Reference standards for vaccine-producing laboratories

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Summary
The inherent variability of the biological systems used for production of vaccines is much greater than that encountered in the production of pharmaceuticals. Therefore, producers of vaccines must take particular care to ensure that the different batches of a vaccine are of reasonable consistency and that the immunising activity of each batch is equivalent to that of the vaccine originally shown to be effective in the target species. This struggle for consistency has been the main motive for developing the range of reference standards which serve as fixed reference points during the production and quality control of a vaccine.

Keywords

Introduction
Reference standards are necessary for the production and control of virtually all vaccines. Although there is great variation in the types of vaccines produced, at some stage in production the use of reference sera, reference antigens or other reference preparation will almost always be necessary. Since these standards are key features in production, they have to be properly established, maintained and monitored to avoid drift in the quality of the vaccine that would otherwise go unnoticed.

The fact that reference standards are the fixed points for judging the consistency of the manufacturing and quality control of vaccines indicates the importance of establishing and maintaining the standards properly and according to well-defined procedures. Reliance on a poor standard can give a false sense of security: the producer would be like the captain of a ship taking guidance from a lighthouse or beacon that is itself moving. For these reasons, as much – or probably more – care must go into the preparation, testing and maintenance of standards as into the preparation of the vaccine itself, if consistent quality is to be maintained during long-term production.

Reference standards can be either ‘primary standards’ or ‘secondary standards’. Primary standards are often established and distributed by public institutions, particularly those with responsibilities for licensing and control of biologicals. Secondary standards are established by calibration against a primary standard that will continue to be available for recalibration or preparation of future secondary standards. When a producer prepares a reference standard for internal use in the production facility, this may be either a primary standard or a secondary standard; such a standard is often referred to as an ‘in-house standard’ and, if it is a secondary standard, it will often be called a ‘working standard’.

Sources of reference standards
Many reference standards are available from public institutions such as national veterinary laboratories. Such standards provide a common basis for test methods to be applied by producers and control authorities, and play an important role in standardisation. The World Health Organisation (WHO) and the Office International des Epizooties (OIE) have also established collections of reference standards that can be of use to producers of veterinary vaccines. In Europe, where quality standards for veterinary vaccines are included in the European Pharmacopoeia, the latter also provides a number of reference standards. Type-culture collections are a valuable source of reference strains for viruses, bacteria and cell cultures. Standards provided by public institutions may be intended for use by
Establishment and maintenance of a collection of reference standards

Facilities for processing, filling and long-term cold storage are needed for collections of reference standards. Laboratory facilities for control purposes will usually also be necessary. Certain operations can be subcontracted, for example filling of vials or ampoules. Since vaccine production depends on the availability of reference standards, storage at more than one site should be envisaged to protect against accidental destruction of the stock by fire or power failure with consequent loss of cold-storage.

If reference standards are to be provided to different producers from a central source (for example, a national veterinary laboratory) the question of transport logistics must also be addressed. Reference standards may have to be shipped at refrigerator temperature (+5°C) or freezer temperature (−20°C to −80°C). Organising such transport and ensuring maintenance of the cold chain requires careful planning and a thorough knowledge of the working methods of the carriers to anticipate the parts of the process that can (and inevitably will, sooner or later) go wrong. International distribution poses special problems because of possible extra delays at customs and restrictions on transport of materials of biological origin. If such restrictions are prohibitive, then local stock-holding can be envisaged and it is then necessary to ensure storage in identical conditions to maintain identity of the different fractions of a given reference standard.

Record keeping is an essential part of the maintenance of a collection of standards. ISO Guide 34: Quality system guidelines for the production of reference materials gives an account of the principles involved (1). A record should be kept for each standard and should be properly updated in accordance with procedures established at the same time as the standard. Full information on the storage conditions is an important requirement. The quality of the standards is guaranteed in part by correct storage and it is therefore important for the custodian to be able to demonstrate with appropriate records that the correct conditions have been used throughout the life of the batch. Record keeping by users of standards is also important. The dates of receipt, the batch numbers, the storage conditions and the date of use should be noted.

Units

Where a standard has a value assigned to it for some property, the units used are usually arbitrary and unless there have been studies in a number of laboratories, they must often be assumed to be applicable only within the test system used for establishing the standard. For example, unless there is a common reference standard, antibody levels measured in ‘enzyme-linked immunosorbent assay (ELISA) units’ will hardly be comparable between laboratories. Public standards often have officially recognised units; the WHO assigns values in International Units to the standards it issues and the existence of such units provides a valuable basis for standardisation.

The units assigned to biological reference standards are usually to be considered as units of biological activity. Strictly speaking, expression of the biological activity of a test preparation in terms of arbitrary units by comparison with a reference standard is valid if the two preparations are identical in composition, or if one can be considered to be a ‘dilution’ of the other, but not necessarily in other circumstances. In practice, the two preparations rarely fulfil this condition but if the test preparation is of consistent composition and the usual conditions of statistical validity for analysis of the results are met, then comparison with the reference standard will provide a reproducible and meaningful measure of the biological activity of the preparation within a given test system. It is extremely important to remember that any change in the method of production of the test material or any change in the test system will necessitate revalidation of the reference material. If there is a change in the method of production, it will probably be necessary to repeat target-animal testing and establish correlation with the reference material again. Where there is a change in the test system, comparison of the results in the old and new test systems is essential to verify the equivalence of the units, but in itself this may not be sufficient; and an external validation criterion is preferable; for potency testing, target-animal tests may need to be repeated if there is no accepted correlate of efficacy to serve as a basis.
Regulatory assessment of a standard

When regulatory approval is sought for a vaccine, the supporting file must contain information on the standards to be used. Assessment of the standard has to be seen as an integral part of the regulatory process. If the reference standard is available from a public institution, the assessment will usually be simplified since it can be assumed that the standard has been established in accordance with the accepted guidelines and continuing supplies of a suitable preparation will be available. The suitability of the public standard for the particular purpose and the particular vaccine must nevertheless be assessed from the information provided with the standard. When the reference standard has been prepared by the vaccine producer, a more thorough assessment is needed to ensure that the points listed below under 'Suitability of reference standards' are met.

Suitability of reference standards

When the establishment of a standard is envisaged, a number of critical questions have to be answered, as follows:

a) Is the standard representative of the activity to be measured?

b) Is the standard sufficiently stable?

c) Does the standard possess the required purity (freedom from contaminating bacteria and viruses) and specificity (freedom from unwanted antigens or antibodies) for the intended use?

d) How will the standard be qualified as suitable for the intended purpose?

e) For standards used in potency testing, is there sufficient information on the correlation of the activity being measured and the immunising potency in the target animal?

f) How will the potency, or other declared value, be assigned?

A batch of candidate reference standard can only be regarded as qualified when all the relevant questions in the above list have been addressed and the necessary testing performed.

Presentation of standards

The WHO recommends the use of sealed glass ampoules for its standards (7). This requires special equipment for sealing large numbers of ampoules by fusion of glass. Glass ampoules have the advantage that there is virtually no exchange between the contents of the ampoule and the surrounding atmosphere, and storage at low temperature does not affect the quality of the closure. It will therefore give the best guarantee of stability where this is an important factor. Many standards, such as reference sera, which are known to have good stability are presented in vials with elastomer closures and this has been found to give satisfactory results.

Stability of reference standards

The use of reference standards as fixed points in the manufacturing and control process implies that these standards must be stable. This presents considerable problems for biological preparations and stability considerations are often a key element in deciding on the type of standard to use. Preparing the standard in a stable form can present difficulties and stability testing of the standard once prepared requires specially designed techniques.

Many vaccines are presented in liquid form, particularly if they contain an adjuvant, and cannot be frozen without adversely affecting the immunogenic activity. The period of validity of such vaccines ranges from one to five years and this is rarely sufficient for use as a standard. In addition, even if renewal of the standard at five-yearly intervals is practically possible, when recalibration against the existing standard is used then there may be a drift in the potency acceptance limit on the basis of the standard.

Live virus vaccines can usually be freeze-dried without unacceptable loss of viability and when stored at low temperature in this form experience has shown that these vaccines have good stability. They are frequently stored at -70°C or, where possible, over liquid nitrogen.

Inactivated virus vaccines often cannot be freeze-dried because of the presence of an adjuvant. Freeze-drying often causes changes that affect the potency of the vaccine after reconstitution. It is sometimes possible to stabilise the vaccine. In this case, the standard and the test preparation will have a different composition which may invalidate the potency test if the stabiliser affects the activity of the product. Nevertheless, such standards can often be established successfully.

With inactivated bacterial vaccines, freeze-drying can affect the antigenic properties of the vaccine which usually leads to loss of immunogenicity, and this may be critical for preparation of a stable standard.

Reference sera can usually be freeze-dried without affecting the biological activity noticeably; the other proteins present act as stabilisers for the immunoglobulins and these preparations have been found to have remarkably good stability when stored at low temperature in the dry state.
For some reference standards, stability is not a critical feature. Preparations of live bacteria and viruses used for characterisation purposes rather than for quantitative determinations will usually be subcultured by the user laboratory and some loss during storage is of little importance.

**Stability testing**

Testing standards for stability presents particular problems because the very need for a standard usually implies that there is no absolute measure of the activity represented by the standard. Changes in activity of the standard over time without such an absolute measure are difficult to detect. One strategy that has been developed is the use of accelerated degradation (2, 3, 4, 5). The reference standard is maintained at elevated temperature, or at a range of elevated temperatures, and after a sufficiently long period of time (weeks or months) the samples are compared with a sample stored at as cool a temperature as possible. Using special techniques for statistical analysis, a prediction of the rate of decline in the activity can be made and this will give a good indication of the suitability and 'shelf-life' of a standard. This technique can give useful results only if there is some relevant property of a standard that can be measured with sufficient precision for any effects of the accelerated degradation to be apparent against the background variability of the assay method.

For many standards, accelerated degradation studies will not be possible and other methods of monitoring have to be envisaged when the standard is established. Trend analysis during use of the standard may give useful information on the stability of a standard. For example, if a standard vaccine is used in a challenge test, the ED$_{50}$ (statistical dose required to protect 50% of the experimental animals) of the standard plotted over time for succeeding potency tests should be noted. This is not an absolute measure since there will be variations in the test system, for example, the test animals and the exact challenge dose. Abrupt changes will often not indicate a change in the standard, but an upward trend for the ED$_{50}$ will probably correspond to a decline in activity; such a trend will only be seen over a fairly long period.

Physicochemical methods have also been applied for stability testing (6). Again, these are not absolute methods but, if applied over a period, they can give some assurance that the standard has good stability; physicochemical changes may be evident before there is any noticeable change in biological activity.

For freeze-dried standards presented in vials with elastomer stoppers, periodic determination of water is recommended as a part of the monitoring programme.

When a standard is established, it is essential that a decision is made on any future monitoring and on the length of time for which the standard will be kept in use.

**Information accompanying the standard**

When a standard is provided by a public institution, it must be accompanied by information concerning its intended use, assigned values (where applicable) and any other information needed for proper use of the standard. Standards are established for one or more specific purposes as stated in the accompanying information and use for other purposes is not always possible or advisable. The institution providing the standard will usually be willing to advise users on this matter, and it is essential to seek such advice if a standard is being used other than as stated.

In addition to the minimum amount of information needed for proper use of the standard, it will often be of interest for users to have some further background on the composition of the standard, its method of preparation and the test methods used for qualification. Where relevant, the standard should also be accompanied by a declaration regarding freedom from extraneous agents, particularly exotic pathogens, and any precautions needed in handling should also be stated.

**Calibration of working standards**

National veterinary laboratories and other institutions which provide reference standards may restrict the amounts supplied to producers in order to ensure that stocks of a scarce material will last for a reasonable length of time. This may oblige the producer to prepare a secondary working standard, and the method chosen for calibration is of critical importance. The laboratory which provided the standard will usually be willing to advise on the method to be used for calibrating the working standard. For quantitative tests, it is usually essential to calibrate the working standard in the test system to be used for control of batches of the vaccine. When the working standard is in use, it is good practice to recheck the calibration at regular intervals by testing the primary standard in parallel. The frequency with which this has to be done depends on the nature of the test system.

**Typical uses of reference standards**

Most reference standards used in vaccine production and control are preparations of either antigens or antibodies. The main uses can be summarised as follows:

a) identification of bacteria or viruses in seed lots or production batches (reference sera or reference strains of the bacterium or virus)

b) controls for tests for extraneous agents (reference sera or reference strains of the bacterium or virus)
Standards for potency testing of live vaccines

For live vaccines, reference standards are needed mainly for internal validation of the test system. Potency testing of routine batches of live vaccines usually consists of measurement of the number of viable units per dose; for bacteria the viable count is determined by culture and for viruses the titre is determined by inoculation into a suitable substrate, such as cell cultures or eggs. The number of viable units is regarded as a reliable indicator of potency when the production method is well standardised with the vaccinal strains managed in a seed-lot system. For each vaccine, it is necessary during development studies to determine the minimum titre per dose that will provide immunity in virtually all healthy animals; future batches of vaccine must be shown to have a sufficiently high titre at the time of release to ensure that the minimum per dose will be guaranteed until the end of the period of validity.

When virus titre is used, it is essential that in every test a reference standard is titrated alongside the test vaccine. If the reference standard is an official one, it will have a nominal titre assigned following testing in a number of laboratories, preferably using a common protocol. Such multi-user official standards that can serve for a number of producers and for the official control laboratory can usually be established for viral vaccines; strain differences rarely necessitate the creation of different standards for different strains. For establishing the assigned titre, approximately six laboratories is the minimum needed to obtain a reliable result. If no public standard is available, the producer must establish an in-house standard consisting of a batch or part of a batch of vaccine set aside for this purpose. The nominal titre of the in-house standard will be assigned as the average of all titrations performed on it and is therefore an historical average gradually tending to the 'true' value. When the reference standard is titrated alongside a test vaccine, this constitutes a validity check to ensure that the substrate is neither more nor less sensitive to the virus than when the test system was set up and the minimum immunising virus titre per dose was established.

The producer must establish the range around the assigned value within which a titration can be considered valid. This should be as narrow as possible and may also be modified as data from tests accumulate. A typical value would be ±0.5 log_{10}, which may seem a wide tolerance – it covers a range from approximately a third to three times the assigned titre – but in practice, better repeatability of virus titrations is difficult to achieve. The expression of virus titre of the test preparation with reference to the standard is not common practice. It might be expected to lead to better reproducibility between laboratories but this improvement is not always found.

For live bacterial vaccines, such reference standards are useful for checking the medium to be used but once the suitability of the medium has been established, inclusion of a reference in routine testing is not usually needed, unless the micro-organism is known to be particularly fastidious or sensitive to inhibitors that may be present in media.

Standards for potency testing of inactivated vaccines

For potency testing of inactivated vaccines, a number of test models are currently used depending on the type of vaccine, and many of these include a reference standard since this will usually give more reliable and reproducible result.

**Challenge models**

Some inactivated vaccines are routinely tested for potency using challenge models in laboratory animals or occasionally in the target species. Where a stable reference standard vaccine can be prepared, this will usually be used in such a test. This type of model, which involves comparison of the test vaccine with a reference standard of known efficacy in the target animal, is preferable to a model in which protection of a defined proportion of the test animals is required because of the uncontrollable variability in test animals. Vaccines for rabies and for swine erysipelas are tested for potency in this way by vaccination of mice followed by virulent challenge; the potency of the test preparation is expressed relative to the standard by comparing the degrees of protection. For some clostridial vaccines, there is no stable reference vaccine and the pass criterion for the potency test is expressed as an absolute proportion of protection for the test animals.

**Serological models**

Serological models will usually be preferred to challenge models since they cause less distress to the test animals; however, their use requires establishment of correlation between the immune response that can be measured after vaccination of laboratory animals and the immunising activity in the target animal. Universally valid correlation has been established in a few cases, but more often producers have to demonstrate the correlation for their particular vaccine, and
the reference serum established for batch potency testing is therefore applicable only to this vaccine. Where a serological model is used, the reference serum is best prepared using a vaccine of minimum acceptable potency that has been tested in the target animal. The acceptance level for production batches can then be set in terms of the antibody level of the reference serum; normally the production batches should produce antibody levels that are not less than those found for the reference serum produced in the batch tested in animals.

The three Rs: replacement, reduction and refinement of the use of experimental animals

Vaccine production and control still require the use of relatively large numbers of animals and there has been less progress in the three Rs in this field than in some others. Scientific and technical advances of recent years do hold a lot of promise for progress but frequently the availability of suitable reference standards will be essential for introduction of new methods. For routine potency testing, serological methods cause less distress than challenge tests but require correlation studies and the use of a reference standard. In many instances, the necessary knowledge on serological correlates of protection is not available, but where it is every attempt should be made to establish the necessary reference standards needed for introduction of a serological model. For tetanus vaccine, the challenge test can now be replaced by a serological test in guinea-pigs or rabbits. A homologous reference serum (i.e., a serum originating from the test species) is needed in each case. The present international standard consists of equine serum and can be used for calibration of the guinea-pig or rabbit reference sera to give some continuity in the units. For other clostridial vaccines in which toxin-neutralisation tests in mice are used for determining antibody levels, alternative, in vitro tests have been developed and now have their place within the control scheme for these products; they may not completely replace the test in mice but will be widely used for routine testing, thus leading to a decrease in the overall numbers of animals needed. The availability of a reference serum for such tests will promote their introduction. Similar developments can be expected for other vaccines, for example the swine erysipelas vaccine. The provision of suitable reference standards by public institutions will thus be an important factor in the promotion of animal welfare.

Standards de référence pour les laboratoires fabriquant des vaccins

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Résumé
La variabilité inhérente aux systèmes biologiques utilisés pour la mise au point de vaccins est bien plus importante que pour la fabrication de produits chimiquement définis. Dès lors, les fabricants de vaccins doivent veiller à ce que les différents lots d'un même vaccin soient raisonnablement homogènes et que le pouvoir immunogène de chaque lot soit équivalent à celui du vaccin ayant, au départ, fait la preuve de son efficacité chez l'espèce à laquelle il est destiné. Ce souci d'homogénéité a été le principal facteur à l'origine du développement d'une série de standards de référence applicables lors de l'élaboration et des contrôles de qualité d'un vaccin.

Mots-clés
Estándares de referencia para los laboratorios fabricantes de vacunas

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Resumen
Los sistemas biológicos utilizados para fabricar vacunas presentan un grado de heterogeneidad intrínseca mucho mayor que los procesos de fabricación de productos farmacéuticos. Por este motivo, los fabricantes de vacunas deben extremar las precauciones para garantizar que los distintos lotes de una misma vacuna guarden un nivel razonable de homogeneidad, y que la actividad inmunógena de cada lote sea equivalente a la de la vacuna original con la que se hicieron los ensayos sobre la especie destinataria. Es este esfuerzo permanente por asegurar la homogeneidad lo que ha propiciado el desarrollo de estándares de referencia que sirven para encuadrar y dirigir todo el proceso de fabricación y de control de calidad de una vacuna.

Palabras clave

References