Vero cell cytotoxicity assay. Commercial assays are available.

DNA probes, PCR assays and microarrays have also been developed to detect other genes in VTEC shown to be associated with virulence in humans, including *eae* (encoding for intimin), *fliC* (encoding the H7 antigen), O157 *rfb* (encoding O157 LPS), *uidA* (the mutant glucuronidase gene in beta-glucuronidase-negative *E. coli* O157:H7) and *katP* (a gene carried on the large plasmid of *E. coli* O157:H7 encoding a novel catalase peroxidase) (2, 27, 29, 33). A variety of multiplex assays has been developed to detect simultaneously several diagnostic genes. These assays are of value in the characterisation of pure cultures. On mixed populations of bacteria in food or faeces samples, they may have a use in identifying samples to which isolation procedures should be targeted.
• Screening faeces for *Escherichia coli* O157:H7

*Escherichia coli* O157:H7 is the VTEC of greatest public health concern in most countries. Its carriage in the intestinal tract of healthy animals, particularly cattle, represents a source of direct and indirect infection to humans. Screening relies on cultural techniques designed to overcome the problems of isolating low numbers of organisms, possibly in a stressed state, from a competing background flora followed by identification of suspect colonies and demonstration of known virulence characteristics. These methods are still evolving and the following is a description of the methods routinely employed in one national veterinary laboratory. Suitable precautions should be taken to avoid human infection (see Chapter 1.1.2).

**a) Pre-enrichment**

i) Transport faeces in sterile, leak proof, closed containers at 4°C and culture as soon as possible, preferably within 2 hours of collection. Faeces intended for long-term storage should be frozen at −70°C.

ii) Mix faeces at a dilution of 1/10 in warmed buffered peptone water (BPW) in a labelled container.

iii) Incubate at 37°C±2°C for 6 hours.

iv) Include positive and negative control cultures.

**b) Immunomagnetic separation**

i) Use of Dynabeads® anti-*E. coli* O157 product 710.04 (Dynal Biotech, ASA, Oslo, Norway) meets the requirements of AFNOR (DYN 16/02-0696 and DIN 10167); it is cited in the USA Food and Drug Administration’s Bacteriological Analytical Manual (www.cfsan.fda.gov/~ebam/bam-4a.html) and the Health Canada Compendium of Analytical Methods (www.hc-sc.gc.ca/food-aliment/mh-dm/mhe-dm/compendium/volume_3/e_mflp9001.html) and is the official method of the Japanese Health Ministry.

ii) Following the instructions of the manufacturers, carry out immunomagnetic separation (IMS) on the pre-enriched samples using the manual (MIMS) or automated (AIMS) method. Care should be taken to mix the beads well before use and to avoid cross-contamination between prepared tubes. If using the manual method, adherence to the instructions for careful washing of the bead–bacteria complexes is essential.

iii) After the final wash, use a micropipette to transfer 50 µl of each bead–bacteria suspension to a labelled sorbitol MacConkey agar plate containing cefixime and potassium tellurite (CT-SMAC) (35) taking care to avoid cross-contamination.

iv) Using a sterile swab, spread the drop over one-third to one-half of the plate to break up the complexes. Using a sterile 10 µl loop, dilute the bead–bacteria complexes further over one quadrant by streaking out at right angles from the previously streaked area. Using a second sterile loop, streak out at right angles from this quadrant into the final un-streaked area of the plate to obtain single colonies. Incubate at 37°C±2°C for 16–18 hours (sorbitol-fermenting colonies lose colour after this time and may be confused with non-sorbitol fermenting *E. coli* O157). An alternative method for isolating sorbitol-negative colonies is to spread the entire inoculum over the surface of a dry CT-SMAC plate with a sterile bent rod.

**c) Colony identification**

i) Pick off up to 10 white, sorbitol-negative colonies per plate and test by O157 latex agglutination following the manufacturer's instructions (include appropriate positive and negative control organisms and latex control).

ii) Subculture agglutination-positive colonies on to solid medium without antibiotics (e.g. 5% sheep blood agar). Streak to obtain single colonies. Incubate at 37°C±2°C overnight.

**d) Confirmation of *Escherichia coli***

i) Inoculate o-nitrophenyl beta-D-galactopyranoside (ONPG) broth. Set up positive and negative controls. Incubate overnight, aerobically at 37°C. *Escherichia coli* produce a positive result indicated by a change to yellow colouration confirming beta-galactosidase activity.

ii) Place a circle of 0.45 µm cellulose nitrate membrane filter paper on to a plate of tryptone bile agar (TBA) using sterile forceps. Use a 1 µl loop to remove a loopful of growth to be tested and inoculate a pea-sized area on the surface of the Millipore filter. Set up positive and negative controls. Incubate at 44°C for at least 17 hours. Transfer the membrane to filter paper soaked with indole reagent for the detection of the use of tryptophan. *Escherichia coli* show a positive reaction indicated by a purple/pink colouration.
iii) A commercial reagent for detection of indole is available. The reagent is placed on to filter paper and a portion of the colony rubbed into the reagent spot. This requires less than 5 minutes and can be backed up by the described test if suspicious colonies appear negative. Indole medium is also available commercially.

iv) Alternatively, use commercially available biochemical test kits to confirm E. coli.

e) Somatic determination (24)

i) Using a sterile loop, pick off a single colony obtained by the colony-identification method described in point c above and subculture into 4 ml of Schlecht broth (31). Incubate at 37°C±2°C overnight.

ii) Boil the Schlecht broth for a minimum of 1 hour at 100°C.

iii) Dispense 25 µl of 0.85% saline into wells 2 to 12 of a U-well microtitre plate. Dispense 50 µl of O157 antiserum into well 1. Make a doubling-dilution series of the antiserum to 1/1024, discarding 25 µl after mixing well. Add 50 µl of boiled broth suspension to wells 1 to 12. Cover the plate to prevent evaporation and incubate at 37°C for 6 hours. Use a black background to identify agglutination in the wells.

f) Vero cell assay

i) Using a sterile loop, pick off a single colony obtained by the colony-identification method described in point c above and subculture into 4 ml of Mundell (26) broth. Incubate at 37°C±2°C overnight.

ii) Set up broths with control strains of organisms producing no toxin, thermolabile enterotoxin (LT), cytotox necrotising factor (CNF) and verocytotoxin (VT). Incubate at 37°C±2°C overnight.

iii) Dispense Vero cells (African green monkey kidney cells, reference ATCC CCL81, seeding rate 2 ×10^5/ml) into flat-well microtest plates, 200 µl to each well, 24 hours before inoculation. Incubate at 37°C±2°C in 5% CO_2 for 24 hours.

iv) Add 100 µl of a 400,000 units/ml solution of polymyxin B sulphate in sterile distilled water to each overnight broth culture. Incubate at 37°C±2°C for 5 hours.

vi) Centrifuge the broths at 3000 rpm for 30 minutes.

vii) Remove supernatants into labelled sterile containers (approximately 1.5 ml required).

viii) Place the Vero cell plate on a numbered worksheet to identify each well. Inoculate 10 µl of prepared supernatant into the relevant well of Vero cells. Return Vero cells to the CO_2 incubator and incubate for 3 days.

ix) Examine cells after 24 hours, 48 hours and 72 hours to observe any cytopathic effect. Compare with positive and negative test controls. With VT-positive samples, the cell sheet becomes disintegrated with blackened, shrivelled cells observed between 24 and 72 hours.

g) Multiplex PCR for VT1, VT2 and eae (1, 15, 32)

Multiplex PCR is used to confirm the presence of virulence determinants using primers as shown below:

<table>
<thead>
<tr>
<th>Target gene</th>
<th>Accession no.</th>
<th>Primer sequence</th>
<th>Nucleotide position</th>
<th>Amplicon size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>VT1</td>
<td>M19437</td>
<td>F (5'-CGC-TCT-GCA-ATA-GGT-ACT-CC-3')</td>
<td>287–306</td>
<td>256</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R (5'-CGC-TGT-TGT-ACC-TGG-AAA-GG-3')</td>
<td>522–541</td>
<td></td>
</tr>
<tr>
<td>VT2</td>
<td>X07865</td>
<td>F (5'-TCC-ATG-ACA-ACG-GAC-AGC-AG-3')</td>
<td>623–642</td>
<td>185</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R (5'-GC-TTG-TTC-TGG-GAC-AGG-AGG-3')</td>
<td>788–807</td>
<td></td>
</tr>
<tr>
<td>eaeA</td>
<td>X60439</td>
<td>F (5'-GC-TTA-GTG-CTG-CTG-AGT-CTG-AG-3')</td>
<td>271–293</td>
<td>618</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R (5'-CCA-GTG-AAC-TAC-CGT-CAA-AGA-3')</td>
<td>871–890</td>
<td></td>
</tr>
</tbody>
</table>

i) Using a sterile loop, pick off a single colony obtained by the colony-identification method described in point c above and subculture into 1 ml of Luria-Bertani broth. Set up three appropriate control broths. Incubate at 37°C±2°C overnight.

ii) Boil the broths for 15 minutes at 100°C. Remove from waterbath and allow to cool.
iii) Prepare master mix for 48 µl per sample containing:

- 1 × Saiki buffer (50 mM KCl; 10 mM Tris, pH 8.5; 100 µg/ml gelatin); 3 mM MgCl₂; 0.5 U Taq polymerase; 25 pmoles of each primer (forward and reverse primers for VT1, VT2 and eaeA); 0.2 mM each of dATP, dCTP, dGTP and dTTP.

iv) Mix by inverting tubes and dispense 48 µl into each PCR reaction tube.

v) Add 2 µl of boiled culture (crude DNA extract) to the bottom of each reaction tube (include three control extracts and a media blank).

vi) Run the PCR using cycling parameters of initial denaturation at 94°C for 2 minutes; 25 cycles of 94°C for 1 minute, 62°C for 1.5 minutes and 72°C for 2 minutes; with a final extension of 72°C for 5 minutes. The reaction is held at 4°C until required for electrophoresis.

vii) Electrophorese 15 µl of each PCR sample on a 1.5% agarose gel in E buffer (10× strength solution made by adding to distilled water in the following order: 109 g/litre Tris, 55.6 g/litre ortho-boric acid, 9.3 g EDTA, made up to 1 litre with distilled water and adjusted to pH 8.0 with 10 ml concentrated hydrochloric acid diluted in distilled water before use). Run 100 bp step ladder molecular weight marker for comparison.

ix) Stain in ethidium bromide and view by transillumination.

x) Inspect control lanes to identify positions of VT1, VT2 and eae amplicons. Compare with bands present in test sample lanes. Record the results.

2. Serological tests

In humans, serodiagnosis of VTEC can be valuable, particularly later in the course of the disease when the causative organism becomes increasingly difficult to isolate from faeces. LPS has emerged as the antigen of choice, and production of serum antibodies to the LPS of a wide range of prevalent serotypes of VTEC has been demonstrated. Serological tests are not used for diagnosis of animal infection with VTEC. However, it has been shown that exposure of cattle to E. coli O157:H7 infection results in the production of antibodies against the O157 LPS, which persist for months, demonstrable by the indirect ELISA (17). Cross-reactions have been demonstrated between O157-LPS and the LPS antigens of other bacteria including E. coli O55, Salmonella spp., Yersinia enterocolitica, Brucella abortus and V. cholerae non-O1 strains. To reduce cross-reactivity, a blocking ELISA using a monoclonal antibody specific for E. coli O157 as the competing antibody has been developed for detection of serum antibodies to O157 antigen in cattle (20). Serum antibodies to VT1, but not to VT2, have been demonstrated in cattle by toxin neutralisation tests in Vero cell assays (17). Other studies have shown greater prevalence of VT1 neutralising antibodies in cattle sera than VT2 which may be explained by the greater prevalence of VT1-producing VTEC in cattle and/or the lesser immunogenicity of VT2.

C. REQUIREMENTS FOR VACCINES AND DIAGNOSTIC BIOLOGICALS

There are currently no vaccines available to control zoonotic VTEC. Various approaches to the immunological control of EHEC infections in humans are being explored (23). These are aimed at preventing colonisation, intestinal disease or the serious sequelae of haemolytic uraemic syndrome and thrombotic thrombocytopenic purpura. They include the use of conjugate vaccines (e.g. O157 polysaccharide linked to the B-subunit of VT1 and VT2 as carrier proteins), live-vector vaccines, toxoid vaccine or passive immunisation with hyperimmune globulin or monoclonal antibodies against VT. However, were an effective vaccine to become available, there is debate about the social, political and economic consequences of widespread vaccination of people against pathogens in their food. As animals, mainly cattle, are thought to be the reservoirs of infection for the human population, a novel strategy being explored is to vaccinate cattle in order to reduce colonisation with pathogenic VTEC and thereby reduce contamination of food and the environment (i.e. to make food safer as opposed to protecting people against their food). One approach is to use a live, toxin-negative colonising strain as an oral vaccine to induce antibodies against surface components, and another is to deliver colonisation factors, such as intimin, as an edible vaccine in transgenic plants (12).

REFERENCES


* * *

NB: There is an OIE Reference Laboratory for *Escherichia coli* (see Table in Part 3 of this Terrestrial Manual or consult the OIE Web site for the most up-to-date list: www.oie.int).
CHAPTER 2.9.12.

ZOONOSES TRANSMISSIBLE FROM NON-HUMAN PRIMATES

For standards for testing non-human primates, please consult the following document:


http://www.lal.org.uk/pdffiles/LAfel5.pdf

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PART 3

OIE REFERENCE EXPERTS

AND DISEASE INDEX
LIST OF OIE REFERENCE LABORATORIES IN 2008

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1 For certain diseases, two institutions have been jointly designated as OIE Reference Laboratory or Collaborating Centre. Where this occurs, the superscript (1) or (2) will be placed after the name of the designated Reference Expert. Pour certaines maladies, 2 instituts ont été désignés conjointement comme laboratoire ou centre collaborateur de référence de l’OIE. Dans ce cas les mentions (1) et (2) sont placées après les noms des experts de référence désignés. Para algunas enfermedades, se designaron conjuntamente dos instituciones como Laboratorios de Referencia o Centros Colaboradores. En tales casos los Expertos de Referencia vienen señalados con los respectivos índices notescritas (1) y (2)
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Antimicrobial resistance – Résistance microbienne – Resistencia microbiana

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Aujeszky’s disease – Maladie d’Aujeszky – Enfermedad de Aujeszky

Dr Philippe Vannier
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Avian chlamydiosis – Chlamydiose aviaire – Clamidiosis aviar

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Jena, Naumburger Str. 96a, 07743 Jena
ALLEMAGNE GERMANY ALEMANIA
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Email: konrad.sachse@fli.bund.de

2 Subject to confirmation by the International Committee in May 2008.
3 To be advised – please refer to the OIE website
<table>
<thead>
<tr>
<th><strong>Avian mycoplasmosis – Mycoplasmose aviaire – Micoplasmosis aviar (Mycoplasma gallisepticum, M. synoviae)</strong></th>
</tr>
</thead>
</table>
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The University of Georgia, College of Veterinary Medicine, Department of Avian Medicine  
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<table>
<thead>
<tr>
<th><strong>Avian tuberculosis – Tuberculose aviaire – Tuberculosis aviar</strong></th>
</tr>
</thead>
</table>
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TCHEQUE (Rép)  CZECH (Rep.)  CHECA (Rep.)  
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Email: pavlik@vri.cz |

<table>
<thead>
<tr>
<th><strong>Bacterial kidney disease – Rénibactériose – Renibacteriosis (Renibacterium salmoninarum)</strong></th>
</tr>
</thead>
</table>
| Dr James R. Winton  
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<table>
<thead>
<tr>
<th><strong>Bee diseases – Maladies des abeilles – Enfermedades de las abejas</strong></th>
</tr>
</thead>
</table>
| Mr Jean-Paul Faucon  
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<table>
<thead>
<tr>
<th><strong>Bluetongue – Fièvre catarrhale du mouton – Lengua azul</strong></th>
</tr>
</thead>
</table>
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### Bovine babesiosis – Babésiose bovine – Babesiosis bovina

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### Bovine genital campylobacteriosis – Campylobactériose génitale bovine – Campilobacteriosis genital bovina

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### Bovine spongiform encephalopathy – Encéphalopathie spongiforme bovine – Encefalopatía espongiforme bovina

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**Prof. Andreas Zurbriggen**  
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Email: TSEeucrl@vla.defra.gsi.gov.uk
### Bovine tuberculosis – Tuberculose bovine – Tuberculosis bovina

<table>
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<tr>
<th>Name</th>
<th>Address</th>
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<th>Email</th>
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<tbody>
<tr>
<td>Dr Amelia Bernardelli</td>
<td>Gerencia de Laboratorios (GELAB) del Servicio Nacional de Sanidad y Calidad, Agroalimentaria (SENASA) Av. Alexander Fleming 1653, 1640 Martinez - Pcia de Buenos Aires ARGENTINE ARGENTINA ARGENTINA</td>
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<td>Email: <a href="mailto:abernard@senasa.gov.ar">abernard@senasa.gov.ar</a> or <a href="mailto:dilab@inea.com.ar">dilab@inea.com.ar</a></td>
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<tbody>
<tr>
<td>Dr Debby V. Cousins</td>
<td>Australian Reference Laboratory for Bovine Tuberculosis, Agriculture Western Australia Locked Bag N° 4, Bentley Delivery Centre, Bentley WA 6983 AUSTRALIE AUSTRALIA AUSTRALIA</td>
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<td>Email: <a href="mailto:dcousins@agric.wa.gov.au">dcousins@agric.wa.gov.au</a></td>
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<tr>
<td>Mme María Laura Boschirolli-Cara</td>
<td>AFSSA Alfort, Unité Zoonoses Bactériennes, Laboratoire d’études et de recherches en pathologie animale et zoonoses, 23 avenue du Général de Gaulle, 94706 Maisons-Alfort Cedex FRANCE FRANCE FRANCIA</td>
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<td>Email: <a href="mailto:ml.boschirolli@afssa.fr">ml.boschirolli@afssa.fr</a></td>
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<tr>
<td>Mr Keith Jahans</td>
<td>VLA Weybridge, New Haw, Addlestone, Surrey KT15 3NB ROYAUME-UNI UNITED KINGDOM REINO UNIDO</td>
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### Bovine viral diarrhoea – Diarrhée virale bovine – Diarrea viral bovina

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<th>Name</th>
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<tr>
<td>Dr Peter D. Kirkland</td>
<td>Elizabeth Macarthur Agriculture Institute (EMAI) AUSTRALIA AUSTRALIE AUSTRALIA</td>
<td>Tel.: (61-2) 46.40.63.31 – Fax: (61-2) 46.40.64.29</td>
<td>Email: <a href="mailto:peter.kirkland@dpi.nsw.gov.au">peter.kirkland@dpi.nsw.gov.au</a></td>
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<tr>
<td>Dr Dirk Deregt</td>
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<td>Email: <a href="mailto:deregtd@inspection.gc.ca">deregtd@inspection.gc.ca</a></td>
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<tr>
<td>Dr Trevor W. Drew</td>
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### Brucellosis – Brucellose – Brucelosis (Brucella abortus, Brucella melitensis, Brucella suis)

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<tr>
<td>Dr Ana Maria Nicola</td>
<td>Gerencia de Laboratorios (GELAB), Servicio Nacional de Sanidad y Calidad Agroalimentaria (SENASA) Av. Alexander Fleming 1653, 1640 Martínez, Pcia. de Buenos Aires ARGENTINE ARGENTINA ARGENTINA</td>
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<td>Email: <a href="mailto:anicola@senasa.gov.ar">anicola@senasa.gov.ar</a></td>
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<tr>
<td>Dr Klaus Nielsen</td>
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</tbody>
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4 Subject to confirmation by the International Committee in May 2008.
List of OIE Reference Laboratories in 2008

**Brucellosis – Brucellose – Brucelosis (Brucella abortus, Brucella melitensis, Brucella suis)**

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Channel catfish virus disease – Herpèsvirose du poisson-chat – Virosis del bagre de canal

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Chronic wasting disease – Cachexie chronique – Caquexia crónica

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Classical swine fever – Peste porcine classique – Peste porcina clásica

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Contagious agalactia – Agalaxie contagieuse – Agalaxia contagiosa

Dr Robin A.J. Nicholas
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Contagious bovine pleuropneumonia – Péripneumonie contagieuse bovine – Perineumonía contagiosa bovina

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Contagious caprine pleuropneumonia – Pleuropneumonie contagieuse caprine – Pleuroneumonía contagiosa caprina

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Contagious equine metritis – Métrite contagieuse équine – Metritis contagiosa equina

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Email: NVSL_Concerns@aphis.usda.gov

5 To to be advised – please refer to the OIE website
Control of Veterinary Medicinal Products in Sub-Saharan Africa – Contrôle des médicaments vétérinaires en Afrique subsaharienne – Control de los Medicamentos Veterinarios en África subsahariana

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Crayfish plague – Peste de l’écrevisse – Plaga del cangrejo de río (Aphanomyces astaci)

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Dourine – Dourine – Durina

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Echinococcosis/hydatidosis – Echinococcose/hydatidose – Equinococosis/hidatidosis

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Enteric septicemia of catfish – Entérosepticémie du poisson-chat– Septicemia entérica del bagre (Edwardsiella ictaluri)

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List of OIE Reference Laboratories in 2008

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**Enzootic abortion of ewes (ovine chlamydiosis) – Avortement enzootique des brebis (chlamydiose ovine) – Aborto enzoótico de las ovejas (clamidiosis ovina)**

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**Enzootic bovine leukemia – Leucose bovine enzootique – Leucosis bovina enzoótica**

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**Epizootic haematopoietic necrosis – Nécrose hématopoïétique épizootique – Necrosis hematopoyética epizoótica**

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**Epizootic ulcerative syndrome – Syndrome ulcératif épizootique – Síndrome ulcerante epizoótico**

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6 Subject to confirmation by the International Committee in May 2008.
Equine encephalomyelitis (Eastern) – Encéphalomyélite équine de l’Est – Encefalomielitis equina del Este
Equine encephalomyelitis (Western) – Encéphalomyélite équine de l’Ouest – Encefalomielitis equina del Oeste

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Equine infectious anaemia – Anémie infectieuse des équidés – Anemia infecciosa equina

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Equine influenza – Grippe équine – Gripe equina

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Equine piroplasmosis – Piroplasmosé équine – Piroplasmosis equina

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OIE Terrestrial Manual 2008 1319
Equine rhinopneumonitis – Rhinopneumonie équine – Rinoneumonía equina

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Equine viral arteritis – Artérite virale équine – Arteritis viral equina

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Escherichia coli – Escherichia coli – Escherichia coli

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Foot and mouth disease – Fièvre aphteuse – Fiebre aftosa

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7 Subject to confirmation by the International Committee in May 2008.
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8 Subject to confirmation by the International Committee in May 2008.
Hendra and Nipah virus diseases – Maladies dues aux virus Hendra et Nipah – Enfermedades causadas por los virus Hendra y Nipah

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9 Subject to confirmation by the International Committee in May 2008.
<table>
<thead>
<tr>
<th>Infection with Bonamia exitiosa – Infection à Bonamia exitiosa – Infección por Bonamia exitiosa</th>
<th>Infection with Bonamia ostreae – Infection à Bonamia ostreae – Infección por Bonamia ostreae</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dr Isabelle Arzul</td>
<td>Dr Eugene M. Burreson</td>
</tr>
<tr>
<td>IFREMER, Laboratoire de Génétique et Pathologie, BP 133, 17390 La Tremblade</td>
<td>Director for Research and Advisory Services, Virginia Institute of Marine Science, College of William and Mary</td>
</tr>
<tr>
<td>FRANCE FRANCE FRANCIA</td>
<td>P.O. Box 1346, Gloucester Point, VA 23062 ÉTATS-UNIS D’AMÉRIQUE UNITED STATES OF AMERICA ESTADOS UNIDOS DE AMÉRICA</td>
</tr>
<tr>
<td>Tel: (33-5) 46.76.26.10 – Fax: (33-5) 46.76.26.11</td>
<td>Tel: (1.804) 684.70.15 – Fax: (1.804) 684.77.96</td>
</tr>
<tr>
<td>Email: <a href="mailto:isabelle.arzul@ifremer.fr">isabelle.arzul@ifremer.fr</a></td>
<td>Email: <a href="mailto:gene@vims.edu">gene@vims.edu</a></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Infection with Haplosporidium nelsoni – Infection à Haplosporidium nelsoni – Infección por Haplosporidium nelsoni</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dr Eugene M. Burreson</td>
</tr>
<tr>
<td>Director for Research and Advisory Services, Virginia Institute of Marine Science, College of William and Mary</td>
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<td>Tel: (1.804) 684.70.15 – Fax: (1.804) 684.77.96</td>
</tr>
</tbody>
</table>

<table>
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<tr>
<th>Infection with Marteilia refringens – Infection à Marteilia refringens – Infección por Marteilia refringens</th>
<th>Infection with Marteilia sydneyi – Infection à Marteilia sydneyi – Infección por Marteilia sydneyi</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dr Isabelle Arzul</td>
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</tr>
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<td>IFREMER, Laboratoire de Génétique et Pathologie, BP 133, 17390 La Tremblade</td>
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<tr>
<td>Email: <a href="mailto:isabelle.arzul@ifremer.fr">isabelle.arzul@ifremer.fr</a></td>
<td>Email: <a href="mailto:isabelle.arzul@ifremer.fr">isabelle.arzul@ifremer.fr</a></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Infection with Mikrocytos mackini – Infection à Mikrocytos mackini – Infección por Mikrocytos mackini</th>
</tr>
</thead>
<tbody>
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</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Infection with Perkinsus marinus – Infection à Perkinsus marinus – Infección por Perkinsus marinus</th>
<th>Infection with Perkinsus olseni – Infection à Perkinsus olseni – Infección por Perkinsus olseni</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dr Eugene M. Burreson</td>
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<td>Email: <a href="mailto:gene@vims.edu">gene@vims.edu</a></td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>Infection with Xenohaliotis californiensis – Infection à Xenohaliotis californiensis – Infección por Xenohaliotis californiensis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prof. Carolyn Friedman</td>
</tr>
<tr>
<td>Friedman Shellfish Health Laboratory, School of Aquatic and Fishery Sciences, University of Washington</td>
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<tr>
<td>Box 355020, Seattle, Washington 98195</td>
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<td>Tel: (1.206) 543.95.19 – Fax: (1.206) 616.88.89</td>
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</tr>
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<tbody>
<tr>
<td>Dr Dirk Deregt(1)</td>
</tr>
<tr>
<td>Canadian Food Inspection Agency, Animal Diseases Research Institute</td>
</tr>
<tr>
<td>P.O. Box 640, Lethbridge, Alberta T1J 3Z4</td>
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<tr>
<td>CANADA CANADA CANADÁ</td>
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<tr>
<td>Tel: (1.403) 382.55.00 – Fax: (1.403) 381.12.02</td>
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<td>et hématopoïétique infectieuse – Necrosis hipodérmica u hematopoyética infecciosa</td>
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</tbody>
</table>

OIE Terrestrial Manual 2008
### Infectious salmon anaemia – Anémie infectieuse du saumon – Anemia infecciosa del salmón

**Dr Birgit Dannevig**  
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### Koi herpesvirus disease – Herpèsvirose de la carpe koi – Herpesvirosis de la carpa koi

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10 Subject to confirmation by the International Committee in May 2008.
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<tbody>
<tr>
<td>Dr William A. Ellis</td>
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<tr>
<td>To be advised11</td>
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</tr>
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<td>Dr Venugopal K. Nair</td>
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<tr>
<td>Dr Aly M. Fadly</td>
<td>USDA, ARS, Avian Disease and Oncology Laboratory&lt;br&gt;33606 East Mount Hope Road, East Lansing, Michigan 48823&lt;br&gt;ÉTATS-UNIS D’AMÉRIQUE UNITED STATES OF AMÉRICA ESTADOS UNIDOS DE AMÉRICA&lt;br&gt;Tel: (1.517) 337.68.29 – Fax: (1.517) 337.67.76&lt;br&gt;Email: <a href="mailto:fadly@msu.edu">fadly@msu.edu</a></td>
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11 To to be advised – please refer to the OIE website
### New world screwworm (Cochliomyia hominivorax) – Myiase à Cochliomyia hominivorax – Miasis por Cochliomyia hominivorax

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### Oncorhynchus masou virus disease – Herpèsvirose du saumon masou – Herpesvirosis del salmón masou

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### Ovine epididymitis – Epididymite ovine – Epididimitis ovina (Brucella ovis)

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<table>
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<tr>
<th>Laboratory Name</th>
<th>Address</th>
<th>Telephone</th>
<th>Facsimile</th>
<th>Email Address</th>
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<td>Dr Klaus Nielsen</td>
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<td>Dr Judy A. Stack</td>
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<tr>
<td><strong>Paratuberculosis – Paratuberculose – Paratuberculosis</strong></td>
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<tr>
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Porcine reproductive and respiratory syndrome – Syndrome dysgénésique et respiratoire du porc –
Síndrome disgenésico y respiratorio porcino

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Rabbit haemorrhagic disease – Maladie hémorragique du lapin – Enfermedad hemorrágica del conejo

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Tel: (33 (0)4) 67.59.37.98 – Fax: (33 (0)4) 67.59.38.50
Email: genevieve.libeau@cirad.fr

Dr John Anderson
Institute for Animal Health, Pirbright Laboratory
Ash Road, Pirbright, Woking, Surrey GU24 ONF
ROYAUME-UNI UNITED KINGDOM REINO UNIDO
Tel: (44.1483) 23.24.41 – Fax: (44.1483) 23.24.48
Email: john.anderson@bbsrc.ac.uk

Salmonellosis – Salmonelloses – Salmonelosis

Dr Cornelius Poppe
Laboratory for Foodborne Zoonoses, Guelph Laboratory, Health Canada, Public Health Agency of Canada
110 Stone Road West, Guelph, Ontario, N1G 3W4
CANADA CANADA CANADÁ
Tel: (1.519) 821.97.02 – Fax: (1.519) 822.22.80
Email: cpoppe@sympatico.ca
List of OIE Reference Laboratories in 2008

**Salmonellosis – Salmonelloses – Salmonelosis**

**Dr Matthias Hartung**  
Bundesinstitut für Risikobewertung (Federal Institute for Risk Assessment)  
P.O. Box 330013, 14191 Berlin  
ALEMAGNE  GERMANY  ALEMANIA  
Tel: (49.30) 84.12.22.12 – Fax: (49.30) 84.12.29.52  
Email: m.hartung@bfr.bund.de

**Dr Antonia Ricci**  
Istituto Zooprofilattico Sperimentale delle Venezie, National Reference Laboratory for Salmonella  
Viale Dell’Università 10, 35020 Legnaro (PD)  
ITALIE  ITALY  ITALIA  
Tel: (39.049) 808.42.96 – Fax: (39.049) 883.02.68  
Email: aricci@izsvenezie.it

**Dr Robert H. Davies**  
VLA Weybridge  
New Haw, Addlestone, Surrey KT15 3NB  
ROYAUME-UNI  UNITED KINGDOM  REINO UNIDO  
Tel: (44-1932) 35.73.61 – Fax: (44-1932) 35.75.95  
Email: r.h.davies@vla.defra.gsi.gov.uk

---

**Scrapie – Tremblante – Prurigo lumbar**

**Dr Aru Balachandran**  
Canadian Food Inspection Agency, Ottawa Laboratory  
3851 Fallowfield Road, P.O. Box 11300, Station H, Nepean, Ontario K2H 8P9  
CANADA  CANADA  CANADÁ  
Tel: (1.613) 228.66.98 – Fax: (1.613) 228.66.69  
Email: balachandran@inspection.gc.ca

**Prof. Andreas Zurbriggen**  
Institute of Animal Neurology, University of Bern  
Bremgartenstrasse 109A, 3012 Bern  
SUISSE  SWITZERLAND  SUIZA  
Tel: (41.31) 631.25.09 – Fax: (41.31) 631.25.38  
Email: andreas.zurbriggen@itn.unibe.ch

**Dr Marion Simmons**  
VLA Weybridge  
New Haw, Addlestone, Surrey KT15 3NB  
ROYAUME-UNI  UNITED KINGDOM  REINO UNIDO  
Tel: (44.1932) 35.95.12 – Fax: (44.1932) 35.73.27  
Email: TSEeucrl@vla.defra.gsi.gov.uk

---

**Sheep pox and goat pox – Clavelée et variole caprine – Viruela ovina y viruela caprina**

**Dr Hamid Reza Varshovi**  
RAZI Vaccine and Serum Research Institute  
P.O. Box 19171/148, Hessarak Karadj, Teheran  
IRAN  IRAN  IRÁN  
Tel: (98.21) 311.79.08 – Fax: (98.261) 455.46.58  
Email: int@rvsri.com or hr_varshovi@yahoo.com

**Dr Eeva Tuppurainen**  
Institute for Animal Health, Pirbright Laboratory  
Ash Road, Pirbright, Woking, Surrey GU24 ONF  
ROYAUME-UNI  UNITED KINGDOM  REINO UNIDO  
Tel: (44.1483) 23.24.41 – Fax: (44.1483) 23.24.48  
Email: eeva.tuppurainen@bbsrc.ac.uk
List of OIE Reference Laboratories in 2008

Spherical baculovirosis (Penaeus monodon-type baculovirus) – Baculovirose sphérique (Baculovirus spécifique de Penaeus monodon) – Baculovirosis esférica (baculovirus de tipo Penaeus monodon)

Dr Grace Lo
Department and Institute of Zoology, National Taiwan University
1, Sec. Roosevelt Road, Taipei
TAIPEI CHINA TAIPEI CHINA TAIPEI CHINA
Tel: (886.2) 23.63.35.62 – Fax: (886.2) 23.63.81.79
Email: gracelow@ccms.ntu.edu.tw

Prof. Donald V. Lightner
Aquaculture Pathology Laboratory, Department of Veterinary Science and Microbiology, University of Arizona
Building 90, Room 202 Pharmacy/Microbiology, Tucson, AZ 85721
ÉTATS-UNIS D’AMÉRIQUE UNITED STATÉS OF AMÉRICA ESTADOS UNIDOS DE AMÉRICA
Tel: (1.520) 621.84.14 – Fax: (1.520) 621.48.99
Email: dvl@u.arizona.edu

Spring viraemia of carp – Virémie printanière de la carpe – Viremia primaveral de la carpa

Dr Peter Dixon
The Centre for Environment, Fisheries and Aquaculture Science (CEFAS), Weymouth Laboratory
Barrack Road, The Nothe, Weymouth, Dorset, DT4 8UB
ROYAUME-UNI UNITED KINGDOM REINO UNIDO
Tel: (44.1305) 20.66.25 – Fax: (44.1305) 20.66.01
Email: peter.dixon@cefas.co.uk

Surra – Surra – Surra (Trypanosoma evansi)

Dr Filip Claes
Institute of Tropical Medicine Antwerp, Department of Parasitology
Nationalstraat 1.55, B-2000 Antwerpen
BELGIQUE BELGIUM BELGICA
Tel: (32.3) 247.65.34 – Fax: (32.3) 247.63.73
Email: fclaes@itg.be

Prof. Noboru Inoue
National Research Center for Protozoan Diseases, Obihiro University of Agriculture and Veterinary Medicine
Inada-cho Nishi 2-13, Obihiro, Hokkaido 080-8555
JAPON JAPAN JAPÓN
Tel: (81.155) 49.56.47 – Fax: (81.155) 49.56.43
Email: protozoa@obihiro.ac.jp

Swine vesicular disease – Maladie vésiculeuse du porc – Enfermedad vesicular porcina

Dr Emiliana Brocchi
Istituto Zooprofittlativo Sperimentale della Lombardia e dell’Emilia Romagna ‘B. Ubertini’
Via A. Bianchi n° 9, 25124 Brescia
ITALIE ITALY ITALIA
Tel: (39.030) 229.03.10 – Fax: (39.030) 229.03.69
Email: emiliana.brocchi@bs.izs.it

Dr David Paton
Institute for Animal Health
Ash Road, Pirbright, Woking, Surrey GU24 ONF
ROYAUME-UNI UNITED KINGDOM REINO UNIDO
Tel: (44.1483) 23.24.41 – Fax: (44.1483) 23.24.48
Email: david.paton@bbsrc.ac.uk

Taura syndrome – Syndrome de Taura – Síndrome de Taura

Prof. Donald V. Lightner
Aquaculture Pathology Laboratory, Department of Veterinary Science and Microbiology, University of Arizona
Building 90, Room 202 Pharmacy/Microbiology, Tucson, AZ 85721
ÉTATS-UNIS D’AMÉRIQUE UNITED STATÉS OF AMÉRICA ESTADOS UNIDOS DE AMÉRICA
Tel: (1.520) 621.84.14 – Fax: (1.520) 621.48.99
Email: dvl@u.arizona.edu
Tetrahedral baculovirosis – Baculovirese tetraédrique – Baculovirosis tetraédrica (Baculovirus penaei)

Prof. Donald V. Lightner
Aquaculture Pathology Laboratory, Department of Veterinary Science and Microbiology, University of Arizona
Building 90, Room 202 Pharmacy/Microbiology, Tucson, AZ 85721
ÉTATS-UNIS D’AMÉRIQUE UNITED STATES OF AMERICA ESTADOS UNIDOS DE AMÉRICA
Tel: (1.520) 621.84.14 – Fax: (1.520) 621.48.99
Email: dvl@u.arizona.edu

Transmissible gastroenteritis – Gastro-entérite transmissible – Gastroenteritis transmisible

Dr Linda J. Saif
Food Animal Health Research Program, Ohio Agricultural Research and Development Center, The Ohio State University
1680 Madison Avenue, Wooster, OH 44691-4096
ÉTATS-UNIS D’AMÉRIQUE UNITED STATES OF AMERICA ESTADOS UNIDOS DE AMÉRICA
Tel: (1.330) 263.37.44 – Fax: (1.330) 263.36.77
Email: saif.2@osu.edu

Trichinellosis – Trichinellose – Triquinelosis

Dr Alvin Gajadhar
Canadian Food Inspection Agency, Centre for Animal Parasitology
116 Veterinary Road, Saskatoon, Saskatchewan S7N 2R3
CANADA CANADA CANADÁ
Tel: (1.306) 975.53.44 – Fax: (1.306) 975.57.11
Email: agajadhar@inspection.gc.ca

Dr Edoardo Pozio
Istituto Superiore di Sanita, Laboratorio di Parasitologia
Viale Regina Elena 299, 00161 Roma
ITALIE ITALY ITALIA
Tel: (39.06) 49.90.23.04 – Fax: (39.06) 49.38.70.65
Email: pozio@iss.it

Trypanosomosis (tsetse-transmitted) – Trypanosomose (transmise par tsé-tsé) – Tripanosomosis (transmitida por tsseté)

Dr Marc Desquesnes
CIRAD-EMVT
Programme Santé animale, TA30/G Campus international de Baillarguet, 34398 Montpellier Cedex 5
FRANCE FRANCE FRANCIA
Tel: (33(0)4) 67.59.37.24 – Fax: (33(0)4) 67.59.37.98
Email: marc.desquesnes@cirad.fr

Tularemia – Tularémie – Tularemia

Dr Torsten Mörner
National Veterinary Institute, Department of Wildlife
751 89 Uppsala
SUÈDE SWEDEN SUECIA
Tel: (46.18) 67.40.00 – Fax: (46.18) 67.46.90
Email: torsten.morner@sva.se

Turkey rhinotracheitis – Rhinotraéchite de la dinde – Rinoatraqueitis del pavo

Dr Nicolas Eterradossi
AFSSA Ploufragan, Unité de virologie, immunologie et parasitologie aviaires et cunicoles
BP 53, 22440 Ploufragan
FRANCE FRANCE FRANCIA
Tel: (33 (0)2) 96.01.62.22 – Fax: (33 (0)2) 96.01.62.63
Email: n.eterradossi@ploufragan.afssa.fr
### List of OIE Reference Laboratories in 2008

<table>
<thead>
<tr>
<th>Disease</th>
<th>Reference</th>
<th>Address</th>
<th>Phone</th>
<th>Fax</th>
<th>Email</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Venezuelan equine encephalomyelitis</strong></td>
<td>Dr Eileen N. Ostlund</td>
<td>Diagnostic Virology Laboratory, National Veterinary Services Laboratories P.O. Box 844, Ames, IA 50010 ÉTATS-UNIS D'AMÉRIQUE UNITED STATES OF AMERICA ESTADOS UNIDOS DE AMÉRICA Tel: (1.515) 663.75.51 – Fax: (1.515) 663.73.48 Email: <a href="mailto:eileen.n.ostlund@aphis.usda.gov">eileen.n.ostlund@aphis.usda.gov</a></td>
<td>(1.515) 663.75.51</td>
<td>(1.515) 663.73.48</td>
<td><a href="mailto:eileen.n.ostlund@aphis.usda.gov">eileen.n.ostlund@aphis.usda.gov</a></td>
</tr>
<tr>
<td><strong>Vesicular stomatitis</strong></td>
<td>Dr Ingrid Bergmann</td>
<td>Centro Panamericano de Fiebre Aftosa OPS/OMS Av. President Kennedy 7778, Sao Bento, Duque de Caxias, ZC 20054-40 Rio de Janeiro BRESIL BRAZIL BRASIL Tel: (55.21) 36.61.90.56 – Fax: (55.21) 36.61.90.01 Email: <a href="mailto:ibergman@panaftosa.ops-oms.org">ibergman@panaftosa.ops-oms.org</a></td>
<td>(55.21) 36.61.90.56</td>
<td>(55.21) 36.61.90.01</td>
<td><a href="mailto:ibergman@panaftosa.ops-oms.org">ibergman@panaftosa.ops-oms.org</a></td>
</tr>
<tr>
<td><strong>Viral encephalopathy and retinopathy</strong></td>
<td>Dr Sabrina L. Swenson</td>
<td>National Veterinary Services Laboratories P.O. Box 844, Ames, IA 50010 ÉTATS-UNIS D'AMÉRIQUE UNITED STATES OF AMERICA ESTADOS UNIDOS DE AMÉRICA Tel: (1.515) 663.75.51 – Fax: (1.515) 663.73.48 Email: <a href="mailto:sabrina.l.swenson@aphis.usda.gov">sabrina.l.swenson@aphis.usda.gov</a></td>
<td>(1.515) 663.75.51</td>
<td>(1.515) 663.73.48</td>
<td><a href="mailto:sabrina.l.swenson@aphis.usda.gov">sabrina.l.swenson@aphis.usda.gov</a></td>
</tr>
<tr>
<td><strong>Viral haemorrhagic septicaemia</strong></td>
<td>Dr Giuseppe Bovo</td>
<td>Istituto Zooprofilattico Sperimentale delle Venezie, Dipartimento di Ittiovirologia, Via Romea 14/A, 35020 Legnaro PD ITALIE ITALY ITALIA Tel: (39.049) 808.42.48 – Fax: (39.049) 808.43.92 Email: <a href="mailto:gbovo@zsvenezie.it">gbovo@zsvenezie.it</a></td>
<td>(39.049) 808.42.48</td>
<td>(39.049) 808.43.92</td>
<td><a href="mailto:gbovo@zsvenezie.it">gbovo@zsvenezie.it</a></td>
</tr>
<tr>
<td><strong>West Nile Encephalitis</strong></td>
<td>Dr Niels Jørgen Olesen</td>
<td>National Veterinary Institute, Technical University of Denmark Hangøvej 2, 8200 Aarhus N DANEMARK DENMARK DINAMARCA Tel: (45) 72.34.68.31 – Fax: (45) 72.34.69.01 Email: <a href="mailto:njo@vet.dtu.dk">njo@vet.dtu.dk</a></td>
<td>(45) 72.34.68.31</td>
<td>(45) 72.34.69.01</td>
<td><a href="mailto:njo@vet.dtu.dk">njo@vet.dtu.dk</a></td>
</tr>
</tbody>
</table>

**West Nile Encephalitis**

Dr Eileen N. Ostlund Diagnostic Virology Laboratory, National Veterinary Services Laboratories P.O. Box 844, Ames, IA 50010 ÉTATS-UNIS D'AMÉRIQUE UNITED STATES OF AMERICA ESTADOS UNIDOS DE AMÉRICA Tel: (1.515) 663.75.51 – Fax: (1.515) 663.73.48 Email: eileen.n.ostlund@aphis.usda.gov
White spot disease – Maladie des points blancs – Enfermedad de las manchas blancas

Prof. Donald V. Lightner
Aquaculture Pathology Laboratory, Department of Veterinary Science and Microbiology, University of Arizona
Building 90, Room 202 Pharmacy/Microbiology, Tucson, AZ 85721
ÉTATS-UNIS D’AMÉRIQUE UNITED STATES OF AMÉRICA ESTADOS UNIDOS DE AMÉRICA
Tel: (1.520) 621.84.14 – Fax: (1.520) 621.48.99
Email: dvl@u.arizona.edu

Dr Grace Lo
Department and Institute of Zoology, National Taiwan University
1, Sec. Roosevelt Road, Taipei
TAIPEI CHINA TAIPEI CHINA TAIPEI CHINA
Tel: (886.2) 23.63.35.62 – Fax: (886.2) 23.63.81.79
Email: gracelow@ccms.ntu.edu.tw

West tail disease– Maladie des queues blanches – Enfermedad de la cola blanca

Dr A. Sait Sahul Hameed12
Aquaculture Biotechnology Division, Department of Zoology, C. Abdul Hakeem College (Affiliated to Thiruvalluvar University, Tamil Nadu), Melvisharam-632 509, Vellore Dt. Tamil Nadu, INDIA INDE INDIA
Tel: (91-4172) 266.187 (Off), (91-4172) 269.487 (Dir) – Fax: (91-4172) 269.487
E-mail: cah_sahul@hotmail.com

Yellowhead disease – Maladie de la tête jaune – Enfermedad de la cabeza amarilla

Dr Peter Walker
CSIRO, Aquaculture and Aquatic Animal Health (AAHL)
5 Portarlington Road, Private Bag 24, Geelong 3220, Victoria
AUSTRALIE AUSTRALIA AUSTRALIA
Tel: (61.3) 52.27.54.65 – Fax: (61.3) 52.27.55.55
Email: peter.walker@csiro.au

12 Subject to confirmation by the International Committee in May 2008.
LIST OF OIE COLLABORATING CENTRES IN 2008

Veterinary Medicinal Products – Médicaments veterinaires – Medicamentos veterinarios

- AFSSA Fougères,
  Agence nationale du médicament vétérinaire,
  B.P. 203, 35302 Fougères Cedex
  FRANCE  FRANCIA
  Tel: 33 (0)2 99.94.78.78/78.71
  Fax: 33 (0)2 99.94.78.99
  E-mail: p.dehaumont@anmv.afssa.fr
  http://www.anmv.afssa.fr

ELISA and Molecular Techniques in Animal Disease Diagnosis – Diagnostic des maladies animales par l’ELISA et les techniques moléculaires – Diagnóstico de enfermedades animales por el método ELISA y las técnicas moleculares

- FAO/IAEA Centre for ELISA and Molecular Techniques in Animal Disease Diagnosis
  International Atomic Energy Agency,
  Wagramerstrasse 5
  P.O. Box 100, A-1400 Wien
  AUTRICHE AUSTRIA
  Tel: (43.1) 260.02.83.55
  Fax: (43.1) 260.02.82.22
  E-mail: a.diallo@iaea.org

Diagnosis, Epidemiology and Control of Animal Diseases in Tropical Regions – Diagnostic, épidémiologie et contrôle des maladies animales en régions tropicales – Diagnóstico, epidemiología y control de las enfermedades animales en las regiones tropicales

- CIRAD/EMVT
  Campus international de Baillarguet, TA 30/B
  Montferriez-sur-Lez
  B.P. 5035
  34398 Montpellier Cedex 5
  FRANCE  FRANCIA
  Tel: 33 (0)4 67.61.58.01
  Fax: 33 (0)4 67.61.55.70
  E-mail: emmanuel.camus@cirad.fr

Surveillance and Control of Animal Diseases in Africa – Surveillance et contrôle des maladies animales en Afrique – Vigilancia y control de enfermedades animales en África

- Onderstepoort Veterinary Institute
  Agricultural Research Council
  Private Bag X05
  Onderstepoort 0110
  SOUTH AFRICA AFRIQUE DE SUD SUDÁFRICA
  Tel: (27.12) 529.91.06
  Fax: (27.12) 565.46.64
  E-mail: musoket@arc.agric.za

Animal Disease Surveillance Systems and Risk Analysis – Systèmes de surveillance des maladies animales et analyse des risques – Sistemas de vigilancia de enfermedades animales y análisis de riesgos

- Centers for Epidemiology and Animal Health
  USDA-APHIS-VS-CEAH
  2150 Centre Avenue, Building B
  Fort Collins, Colorado 80526-8117
  UNITED STATES OF AMERICA ETATS-UNIS D’AMÉRIQUE ESTADOS UNIDOS DE AMÉRICA
List of OIE Collaborating Centres 2008

Diagnosis and Control of Animal Diseases in Eastern Europe, Central Asia and Transcaucasia – Diagnostic et contrôle des maladies animales en Europe de l’Est, en Asie centrale et en Transcaucase – Diagnóstico y control de las enfermedades animales en Europa oriental, Asia central y Transcaucasia

- Federal Governmental Institution, Federal Centre for Animal Health, FGI-ARRIAH\(^{(1)}\)
  Ministry of Agriculture of the Russian Federation
  600901 Yur’evets, Vladimir
  RUSSIA RUSSIE RUSIA
  Tel: (7.4922) 26.38.77
  Fax: (7.4922) 26.19.14
  E-mail: mail@arriah.ru
  http://www.arriah.ru (russian version)
  http://www.arriah.ru/portal/en (english version)

Food Safety, Diagnosis and Control of Animal Diseases in Eastern Europe, Central Asia and Transcaucasia – Sécurité sanitaire des aliments, diagnostic et contrôle des maladies animales en Europe de l’Est, en Asie centrale et en Transcaucase – Seguridad sanitaria de los Alimentos y el Diagnóstico y Control de las Enfermedades Animales en Europa oriental, Asia central y Transcaucasia

- All-Russian Research Institute for Control\(^{(2)}\)
  Standardisation and Certification of Veterinary Preparations
  Ministry of Agriculture and Food
  5 Zvenigorodskoye shosse,
  123022 Moscou
  RUSSIA RUSSIE RUSIA
  Tel: (7.095) 253.14.91
  Fax: (7.095) 253.14.91
  E-mail: Vgnki-vet@mtu-net.ru

Veterinary Training, Epidemiology, Food Safety and Animal Welfare – Formation vétérinaire, épidémiologie, sécurité sanitaire des aliments et bientraitance animale – Entrenamiento Veterinario, Epidemiología, Seguridad Sanitaria de los Alimentos y Bienestar Animal

- Istituto Zooprofilattico Sperimentale dell’Abruzzo e del Molise ‘G. Caporale’
  Via Campo Boario
  64100 Teramo
  ITALY ITALIE ITALIA
  Tel: (39.0861) 33.22.79
  Fax: (39.0861) 33.22.51
  E-mail: caporale@izs.it

Diagnosis of Animal Diseases and Vaccine Evaluation in the Americas – Diagnostic des maladies animales et évaluation des vaccins dans les Amériques – Diagnóstico de las enfermedades animales y evaluación de las vacunas en las Américas

- National Veterinary Services Laboratories\(^{(1)}\)
  USDA, APHIS, Veterinary Services
  P.O. Box 844
  Ames
  Iowa 50010
  UNITED STATES OF AMERICA ETATS-UNIS D’AMÉRIQUE ESTADOS UNIDOS DE AMÉRICA
  Tel: (1.515) 663.72.66
  Fax: (1.515) 663.73.97
  E-mail: nvsl.concerns@aphis.usda.gov

- Center for Veterinary Biologics\(^{(2)}\)
  USDA, APHIS, Veterinary Services
  P.O. Box 844, Ames, Iowa 50010
  UNITED STATES OF AMERICA ETATS-UNIS D’AMÉRIQUE ESTADOS UNIDOS DE AMÉRICA
  Tel: (1.515) 663.73.31
  Fax: (1.515) 663.76.73
  E-mail: cvb@aphis.usda.gov
• Institute for International Cooperation in Animal Biologics (3)
  College of Veterinary Medicine
  Iowa State University, Ames, Iowa 50011
  UNITED STATES OF AMERICA  ETATS-UNIS D'AMÉRIQUE  ESTADOS UNIDOS DE AMÉRICA
  Tel: (1.515) 294.84.59
  Fax: (1.515) 294.82.59
  E-mail: icab@iastate.edu
  http://www.cfsph.iastate.edu/icab/

Food-Borne Zoonotic Parasites – Les zoonoses parasitaires d'origine alimentaire – Las zoonosis parasitarias de origen alimentario
• Canadian Food Inspection Agency
  Centre for Animal Parasitology
  116 Veterinary Road
  Saskatoon, Saskatchewan S7N 2R3
  CANADA  CANADÁ
  Tel: (1.306) 975.53.44
  Fax: (1.306) 975.57.11
  E-mail: agajadhar@inspection.gc.ca

New Emerging Diseases – Maladies nouvelles et émergentes – Nuevas enfermedades y enfermedades emergentes
• CSIRO, Australian Animal Health Laboratory (AAHL)
  5 Portarlington Road, Private Bag 24, Geelong 3220, Victoria
  AUSTRALIA  AUSTRALIE  AUSTRALIA
  Tel: (61.3) 52.27.50.00
  Fax: (61.3) 52.27.55.55
  E-mail: martyn.jeggo@csiro.au

Training of Official Veterinarians – Formation des vétérinaires officiels – Formación de Veterinarios Oficiales
• Ecole nationale des Services vétérinaires
  1 avenue Bourgelat, BP 83, 69280 Marcy l'Etoile
  FRANCE  FRANCIA
  Tel: (33(0)4) 78.87.25.45
  Fax: (33(0)4) 78.87.25.48
  E-mail: ensv@ensv.vet-lyon.fr

Research and Training in Population Animal Health Diagnosis and Surveillance Systems – Recherche et formation en matière de diagnostic et de systèmes de surveillance des maladies animales – Investigación y Formación en el ámbito del Diagnóstico Zoosanitario y de los Sistemas de Vigilancia de Poblaciones
• International Epilab, National Veterinary Institute
  Technical University of Denmark, Bulowsvej 27, DK-1790 Copenhagen V
  DENMARK  DANEMARK  DINAMARCA
  Tel: (45) 72.34.62.43
  Fax: (45) 72.34.60.01
  E-mail: hvl@dfvf.dk
  http://www.food.dtu.dk

Biotechnology-based Diagnosis of Infectious Diseases in Veterinary Medicine
Diagnostic basé sur la biotechnologie des maladies infectieuses en médecine vétérinaire
Diagnóstico de las enfermedades infecciosas de la medicina veterinaria basado en la biotecnología
• National Veterinary Institute, 751 89 Uppsala
  SWEDEN  SUEDE  SUECIA
  Tel: (46.18) 67.40.00
  Fax: (46.18) 67.46.69
  E-mail: sandor.belak@sva.se

Emerging and Re-emerging Zoonotic Diseases – Zoonoses émergentes et ré-émergentes – Enfermedades Zoonóticas Emergentes y Re-emergentes
• National Center for Infectious Diseases, CDC, Division of Bacterial and Mycotic Diseases
  1600 Clifton Road, Mailstop C12, Room 6034, Building 1, Atlanta, Georgia 30333
  UNITED STATES OF AMERICA  ETATS-UNIS D'AMÉRIQUE  ESTADOS UNIDOS DE AMÉRICA
  Tel: (1-404) 639.38.31
  Fax: (1-404) 639.44.41
  E-mail: nmarano@cdc.gov
List of OIE Collaborating Centres 2008

Zoonoses in Europe – Zoonoses en Europe – Zoonosis en Europa

- Friedrich-Loeffler-Institute, Boddenblick 5a, 17493 Greifswald, Insel Riems
  GERMANY  ALLEMAGNE  ALEMANIA
  Tel: (49-38351) 7-102
  Fax: (49-38351) 7-151
  E-mail: thomas.mettenleiter@fli.bund.de

Training of Veterinary Services – Formation des services vétérinaires – Capacitación de los servicios veterinarios

- Centro Buenos Aires para la Capacitación de los Servicios Veterinarios (CEBASEV)
  Avenida Alexander Fleming 1653 Piso 1, CP 1640 – Martinez, Pcia de Buenos Aires
  ARGENTINA  ARGENTINE  ARGENTINA
  Tel: (54.11) 41.21.53.53
  Fax: (54.11) 41.21.53.60
  E-mail: asilvest@senasa.gov.ar

Information on Aquatic Animal Diseases – Information sur les maladies des animaux aquatiques – Información sobre las enfermedades de los animales acuáticos

- The Centre for Environment, Fisheries and Aquaculture Sciences (CEFAS)
  Weymouth Laboratory, Barrack Road, The Nothe, Weymouth, Dorset, DT4 8UB
  UNITED KINGDOM  ROYAUME-UNI  REINO UNIDO
  Tel: (44.1305) 20.66.26
  Fax: (44.1305) 20.66.27
  E-mail: b.j.hill@cefas.co.uk
  http://www.collabcen.net

Wildlife Disease Surveillance and Monitoring, Epidemiology and Management – Surveillance et suivi, épidémiologie et gestion des maladies des animaux sauvages – Vigilancia y el seguimiento, epidemiología y gestión de las enfermedades de la fauna salvaje

- Canadian Cooperative Wildlife Health Centre (CCWHC),
  Department of Veterinary Pathology, Western College of Veterinary Medicine,
  University of Saskatchewan,
  52 Campus Drive, Saskatoon S7N 5B4
  CANADA  CANADÁ
  Tel.: (+1-800) 567.20.33
  E-mail: ccwhc@usask.ca

Animal Welfare Science and Bioethical Analysis (Asia/Pacific) – Science du bien-être animal et de l'analyse bioéthique (Asie/Pacifique) – Ciencias relativas al bienestar animal y el análisis bioético (Asia/Pacífico)

- Animal Welfare Science and Bioethics Centre (AWSBC)
  Universidad de Massey, Private Bag 11 222, Palmerston North,
  NOUVELLE-ZÉLANDE  NEW ZEALAND  NUEVA ZELANDA
  Tel.: (+6) 350.48.07
  E-mail: d.j.mellor@massey.ac.nz

Epidemiology, Training and Control of Emerging Avian Diseases – Epidémiologie, formation et contrôle des maladies aviaires émergentes – Epidemiología, formación y lucha contra las enfermedades aviares emergentes

- Istituto Zooprofilattico Sperimentale delle Venezie (IZSVe),
  Viale dell’Università 10, 35020 Legnaro, Padua
  ITALIE  ITALY  ITALIA
  Tel.: (+39-049) 808.42.79
  E-mail: dirsan@izsvenezie.it

Research on Emerging Avian Diseases – Recherche sur les maladies aviaires émergentes – Investigación sobre las enfermedades aviares emergentes

- Southeast Poultry and Research Laboratory (SEPL),
  United States Department of Agriculture (USDA), Agricultural Research Service
  934 College Station Road, Athens, Georgia 30605
  UNITED STATES OF AMERICA  ETATS-UNIS D’AMÉRIQUE  ESTADOS UNIDOS DE AMÉRICA
  Tel.: (1-706) 546.3433
  E-mail: dswayne@seprl.usda.gov
List of OIE Collaborating Centres 2008

Training Veterinary Officials, Diagnosing Infectious Animal Diseases and Zoonoses in Tropical Africa – Formation des agents des Services vétérinaires officiels et pour le diagnostic des maladies animales infectieuses et des zoonoses en Afrique tropicale – Formación de veterinarios oficiales, el diagnóstico de epizootias infecciosas y zoonosis en África Tropical

- Ecole Inter-Etats des Sciences et Médecine Vétérinaires (EISMV) de Dakar
  SENEGAL  SÉNÉGAL
  Tel.: (221) 865.10.08
  Fax: (221) 825.42.83
  E-mail: tekoagbo2001@yahoo.fr

Validation, Quality Assessment and Quality Control of Diagnostic Assays and Vaccine Testing for Vesicular Diseases in Europe – Centre collaborateur de l’OIE pour la validation et l’assurance qualité et/contrôle qualité des épreuves diagnostiques et des vaccins contre les maladies vésiculeuses en Europe – la validación y el aseguramiento y control de la calidad de los ensayos de diagnóstico y las pruebas de vacunas para enfermedades vesiculares en Europa

- Centre d’Etudes et de Recherches Vétérinaires et Agrochimiques (CERVA) de Ukkel
  BELGIUM  BELGIQUE  BÉLGICA
  Tel.: (32-2) 379.04.00
  Fax: (32-2) 379.06.66
  E-mail: kris.de.clercq@var.fgov.be

Laboratory Enhancement (Capacity Building) – Renforcement des capacités des laboratoires – Desarrollo de capacidad de los laboratorios

- Australian Animal Health Laboratory (AAHL), Geelong,
  AUSTRALIA  AUSTRALIE  AUSTRALIA
  Tel: (61.3) 52.27.50.14
  Fax: (61.3) 52.27.52.50
  E-mail: peter.daniels@csiro.au

Surveillance and Control of Animal Protozoan Diseases – Surveillance et le contrôle des maladies animales protozoaire – Vigilancia y el control de las enfermedades protozoicas animales

- National Research Center for Protozoan Diseases
  Obihiro University of Agriculture and Veterinary Medicine
  JAPAN  JAPON  JAPÓN
  Tel: (81-155) 49-5641
  Fax: (81-155) 49-56430
  E-mail: igarcpmi@obihiro.ac.jp

Veterinary Services Capacity Building – Renforcement des capacités des services vétérinaires – Desarrollo de capacidad de los servicios veterinarios

- Center for Animal Health and Food Safety (CAHFS), University of Minnesota, 136 Andrew Boss Laboratory, St. Paul, MN 55108,
  UNITED STATES OF AMERICA  ETATS-UNIS D’AMÉRIQUE  ESTADOS UNIDOS DE AMÉRICA
  Tel: (1-612) 625-8709
  Fax: (1-612) 624-4906
  E-mail: cahfs@umn.edu
  Web site: http://www.cahfs.umn.edu

*  
* *

13 Subject to confirmation by the International Committee in May 2008.
### ALPHABETICAL LIST OF DISEASES COVERED IN THIS TERRESTRIAL MANUAL

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