

## AMERICAN FOULBROOD OF HONEY BEES

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### SUMMARY

American foulbrood (AFB) affects the larval stage of the honey bee *Apis mellifera* and other *Apis* spp., and occurs throughout the world. *Paenibacillus larvae*, the causative organism, is a bacterium that can produce over one billion spores in each infected larva. The spores are extremely resistant to heat and chemical agents, and can survive for many years in scales (from diseased dead brood), hive products and equipment. Only the spores are capable of inducing the disease.

**Identification of the agent:** Combs of infected colonies have a mottled appearance due to a mixture of healthy capped brood, uncapped cells containing the remains of diseased larvae, and empty cells. This is not a characteristic of AFB only. Cell cappings of a diseased larva appear moist and darkened, becoming concave and possibly punctured as infection progresses. The larval or pupal colour changes to creamy brown and then to a dark brown with a ropy appearance when drawn out. In some cases the larval remains are rather watery. The diseased brood eventually dries out to form characteristic brittle scales that adhere tightly to the lower sides of the cell. The formation of a pupal tongue is one of the most characteristic but rarely seen signs of the disease and precedes the formation of the scales.

Diagnosis of AFB is based on identification of the pathogenic agent and the presence of clinical signs. The analyst can rely on a broad range of samples. However, in practice, the samples of choice will depend on whether it concerns a suspicious or diseased honey bee colony/apiary, or analysis in the context of an AFB monitoring/prevention programme. Some of the identification methods require a previous culturing step, while others can be performed directly on collected samples. Four solid culture media are recommended: PLA (*Paenibacillus larvae* agar), MYPGP agar, BHIT agar and Columbia sheep blood agar. Two polymerase chain reaction (PCR) protocols are described in this chapter. The first protocol can be used for rapid confirmation of clinical AFB and for identification of bacterial colonies after a cultivation step. The second protocol is a so-called nested PCR that also permits direct analysis of spore solutions. The biochemical profiling of *P. larvae* is based on the catalase test, the production of acid from carbohydrates and the hydrolysis of casein. Further, antibody-based techniques and the microscopic identification of the pathogenic agent are described.

**Serological tests:** There are no serological tests available.

**Requirements for vaccines and diagnostic biologicals:** Monoclonal and polyclonal antibodies produced for the development of diagnostic tests should be sufficiently specific.

### A. INTRODUCTION

American foulbrood (AFB) is an infectious disease of the larval stage of the honey bee *Apis mellifera* and other *Apis* spp., and occurs throughout the world where such bees are kept. *Paenibacillus larvae*, the causative organism, is a Gram positive bacterium that can produce over one billion spores in each infected larva. The bacterium is a round-ended, straight and sometimes curved rod, which varies greatly in size (0.5 µm wide by 1.5 to 6 µm long), occurring singly and in chains and filaments; some strains are motile. The sporangia are often sparse *in vitro*, and the ellipsoidal, central to subterminal spores, which may swell the sporangia, are often found free (16). The spores are extremely heat stable and resistant to chemical agents. Only spores are capable of inducing the disease.

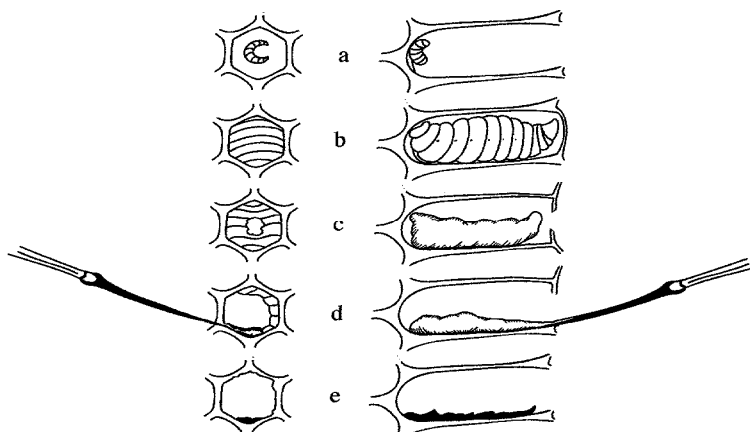
The infection can be transmitted to larvae by nurse bees or by spores remaining at the base of a brood cell. Although the larval stages of worker bees, drones and queens are susceptible to infection, infected queens and drone larvae are rarely seen under natural conditions. The susceptibility of larvae to AFB disease decreases with

increasing age (35); larvae cannot be infected later than 53 hours after the egg has hatched. The mean infective dose ( $LD_{50}$  = spore dose at which 50% of the larvae are killed) needed to initiate infection, though very variable, is 8.49 spores in 24–48 hour-old bee larvae (14). Exchanging combs containing the remains of diseased brood is the most common way of spreading the disease from colony to colony. In addition, feeding or robbing of spore-laden honey or bee bread, package bees and the introduction of queens from infected colonies can also spread the disease. Wax contaminated with the spores of *P. larvae*, which are used in the production of combs foundation, can also spread the disease. The early detection of AFB helps to prevent further spread.

## B. DIAGNOSTIC TECHNIQUES

### 1. Identification of the agent

Diagnosis of AFB is based on identification of the pathogenic agent only. The analyst can rely on a broad range of samples. However, in practice, the samples of choice will depend on whether it concerns a suspicious or diseased honey bee colony/apiary, or analysis in the context of an AFB monitoring/prevention programme. An initial overview of clinical signs of the disease will be provided in this chapter, followed by identification methods that require a previous culturing step, or that can be performed directly on collected samples. The techniques involved are microbiological characterisation, the polymerase chain reaction (PCR), biochemical profiling, antibody-based techniques and microscopy. The analyst should be aware of differences in sensitivity between the presented approaches and should select the most appropriate for a given situation.



**Fig. 1.** Progression of the disease: (a) Point of infection. (b) Larval development to the prepupal stage. (c) Cell contents reduced and capping is drawn inwards or is punctured. (d) Cell contents become glutinous. (e) Residual scale tightly adherent to bottom of cell.

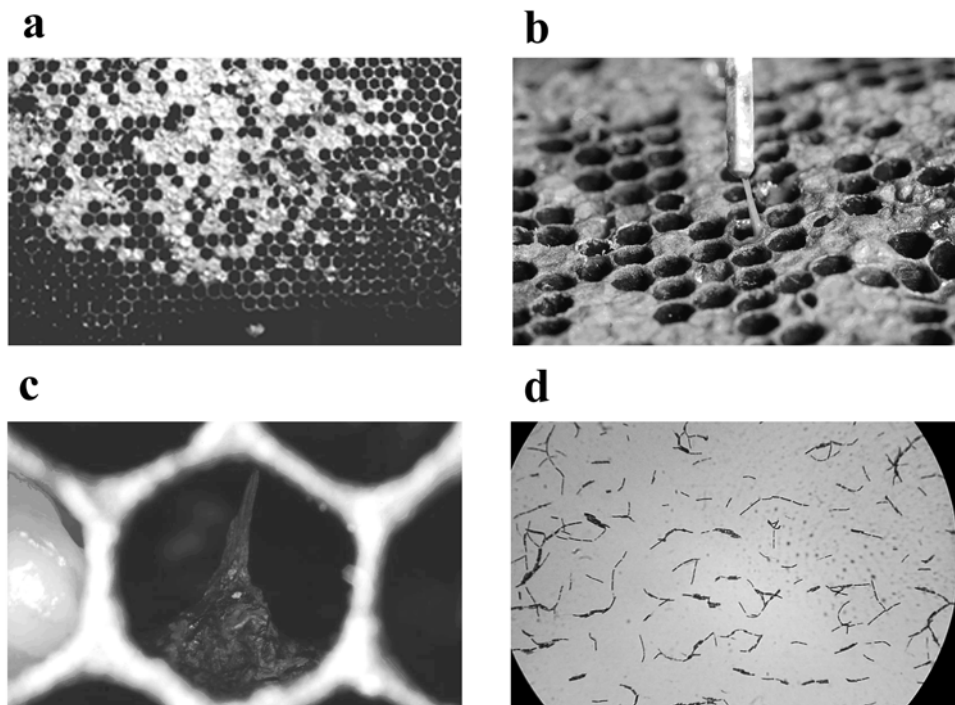
#### a) Epizootology and clinical signs

Spores of *P. larvae* can survive in bee products (honey, wax, dry larval scales) and in the environment for 3 to 10 years and purified spores can survive even more than 70 years (29).

The clinical signs of AFB are very diverse and depend on the genotype involved, the stage of the disease and the strength of the bee colony (and possibly its resistance to AFB). Larvae can be killed rapidly at an early age when they are curled at the base of uncapped brood cells. Adult worker bees will remove these dead larvae leaving only an empty cell (4). Other larvae will die later on in their development, when they are in an upright position, filling most of the brood cell. Often the larvae or pupae will die after brood cell capping.

In severely infected colonies, the combs have a mottled appearance caused by a pattern of healthy capped brood, uncapped cells containing the remains of diseased larvae, and empty cells. The capping of a cell that contains a diseased larva appears moist and darkened and becomes concave and punctured as the infection progresses. Also, the larva or pupa changes colour, first to a creamy and eventually to a dark brown. The larvae can become glutinous in consistency and can be drawn out as threads when a probe is inserted into the larval remains and removed from the cell (match-stick test). This is probably the best-known technique for field diagnosis of the disease, but in some cases the larval remains are rather watery, resulting in a negative match-stick test. Finally, 1 month or more after the larva becomes ropy, the remains of the

diseased brood dry out to form typical hard, dark scales that are brittle and adhere strongly to the lower sides of the cell (Figure 1). If death occurs in the pupal stage, the pupal tongue protrudes from the pupal head, extending to the top of the brood cell or may angle back towards the bottom of the cell. The protruding tongue is one of the most characteristic signs of the disease, although it is rarely seen (Figure 2). The tongue may persist also on the dried scale. European foulbrood needs to be taken into consideration as a differential diagnosis.



**Fig. 2.** Clinical American foulbrood (a-c) and Gram staining (d): (a) Combs have mottled appearance. (b) A matchstick draws out the brown, semi-fluid larval remains in a ropy thread. (c) The formation of a pupal tongue is a very characteristic sign, but rarely seen. (d) Microscopic examination reveals Gram-positive rods, occurring singly and in chains.

## b) Selection of samples

### i) Collection of samples from a suspicious or diseased colony/apiary

While maintaining their colonies, beekeepers often find brood combs with signs of disease. In this case a brood sample can be collected for diagnosis. The brood is sampled by cutting out a piece comb of about 20 cm<sup>2</sup> in size, containing as much of the dead or discoloured brood as possible. An experienced person can collect infected larval/pupal remains directly from the cells with a sterile swab, significantly reducing the sample size and facilitating packaging and sample transportation to the laboratory (see below). When microscopic examination is the method of choice, smears of the remains of diseased larvae can also be made at the apiary (17). After air-drying they can be forwarded to the laboratory.

Every bee colony in the vicinity of such a clinical case of AFB should be considered as suspicious and a broad range of samples should be taken for confirmation. Apart from brood samples, food stores (honey [27, 34], pollen [12] and royal jelly), adult workers (21) and wax debris (32) can be used to detect the presence of *P. larvae* spores. Honey samples can be collected from cells close to the brood with separate disposable spoons to prevent cross-contamination between samples; however, honey may have been sitting in the comb for months at the time of sampling. Adult bees can be shaken or brushed from the combs of the brood chamber or the honey supers into a plastic bag or container. For the most reliable picture of the actual situation, bees from the brood nest (and not the honey supers) should be analysed. Wax debris can be collected at the hive bottom all year round.

### ii) Samples for AFB monitoring/prevention programmes

To prevent the propagation of diseased brood, honey, adult bee and debris samples can be used to detect AFB in colonies where no clinical signs are observed. Routine collection of samples from colonies or from harvested honey can be used as part of an operational or regional AFB detection programme.

Microscopic examination of smears from larvae with no clinical signs is far less sensitive at detecting spores in colonies compared with bacteriological or PCR-based methods. In fact, bacteriological and PCR-based methods will often detect spores in colonies that never develop clinical signs of AFB. High numbers of spores cultured from honey and bee samples using bacteriological methods, however, can often predict the presence of clinical AFB signs at colony, apiary and operational levels.

**b) Packaging and transportation of samples to the laboratory**

Brood comb should be wrapped in a paper bag, paper towel or newspaper and placed in a wooden or heavy cardboard box for transport. Swabs with larval remains can be put into appropriate test tubes with a cap. Holders for microscope slides are commercially available. Adult bees can be kept frozen or submerged in 70% ethanol during transportation, although dried bees are adequate. Food supplies can be put into a test tube or a suitable pot, or wrapped in a plastic bag together with the spoon. Leaking and cross-contamination of the samples must be prevented. If possible, fresh material for laboratory tests should be sent refrigerated.

**c) Sample preparation**

i) Samples for cultivation

In general, an aqueous solution containing *P. larvae* spores should be prepared for further analysis. This spore suspension is heat-shocked at 80°C for 10 minutes or 95–96°C for 3–5 minutes in order to kill other spore-forming microorganisms.

Larval/pupal remains from brood comb are collected with a sterile swab and suspended in 5–10 ml of sterile water or physiological solution (phosphate buffered saline or 0.9% NaCl) in a test tube.

Honey samples to be examined for spores are heated to 45–50°C and shaken to distribute any spores that may be present. Dilution with an equal volume (25 ml) of water permits easier handling. The diluted honey is transferred into 44 mm width dialysis tubing that has been tied at one end. The open end is tied after filling. The tubes are submerged in running water for 18 hours or in a water bath with 3–4 water changes over the same time period. After dialysis, the contents are centrifuged at 2000 *g* for 20 minutes. The supernatant liquid is discarded leaving approximately 1 ml (or less) of residue in each sample. The residue is then resuspended in 9 ml of water (31).

Honey can also be prepared for cultivation without the dialysis step, however this requires longer (30 minutes) and faster (3000 *g*) centrifugation. Likewise, the volume in which the deposit is finally resuspended can be much smaller (200 µl) in order to improve the sensitivity of the test (6).

Direct plating of diluted honey (27) is widely used, but its sensitivity is inferior to that of the centrifugation method as only a fraction of the total volume will be plated out. Whatever the method of choice is, when honey is analysed quantitatively and threshold values are set, the methodology that was used to establish these values should always be strictly followed.

An aqueous filtrate of pollen can be made by thoroughly dispersing 1 g of pollen in 10 ml final volume sterile distilled water and filtering it through Whatman No. 1 paper (12).

When adult bees are dispatched in ethanol, the latter should be decanted and replaced by sterile water or physiological solution before crushing.

Debris and bee wax (1.5 g) should be dissolved in an organic solvent (10 ml): toluene (32), chloroform (19) or diethyl ether (28). The liquid part (2 ml) is then diluted in physiological solution (6 ml). After shaking roughly, this suspension can immediately be plated out (no heat-shock) (32). In another protocol, bee wax is first diluted in water (wax/water 1/10) and heated up to 90°C for 6 minutes. After cooling down, the organic solvent is added (organic solvent/water 1/9) and the mixture is shaken carefully. After 2 minutes standing time, a deposit of a watery solution containing *P. larvae* spores forms (28).

ii) Samples for PCR

Cell/spore suspensions and suspensions containing only spores have to be differentiated, the latter requiring a more complex DNA extraction step (except for the nested PCR).

If the PCR is aimed at identifying bacterial colonies (= cell/spore suspension) after a cultivation step, the pre-treatment is as follows: one colony is suspended in 50 µl of distilled water and heated to 95°C for 15 minutes. Following centrifugation at 5000 *g* for 5 minutes, 1–5 µl of the supernatant is used as template DNA in a PCR 50 µl mixture (9).

For rapid confirmation of clinical AFB, the samples should be prepared as follows: the remains of two diseased honey bee larvae (= cell/spore suspension) are suspended in 1 ml of sterile distilled water and mixed thoroughly. 100 µl of this suspension is diluted with 900 µl distilled water. This dilution is vortexed and 100 µl of it is used to extract DNA by heating and centrifugation (see above) (9).

All aqueous solutions resulting from the sampling of honey, adult bees, debris, bee wax, pollen and royal jelly should be considered as a spore suspension. Here, the extraction of DNA demands another

approach. Indeed, spore suspensions are centrifuged at 6000 **g** and 4°C for 30 minutes. Next, the pellet is subjected to microwave treatment for 5 minutes at maximum power to break the spores, and the released DNA is suspended in 30 µl of 10 mM Tris/HCl, pH 8.0, containing 1 mM EDTA (26).

When spores are to be detected from honey, DNA is serially diluted with sterile distilled water to eliminate PCR inhibition caused by honey (26). Another DNA extraction method, based on lysozyme and proteinase K treatment, has been described (3).

Good results can also be obtained by incubating a pelleted spore suspension in MYPGP broth at 37°C for 2–24 hours. Thereafter, the suspension is centrifuged at 14,500 **g** for 5 minutes, washed with sterile distilled water and resuspended in 200 µl of sterile distilled water. This short incubation step causes spores to germinate, making them sensitive for DNA preparation by heat treatment again (see above) (20).

When the nested PCR is chosen, the spore solution should only be boiled at 100°C for 10 minutes and thereafter centrifuged at 14,500 **g** for 2 minutes. The supernatant can immediately serve as template DNA sample in the nested PCR reaction (20).

#### d) Culture

Several media for cultivating *P. larvae* have been described but best results were obtained with PLA (*Paenibacillus larvae* agar) (30), MYPGP agar (the abbreviation refers to its constituents: Mueller-Hinton broth, yeast extract, potassium phosphate, glucose and pyruvate) (7), BHIT agar (Brain–Heart Infusion medium supplemented with thiamine) (11) and CSA (Columbia sheep blood agar). The formulations of the first two media are as follows:

- PLA

This selective medium combines three different media to comprise the base, to which is added antibiotics and egg yolk supplements (30). Equal quantities (100 ml) of sterile, molten *Bacillus cereus* selective agar base (Oxoid CM617), trypticase soy agar (Merck 5458) and supplemented nutrient agar (SNA) are combined and mixed. SNA is composed of (per litre): nutrient agar 23 g, yeast extract 6 g, meat extract 3 g, NaCl 10 g, Na<sub>2</sub>HPO<sub>4</sub> 2 g: final pH is 7.4 ± 0.2. All solid media are sterilised at 121°C/15 minutes. Nalidixic acid stock solution (18) is prepared by dissolving 0.1 g in 2 ml of 0.1 N NaOH and diluting to 100 ml with 0.01 M phosphate buffer (pH 7.2). Pipemidic acid stock (2) is prepared by dissolving 0.2 g in 2 ml of 0.1 N NaOH and then diluting to 100 ml with the same phosphate buffer. Both antibiotic solutions are filter sterilised.

After the three molten media are combined, 3 ml of stock nalidixic acid, 3 ml of stock pipemidic acid, and 30 ml of 50% egg-yolk suspension (13) is added to form the PLA medium. The PLA medium is poured (20 ml) into sterile Petri dishes and plates are dried before use (45–50°C for 15 minutes).

- MYPGP agar

MYPGP agar is composed of (per litre): Mueller-Hinton broth (Oxoid CM0405) 10 g, yeast extract 15 g, K<sub>2</sub>PO<sub>4</sub> 3 g, glucose 2 g, Na-pyruvate 1 g and agar 20 g (7). Addition of nalidixic acid and pipemidic acid is as above.

If cultivation of *P. larvae* is hampered by the occurrence of fungi, the addition 16.8 µg/ml medium of amphotericin B (Sigma) works very well.

A sterile cotton swab is used to transfer a portion of the sample on to the surface of the solid medium. For a quantitative evaluation, it is recommended to spread a fixed volume of the suspension on the solid agar with a sterile scraper or pipette rather than using cotton swabs.

Inoculated plates are best incubated at 34–37°C for 2–4 days in an atmosphere of 5–10% CO<sub>2</sub> in air, although aerobic incubation will do as well.

#### e) Identification

##### i) Colony morphology

Samples from clinically diseased larvae will result in confluent grown plates after 2–4 days, leading to a subculturing step in order to isolate colonies.

On PLA, colonies of *P. larvae* are small, pale green to yellow (= the same colour as the medium), with a slightly opaque and rough surface; sometimes the centre is raised.

On MYPGP agar, colonies are small, regular, mostly rough, flat or raised and whitish to beige coloured.

On Columbia sheep blood agar, colonies are small, regular, glossy, butyrous and greyish.

*Paenibacillus larvae* colonies with orange to red pigmentation have been described (10, 22).

It is advised to run *P. larvae* reference strains in parallel, for instance LMG 9820 (other designation: ATCC 9545, DSM 7030) for the non-pigmented variant and DSM 16115 or DSM 16116 for the pigmented genotype.

A proven positive brood or honey sample can serve as a positive control for the entire examination.

Colony morphology is not conclusive but might serve to select the bacterial colonies for further identification.

ii) Polymerase chain reaction

PCR reactions are set up as 50 µl mixtures containing 5 µl template DNA (see sample preparation), 50 pmol forward (AFB-F) and reverse primer (AFB-R; primer sequences are given below), 10 nmol of each deoxynucleoside triphosphate and 1–2.5 U of *Taq* polymerase, in the appropriate PCR buffer (provided together with *Taq* polymerase) containing 2 mM MgCl<sub>2</sub> (ref. 9 with modifications). Reducing the volume of the PCR mixtures to 25 µl is possible. Amplification of a specific DNA fragment occurs in a thermocycler under the following PCR conditions: a 95°C (1–15 minutes) step; 30 cycles of 93°C (1 minute), 55°C (30 seconds), and 72°C (1 minute); and a final cycle of 72°C (5 minutes).

Nested PCR comprises an external and an internal amplification step (20). The external amplification is performed using primers PleF and PleR (see below). Each 50 µl PCR reaction contains: 10 µl template DNA (see sample preparation), 1 × PCR buffer (with 1.5 mM MgCl<sub>2</sub>), 0.5 µM PleF primer, 0.5 µM PleR primer, 0.2 mM of each dNTP, additional 0.75 mM MgCl<sub>2</sub>, 1.25 U *Taq* polymerase. A 'touchdown' PCR protocol was performed in which annealing is lowered by 0.5 C/cycle, from 69 to 59°C, for a total of 20 cycles with each annealing step lasting 30 seconds. Twenty more cycles are then performed with the annealing temperature at 59°C for 30 seconds. Denaturation steps are all executed at 94°C (for 30 seconds) and extensions at 72°C (for 45 seconds). Following this, a final extension at 72°C for 5 minutes is performed, and then the reaction is cooled at 4°C. Internal amplification is performed using primers PliF and PliR (see below). Each 50 µl PCR reaction contains 1 µl of the external PCR amplification, 1 × PCR buffer (with 1.5 mM MgCl<sub>2</sub>), 0.5 µM PliF primer, 0.5 µM PliR primer, 0.2 mM of each dNTP, additional 1 mM MgCl<sub>2</sub>, 1.25 U *Taq* polymerase. Cycling conditions are: 94°C (30 seconds), 59°C (30 seconds), 72°C (45 seconds) for a total of 30 cycles followed by 5 minutes at 72°C and then the reaction is cooled at 4°C.

The molecular weights of the PCR products are determined by electrophoresis in a 0.8% agarose gel and staining with ethidium bromide.

Ref.	Name	Sequence	PCR-product size	Specificity level
(9)	AFB-F	5'-CTT-GTG-TTT-CTT-TCG-GGA-GAC-GCC-A-3'	1106 bp	species
	AFB-R	5'-TCT-TAG-AGT-GCC-CAC-CTC-TGC-G-3'		
(20)	PleF	5'-TCG-AGC-GGA-CCT-TGT-GTT-3'	969 bp	species
	PleR	5'-CTA-TCT-CAA-AAC-CGG-TCA-GAG-3'		
	PliF	5'-CTT-CGC-ATG-AAG-TCA-TG-3'	572 bp	
	PliR	5'-TCA-GTT-ATA-GGC-CAG-AAA-GC-3'		

iii) Biochemical tests

*Paenibacillus larvae* can be also be identified by its biochemical profile. The bacteria are catalase negative or weak delayed positive, they have a typical carbohydrate acidification profile with acid from glucose and trehalose, not from arabinose and xylose, and they can hydrolyse casein or milk. Some strains of *P. larvae* can change the biochemical signs.

- Catalase test

A drop of 3% hydrogen peroxide is placed on an actively growing culture on solid medium. Most aerobic bacteria break down the peroxide to water and oxygen, producing a bubbly foam, but *P. larvae* is negative or weak delayed positive for this reaction (15). When Columbia sheep blood agar is used for cultivation, the test cannot be done on the solid medium, as the presence of sheep blood will cause a false-positive reaction. In this case, colonies should be transferred to a clean microscope slide for the execution of the test. Here the evaluation of the test occurs as above with the naked eye.

- Production of acid from carbohydrates (13)

Bacteria are grown in J-broth (per litre: yeast extract 15 g, tryptone 5 g and  $K_2HPO_4$  3 g) in which 0.5% of the test substrate, separately sterilised in aqueous solution, is substituted for the glucose. The carbohydrates used are L (+)-arabinose, D (+)-glucose, D (+)-xylose and D (+)-trehalose. The cultures are tested at 14 days by aseptically removing one ml or less to a spot plate, mixing the sample with a drop of 0.04% alcoholic bromocresol purple, and observing the colour of the indicator. *Paenibacillus larvae* produces acid aerobically from glucose and trehalose. No acid is produced from arabinose and xylose (1).

The use of commercial kits, such as API 50 CHB (5), BBL CRYSTAL (8) and Biolog system (22) for the biochemical characterisation of *P. larvae* can be taken into consideration.

- Hydrolysis of casein (30)

Casein hydrolysis is assayed using milk agar plus thiamine (per litre: agar 20 g, yeast extract 10 g; sterilised at 121°C/15 minutes). Add to each 70 ml cooled medium 30 ml of UHT (ultra heat treated) skimmed milk and 1.5 ml filter sterilised 0.1% thiamine solution. Plates are streaked and examined after 5 days of incubation at  $36 \pm 1^\circ\text{C}$ . *Paenibacillus larvae* hydrolyses casein, hence zones of clearing are observed around bacterial colonies.

iv) Antibody-based techniques

Different antibody-based techniques have been developed for the diagnosis of AFB. Most of them rely on polyclonal rabbit serum developed against pure cultures of *P. larvae*. They can be used for identification of bacterial colonies resulting from a culturing step or for direct examination of suspicious larval remains.

In an immunodiffusion test the antibodies interact with the bacterial antigen during a double diffusion process, leaving precipitation marks behind (25). In the fluorescent antibody technique these antibodies are conjugated with a fluorochrome dye. The resulting fluorescent antibody reacts with a bacterial smear on a slide. Any excess antiserum is washed off and the smear is examined by fluorescence microscopy. *Paenibacillus larvae* stains can be recognised specifically as brightly fluorescing bacteria on a dark background (24, 33, 36). An enzyme-linked immunosorbent assay using a monoclonal antibody specific to *P. larvae* exists (23). A lateral flow device for rapid confirmation of AFB has been commercialised.

v) Microscopy

Two microscopic techniques are commonly used. Gram staining is often done on smears of bacteria from isolated bacterial colonies. *Paenibacillus larvae* is Gram positive. Carbol fuchsin staining is done on larval smears and can confirm clinical AFB based on spore morphology. These techniques are outlined below:

- Gram staining of bacteria

Flood (cover completely) the entire slide with crystal violet. Let the crystal violet stand for about 60 seconds. When the time has elapsed, wash the slide for 5 seconds with water. The specimen should appear blue-violet when observed with the naked eye. Now, flood the slide with the iodine solution. Let it stand for about a minute as well. When the time has expired, rinse the slide with water for 5 seconds and immediately proceed. At this point, the specimen should still be blue-violet. This step involves addition of the decolouriser, ethanol. This step is somewhat subjective because using too much decolouriser could result in a false Gram (-) result. Likewise, not using enough decolouriser may yield a false Gram (+) result. To be safe, add the ethanol drop-wise until the blue-violet colour is no longer emitted from the specimen. As in the previous steps, rinse with the water for 5 seconds. The final step involves applying the counter-stain, safranin. Flood the slide with the dye and let this stand for about a minute to allow the bacteria to incorporate the safranin. Gram-positive cells will incorporate little or no counter-stain and will remain blue-violet in appearance. Gram-negative bacteria, however, take on a pink colour and are easily distinguishable from the Gram positives. Again, rinse with water for 5 seconds to remove any excess of dye. Blot the slide gently with bibulous paper or allow it to air dry before viewing it under the microscope.

- Carbol fuchsin staining of larval smears (17)

Heat-fix smears. Flood the slides with 0.2% carbol fuchsin for 30 seconds. Wash off the stain and allow to air dry or gently blot dry with absorbent material. Examine under the microscope for *P. larvae* spores, which are about  $1.3 \times 0.6 \mu\text{m}$ , ellipsoidal and thick rimmed.

## 2. Serological tests

No serological tests are available.

## C. REQUIREMENTS FOR VACCINES AND DIAGNOSTIC BIOLOGICALS

### 1. Antibody production

VITA diagnostic kit for the early detection of AFB was developed by the Central Science Laboratory Pocket Diagnostic (UK).

When monoclonal or polyclonal *P. larvae*-specific antibodies are produced for the development of a diagnostic test, no cross-reactivity may occur with closely related bacteria or bacteria that commonly occur in beehives, for example against *Paenibacillus alvei*, often found in late phase European foulbrood.

#### o Acknowledgement

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**NB:** There are OIE Reference Laboratories for Bee 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](http://www.oie.int)).