EXPERT REVIEWS

# Replacement, reduction and refinement alternatives to animal use in vaccine potency measurement

*Expert Rev. Vaccines* 8(3), xxx–xxx (2008)

Coenraad FM Hendriksen
*Netherlands Vaccine Institute (NVI), PO 11, 3720 AL Bilthoven, The Netherlands*
*Tel.: + 31 302 742 503*
*coenraad.hendriksen@nvi-vaccin.nl*

Models to measure potency in vaccine research and development and preclinical testing are frequently based on an immunization – challenge procedure in laboratory animals. These models have proven to be very instrumental in scientifically underpinning the correlation of protection of selected vaccine antigens and their efficacy. *In vivo* models in vaccine research and development are, for the time being, irreplaceable, although significant progress has been made in using *in vitro* prescreening tests to evaluate particular immunological parameters. For a long time, *in vivo* potency tests have been similarly relevant for routine vaccine lot-release testing. The design of a potency test defined in most pharmacopeias, relied on a direct or indirect challenge-procedure in laboratory animals. For various reasons, there now is an increased interest in the development of alternatives to the current *in vivo* potency tests. Animal models have their limitations, with respect to their relevance, reliability, costs and moral acceptability. All alternative approaches have in common that they ultimately result in a refinement, reduction or replacement in the use of animals. The new models range from modifications of the existing *in vivo* test procedure (e.g., use of humane end points or serology instead of challenge) to *in vitro* antigen quantification tests. A new paradigm in quality control of vaccines is the consistency approach. This approach is state-of-the-art in quality control of the new generation vaccines, and is now finding its way into quality control of traditional vaccines. The consistency approach implies the use of a set of parameters to constitute a product profile, which is monitored throughout production, and which guarantees that each lot released is similar to a manufacturer-specific vaccine of proven clinical efficacy and safety. Consistency relies heavily on the implementation of quality systems, such as good manufacturing practice and quality assurance, and on the use of *in vitro* analytical tools, such as immunochemical and physicochemical tests.

**Keywords:** 3Rs • consistency • *in vitro* • laboratory animal • quality control • serology • vaccine

Author Proof

Some procedures that are applied in biomedical research and testing hardly undergo any change from their early development onwards. For a long time, this was also true for measurement of vaccine potency. In his studies on the development of an anthrax and rabies vaccine, Louis Pasteur was one of the first researchers to identify the capacity of a vaccine to induce a protective immune response to an immunization and subsequent challenge with the virulent microorganism in the target animal or in a susceptible animal species [1]. Although the model has been modified several times since then in order to improve its relevance and statistical significance, the principle of the immunization – challenge procedure in laboratory animals, either directly or indirectly, is still being used, both in vaccine research and development and routine lot-release testing. There are reasons for a change, particularly for lot-release testing, if the animal model is a surrogate for the target species. By definition, this is the case for vaccines for human application. Major limitations of the challenge models are: the use of virulent microorganisms or toxins that may pose a potential risk to those working in the laboratory; the increasing evidence that some surrogate models for potency testing poorly predict the efficacy of the vaccine in the target species; and above all, that the demonstration of potency requires substantial numbers of

 10.1586/14760584.8.3.xxx © 2009 Expert Reviews Ltd ISSN 1476-0584 

laboratory animals and might inflict high levels of pain and suffering in non-protected animals. Although no absolute figures can be given, it has been estimated that potency testing of vaccines is one of the major purposes for which laboratory animals are required within biomedical research and testing [2]. An indirect reason for a change is that vaccine production technology has been optimized in the last few decades, resulting in increased product standardization, improved product characterization and the implementation of product monitoring systems, such as good manufacturing practice (GMP) and quality assurance (QA).

In vaccine research and development and preclinical testing there will be a continuous need for *in vivo* functional models, such as immunization – challenge, to demonstrate the role of selected antigenic structures in protection, as well as the interaction between vaccine components. However, the contributing role of *in vitro* models for prescreening purposes is increasing and provides an extensive data set before starting *in vivo* research.

This paper will provide a review of new developments in vaccine potency testing, thereby differentiating between vaccine research and development, and lot-release testing. First, an overview will be given of the traditional animal models. Several of these models have now been replaced by serological tests, using immune responses such as antibody induction, as the test parameter. *In vitro* potency testing is an approach that is state-of-the-art for live attenuated vaccines, but which is not yet widespread for inactivated vaccines. Nevertheless, progress has been made in the last decade, which will be summarized and illustrated by a few examples. Second, a paradigm shift in vaccine quality control will be discussed, based on demonstration of consistency in production, which might allow for a more generic replacement of the animal models in vaccine quality control by *in vitro* testing. Finally, attention will be given to factors that might slow down progress in the introduction of alternatives, particularly test validation and harmonization of guidelines. Although the principles discussed in the paper are relevant for both human and veterinary vaccines, the emphasis on examples given is mostly in the area of human vaccines.

## Potency testing in the historical context

Immunobiologicals, such as antisera and vaccines, are complex products produced by complex manufacturing procedures. Consequently, many variables might influence the quality of the product, which makes extensive quality control of each lot produced essential and even mandatory. Testing for potency is one of the main pillars for assuring the quality of the vaccine. Potency can be defined as the measure of one or several parameters that have been shown to be related directly or indirectly with product efficacy (the ability to produce an effective level of protection in the target species) [3].

One of the pioneers in the field of potency testing was Paul Ehrlich (1854–1915). His major area of interest was the standardization of diphtheria and tetanus antisera, products whose therapeutic effect could differ from batch to batch. In 1897, Ehrlich described an assay method that went down in history as the German method [4] or indirect protection test. The method was based on mixing serial dilutions of the antiserum with a fixed amount of diphtheria or tetanus toxin prior to injection of these mixtures into laboratory animals. Depending on the protective antibody titre, subsequent toxin–antitoxin mixtures would vary in their state of neutralization from fully neutralized toxin to free toxin. The parameter being evaluated was the first serum dilution showing incomplete toxin neutralization, demonstrated by the death of the animals within the specified observation period.

In order to increase standardization and to eliminate the influence of test variables (e.g., variation in sensitivity of the individual animals and differences in toxin virulence), Ehrlich decided to use the toxin-neutralizing power of a particular antiserum as a unit for determination of the potency of an unknown antiserum. He employed an arbitrary antiserum of known potency as a standard and, thereby, introduced the regular use of standard preparations as a unit of measure into the control of sera (and vaccines). Until now, the use of a standard preparation is still an integral part of potency testing for most vaccines.

The principle of toxin neutralization, although without the use of a standard preparation, is still leading in the Code of Federal Regulations (CFR) of the US FDA on potency testing of toxoid vaccines for human application, and in the CFR and European Pharmacopoeia for potency testing of toxoid vaccines for veterinary application. For human toxoid vaccines, the potency test described below is used in the monographs of the European Pharmacopoeia, although alternative test systems increasingly rely upon *in vitro* toxin neutralization procedures.

Following the toxin-neutralization test for potency testing of toxoid vaccines, a second potency method was developed in the 1930s. The test principle was comparable to the immunization–challenge procedure introduced by Louis Pasteur, but modified in order to exclude external variables and to improve the statistical power. An important contribution to the test modifications was given by the German scientist Prigge. In an article published in 1937, Prigge settled on a concrete proposal for the potency test based on the knowledge available:

*"Several groups of guinea pigs are injected with increasing amounts of antigen, ranging from a dose that is ineffective in all animals to a dose that is effective in all. Next, the percentage of animals which is protected when injected with toxin is determined. When the percentage of protected animals is plotted against the antigen dose, an S-shaped dose–response curve or 'efficacy' curve is obtained of the vaccine being tested. The effectiveness of the vaccine is now measured by determining that dose with the same effect as a reference dose of the standard preparation, under the same experimental condition"* [5].

He continued by saying that "a proper determination of the efficacy is only possible when the efficacy curves run parallel". Thus, Prigge introduced test parameters such as dose–response, linearity, parallelism and the vaccine dose that induces protection in 50% of the immunized animals ($ED_{50}$). The design of the direct protection test, also called parallel-line bioassay, is very similar to the assay we use today for potency testing of inactivated vaccine products.

## Critical review of the classical potency tests

The toxin neutralization test and the challenge test have dominated vaccine potency testing for decades, without inferior products arriving on the market. The question of course is whether

Author Proof

this shows the significance of the potency tests or the consistency of a high quality level of production. Nevertheless, for various reasons, the interest in new approaches in potency testing is increasing, particularly for lot release testing. Safety is a matter of concern when working with virulent microorganisms. In general, risks are easier to manage in a cell culture lab or analytical lab than in an animal facility. Although procedures can be performed in safety cabinets and animals can be housed in containment systems (e.g., isolators or individually ventilated cages), injection of virulent material or manipulation of contaminated animals exposes animal technicians to additional risks. Animal testing is also laborious, time consuming and expensive. A potency test requires at least 6 weeks, which is an undesirable aspect considering the shelf life of a vaccine often being less than 2 years. The costs of animal tests are particularly influenced by the specific housing conditions required, such as barrier systems and ventilation equipment, and these costs are expected to rise in the near future due to tighter regulations on housing and care.

A reason of increasing importance is public concern about laboratory animal use. In most western countries, the majority of people tend to be negative about animal experimentation, even moreso when experiments involve severe pain and suffering [6]. Vaccine potency testing requires large numbers of animals, frequently over 100 animals per test, and in case of a toxin neutralization test or a direct challenge test, a significant percentage of these animals will show severe clinical signs. As a consequence, politicians and policy makers often emphasize the development of methods that will refine, reduce or replace the use of animals; the so-called 3Rs [7] that now have become the red line in most animal welfare laws and regulations in our western society.

In addition to economic and ethical reasons, there are also scientific reasons to look for alternative approaches. Some of the potency tests, for example, for the rabies vaccine [8] and for the whole-cell pertussis vaccine [9], are notorious for their poor intra- and interlaboratory reproducibility, urging manufacturers to repeat tests. Other potency tests are questioned for their relevance, and as a matter of fact have never been properly validated as regards their predictive value for clinical efficacy. Thus, for tetanus vaccine, studies have demonstrated a lack of correlation with efficacy in clinical studies [10]. Finally, some models are artificial in their experimental design and do not mimic the human situation (Table 1). For instance, in regulatory required whole cell pertussis potency testing, animals are challenged by the intracerebral route and not by the intranasal route, which is the natural portal of entry for *Bordetella pertussis*. Also, potency testing does not provide any information about the effect of booster immunizations or the time interval for weaning of immunity [11].

The limitations described above are of lesser importance for vaccine research and development than they are for lot-release testing. A crucial step in vaccine research and development is the demonstration of a correlate of protection for the antigen(s) used in the vaccine. The only possible way to show protection is in a functional test by performing a challenge procedure. However, demonstration of consistency is more important in lot-release testing and, consequently, different procedures can be used. These include modifications of the existing *in vivo* models, *in vivo* models based on serology, *in vitro* antigen quantification tests, and physicochemical and immunochemical methods (Figure 1).

## Modifications of the *in vivo* potency tests

Modifications of the classical potency tests particularly aim to reduce numbers of animals or to limit the level of suffering. In the 1980s, animal numbers per vaccine dilution in the direct challenge test were often given as a general guidance in the monographs of the pharmacopeias. These numbers were considered necessary to meet the confidence interval requirements for lot release, as given by the pharmacopeias. The range of the confidence interval is determined by the variation in response of the individual animals. Factors influencing variation are diverse and include, amongst others, the health status and microbiological status of the animals, the skills of the animal technicians and food quality. In time, these factors have been optimized, consequently

**Table 1. Variables which might influence the vaccine-induced protective immunity in the target species or in the laboratory animal model (direct challenge procedure).**

| Variable | Target species | Animal model |
|---|---|---|
| Route | Subcutaneously | In some cases intraperitoneal |
| Dose | One dose | Relatively high, but only part of the dose used with target species |
| Schedule | Usual several injections | Usually one injection |
| Immunogenicity | Sufficient to induce protective immunity | May differ from target species because of protective immunity. May not be measurable if the laboratory animal is not sensitive to the pathogen under study |
| Type of immunity | Humoral-cellular | Humoral-cellular |
| Challenge | Natural infection | Artificial infection by an artificial route; may not be possible if animal is not sensitive to the pathogen under study |
| Memory | Recall injections induce memory | Generally only primary immunization |

Adapted from [11] with permission from S Karger AG, Basel, Switzerland.

Author Proof

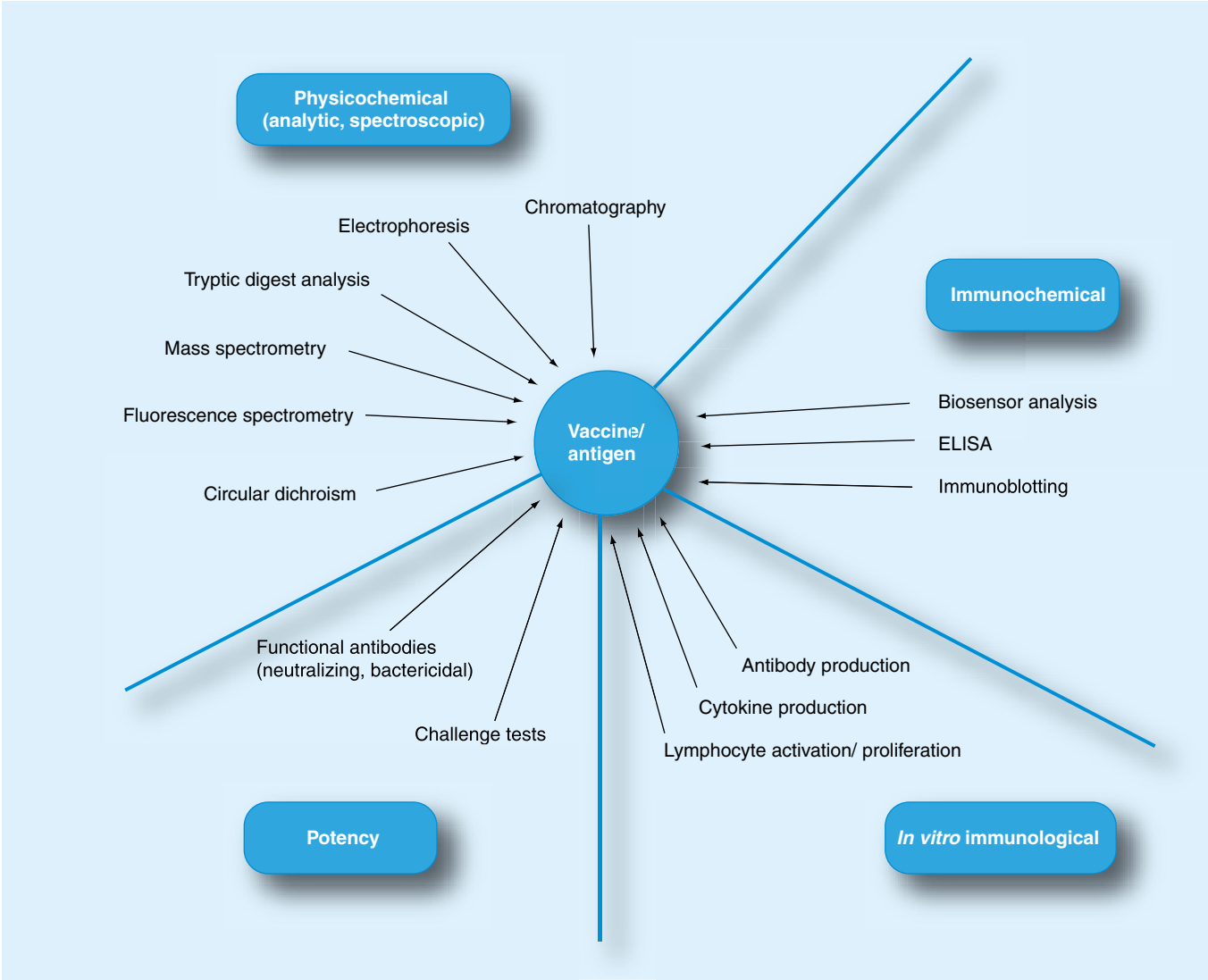


**Figure 1. Possibilities for *in vivo* and *in vitro* analysis of vaccine/protein antigen.**
Adapted from [43].

reducing variation in response and allowing a significant reduction in the number of animals per vaccine dilution, as was shown in several studies [12–14]. Based on the results of these studies, WHO and *Ph.Eur.* have adapted their guidance by indicating that the number of animals to be used should meet the confidence interval criteria specified.

A further reduction in number of animals per test has been achieved in the monographs of the *Ph.Eur.* for diphtheria and tetanus toxoid vaccine by allowing the use of a single-dose potency test instead of a multidose test. As single dose potency testing only shows that the vaccine under study meets the minimum requirement in IU/ml, single dose testing is only an option if consistency in vaccine production has been demonstrated and is being monitored [15].

Refinement can be achieved by replacing crude general end points, such as death or severe clinical findings, by more specific local end points, as has been reported for tetanus (paralytic challenge instead of lethal challenge [16]), diphtheria vaccine (intradermal challenge instead of lethal challenge [17]) and rabies vaccine (peripheral challenge instead of lethal challenge [18]).

A particular approach to limit the level of suffering is the use of humane end points. For many vaccines, pharmacopeias specified (severe) clinical signs or death within the observation period of the potency test as the end point to be met. The idea behind humane end points is to reduce the time interval animals have to suffer by killing the animal in an early stage of disease, keeping in mind the scientific objective of the test. For several products, such as the rabies vaccine [19], the erysipleas vaccine [20] and the whole-cell pertussis vaccine [21], clinical signs or pathophysiological effects have been identified that are predictive for death or severe clinical signs in the observation period, and these end points have been validated in additional studies. Humane end points have been adopted by most pharmacopeias, such as the *Ph.Eur.* and the Code of Federal Regulation. Thus, the *Ph.Eur.* now includes the following statement:

*"…tests must be carried out in such a way as to use the minimum number of animals and to cause the least pain, suffering, distress or lasting harm. The criteria for judging tests in monographs must be applied in the light of this. For example, if it is indicated that an animal is considered to show positive, infected etc. when typical clinical signs or death occur then as soon as sufficient indication of a positive result is obtained the animal in question shall be humanely destroyed or given suitable treatment to prevent unnecessary suffering"* [22].

Implementation of humane end points in the laboratory might face concern, as some fear that replacing an objective end point (death) by a subjective end point (clinical signs) might influence test precision and reproducibility. Another reason is more trivial. Looking for clinical signs requires specific skills of the animal technicians, is time consuming and, consequently, more expensive. However, if properly trained, animal technicians are able to correctly predict the death of the animal within the observation period.

### *In vivo* models based on serology

In case protection to the virulent organism or toxin is based on humoral immunity, a significant modification of the traditional potency test can be achieved by using serology instead of challenge or *in vivo* toxin neutralization. Titration for protective antibodies has been reported in immunoassays such as ELISA or the hemagglutination test. In case of toxin neutralization studies, ELISA or cell culture can be used to measure 'non-neutralized' toxin. Serological approaches are now available for a range of vaccines, including those to a number of clostrial species, diphtheria, pertussis, rabies and leptospiral species. Several of the models have been validated in large scale interlaboratory studies organized by the European Directorate for the Quality of Medicines and HealthCare (EDQM) and, in the case of the tetanus vaccine, in collaboration with the EU European Centre for the Validation of Alternative Methods [23–25]. Based on the outcome of these studies, the *Ph.Eur.* has revised their monographs for these products [26,27], which now include serology as an alternative to using the challenge procedure. Recently, the Steering Committee of the Biological Standardization Programme (BSP) of the EDQM has agreed to start a large-scale multi-laboratory study to the validity of the whole cell ELISA [28] for whole cell pertussis potency testing (Behr-Gross, pers. comm.).

Serology offers a number of advantages in terms of safety, animal welfare, efficiency and test monitoring. First, it is no longer necessary anymore to work with virulent micro-organisms or toxin in the animal facility. Second, as animals are no longer challenged with the virulent microorganism or toxin, pain and distress levels have been reduced from severe to minor. Third, test performance time is shortened by omitting the observation period and using a quantitative end point (antibody titre) instead of a qualitative end point (death or clinical signs). This allows for a substantial reduction in the number of animals required per vaccine dose. A further step in test efficiency can be achieved by combining potency testing of antigens in a combined vaccine preparation, for which a serological approach is available, resulting in an additional reduction in numbers of animals. Systems are now available that allow simultaneous quantification of antibody titres against the various vaccine antigens, such as the multiplex system [29]. Finally, in contrast to challenge procedures, serology allows for storage of test (serum) material. This material can be reused for questions coming from postmarketing surveillance or for retesting of samples from vaccine manufacturers by regulatory authorities. In this case, however, authorities will only confirm the reproducibility of the serological test, without obtaining information about the potency test itself.

### Antigen quantification

*In vitro* antigen quantification is state-of-the-art for lot-release potency testing of live attenuated vaccines or genetically modified live vaccines, either by determining the number of live particles in case of live attenuated bacterial vaccines (e.g., Bacillus Calmette-Guérin, typhoid and cholera) or, in case of live viral vaccines, by virus titration in cell cultures using end points such as plaque formation, cytopathology and, indirectly, virus neutralization by virus-specific serological reagents. Increasingly, *in vitro* antigen quantification tests are also being developed for inactivated viral vaccines. These tests are generally based on binding of key protective antigens to specific antibodies in an *in vitro* immunoassay. Thus, as an alternative to the mouse immunogenicity test for the recombinant hepatitis B vaccine, two types of immunoassays have been developed, one based on direct determination of hepatitis B surface antigen (HbsAg), the other on neutralization of HbsAg and subsequent determination of anti-HbsAg antibodies in ELISA [30,31]. As an alternative to the NIH rabies potency test in mice, ELISAs were developed using monoclonal antibodies directed to the glycoproteins and to the nucleoproteins of the rabies virus [32]. On the condition of proven validity, antigen quantification for rabies vaccine is now accepted by the *Ph.Eur.* for lot-release. In practice, however, the NIH test is still being used, as most validation studies have shown a poor correlation between the NIH data and data from the immunoassays. It is generally believed that this is due to the high variability of the NIH test, rather than to the invalidity of the *in vitro* test [8].

An obstacle in the use of antigen quantification for potency testing of inactivated vaccines is that most products include an adjuvant product, which has to be removed before antigen quantification. Thus, it has to be demonstrated that removal is successful in terms of antigen recovery, as well as in retaining antigen integrity.

### *In vitro* immunological tests

One of the most complex and interactive responses in a vertebrate organism is the immune response, involving various cells of the immune system in a cascade of reactions after contact of the organism with the antigen. This response is highly specific and tailor made both with regard to the type of antigen and the historical context of the exposure of the organism to the antigen. Key actors in the cascade of reactions are antigen

Author Proof

presenting cells (APC), such as monocytes and dendritic cells [33], antibody-producing B lymphocytes, helper T cells, killer T cells and memory B and T cells. These cells are supported by so-called accessory cells (fibroblast, endothelial cells), as well as cell products, such as cytokines, that play an important role in intercellular communication.

Several *in vitro* immunogenicity models are now available, each addressing a functionally relevant part of the immune response. These models vary from simple peripheral blood mononuclear cell (PBMC) cultures to complex co-culture systems, including various types of immune cells [32] as well as accessory cells [33]. Some have shown good correlation between vaccine quality and cytokine profiles after stimulation of PBMCs with tetanus toxoid [32]. However, it is generally believed that *in vitro* immunogenicity models are limited in their availability to correctly mimic the complex immune response [34], particularly when correlates of protection for specific immune parameters have not yet been established. Examples include:

- Each *in vitro* model only represents a particular phase during the development of the immune response. Slight modifications in one model might not be picked up while it might have considerable consequences in the succeeding phase of the immune response;
- Conditions *in vitro* (e.g., antigen dose, cell density and cell–cell interaction) differ substantially from the *in vivo* situation;
- Primary responses are difficult to measure owing to the low number of potential responsive cells. Improvements have been described, such as pulsing of APC with antigen before culturing these cells with B and T lymphocytes;
- *In vitro* models offer the opportunity to use human cells. Although this might seem an advantage, it frequently is not as naive donors are not usually available for many traditional vaccine antigens.

Recently, a complex *in vitro* system (MIMIC™) was introduced by the company VaxDesign. MIMIC is based on three integrated modules: a peripheral tissue model, a lymphoid tissue equivalent module, and a disease model module; each module representing a functional element of the immune response [101]. The MIMIC technology looks promising but still has to demonstrate its relevance in vaccine research and testing.

Considering these limitations, *in vitro* immunogenicity models have been of little help for mandatory required quality control of traditional vaccines to date. However, they play an increasingly important role in vaccine development studies that focus on studying a particular aspect of the immune response, such as the role of APCs for antibody responses [35], or the screening of potential vaccine antigens for immunogenicity.

## The consistency approach in lot-release testing

The discussion on alternatives to vaccine potency testing in the previous sections all started from the paradigm of uniqueness of each lot of vaccine produced. This paradigm has dominated vaccine quality control for decades, particularly with regard to the traditional vaccines. The basic idea behind the uniqueness principle was that quality differences might occur from lot to lot, owing to intrinsic variability of the vaccine production process (i.e., culture, purification, inactivation, filling and formulation) and the complexity of the product itself (i.e., antigenic structure, antigen interactions in case of multicomponent vaccines, additives such as adjuvant and preservatives), urging extensive quality control of each final lot. However, several fundamental changes in the vaccine production process have taken place in the last few decades, offering a platform to reconsider the validity of the uniqueness paradigm:

- Advances made in production technologies (e.g., optimization of culture conditions and improvement of purification procedures) have resulted in more defined products;
- Final lot testing is not a standalone activity, but is supported by extensive in-process testing using *in vitro* immunochemical and physicochemical tests. For instance, the WHO recommendations for diphtheria–tetanus–pertussis vaccines state that "there is a need to support the data generated by a simple potency assay with physical/chemical methods, in order to ensure overall consistency in production" [102];
- Technical improvements of these analytical tools as well as the possibility to apply these tools to a wider range for product characteristics;
- The fact that most vaccine manufacturers have implemented quality systems to the production process, such as the strict rules of current GMP (cGMP) and QA.

These achievements have resulted in increased opportunities to monitor for consistency in production, which, in fact, is the key element of current vaccine manufacturing practice: the consistent production of vaccine lots with similar characteristics to those lots that have been shown to be safe and effective in the target species

**Table 2. Potential physicochemical and immunochemical techniques for quality control of toxoid vaccines.**

| Physicochemical technique | Immunochemical technique (*in vitro*) |
|---|---|
| Circular dichroism | Biosensor analysis |
| Colorimetric assays | ELISA |
| 2D-electrophoresis | Flocculation (Kf and Lf) |
| Differential scanning calorimetry | Immunoblotting |
| Fluorescence | |
| Infrared spectroscopy | |
| Ion exchange chromatography | |
| Isoelectric focussing | |
| Nuclear magnetic resonance | |
| Peptide mapping | |
| SDS-PAGE | |
| Size exclusion chromatography | |

Kf: Flocculation rate in antibody reaction; Lf: Limit of flocculation.
Adapted from [38].

Author Proof

[36]. For more recently developed vaccines (e.g., polysaccharide conjugate vaccines and rDNA vaccines), handling on consistency has resulted in a simplification of release protocols, such as for conventional polysaccharide vaccines, where it has been shown that immunogenicity is largely proportional to their molecular weight. Potency testing, therefore, relies heavily on physical characterization in terms of composition, molecular weight and quantity.

In light of the reasons given above, it is now argued that, for traditional vaccines, the extent of lot release testing should reflect the level of consistency in production that has been obtained with the vaccine [36]. The following performance description has been given for using consistency as a new approach to lot-release testing of the traditional vaccines:

*"The consistency approach implies the use of a set of parameters to constitute a product profile (e.g., antigen content, antigen integrity, purity, etc.) that can replace current release tests. The product profile is established to the satisfaction of the regulators at the time of licensing and is monitored throughout production under a strict quality system. The product profile ensures that each lot released is similar to a manufacturer-specific vaccine of proven clinical efficacy and safety, with respect to all characteristics agreed upon at licensing between manufacturer and regulator"* [36].

In line with the concept of consistency testing, it is believed that components other than testing alone are essential for an operational quality system. These other components include, for instance, a careful validation and maintenance of the manufacturing process and of information regarding the field history of the vaccine, clinical studies (prelicensing and pharmacovigilance data), as well as complete and accurate standard operating procedures. Under these conditions, it is believed that *in vivo* models for potency testing of the final lot can be substituted by a battery of analytical tests that are able to demonstrate equivalence with lots of proven safety and efficacy for a set of critical indicators, preferably also in clinical studies [37]. In this strategy, laboratory animals might only be needed for establishing the product profile. Eventually, and for a restricted period of time, an immunogenicity study in a small set of animals can be performed for lot-release testing in order to provide additional information and confirmation.

The battery of analytical tests should reflect the set of critical indicators and is consequently product specific as well as dynamic in terms of adapting to a change in the product profile. An example of a potential set of physicochemical and *in vitro* immunochemical tests for the quality control of toxoid vaccines has been given by Metz *et al.* (Table 2) [38].

For the time being, implementation of the consistency principle in quality control of traditional vaccines is still a theoretical exercise. A number of fundamental questions still have to be answered [36]:

- Most vaccines include an adjuvant. Little is known about the modulating effect of adjuvant–antigen interaction on the antigen integrity. The same is true for antigen–antigen interactions in combined vaccine products, and for the effect of vaccine impurities and preservatives;
- Additional characterization of protective vaccine antigens by using various technologies, such as biosensor analysis or peptide mapping;
- The vaccine dilution in the final lot: the antigen concentration might be below the level required for some of the *in vitro* physicochemical and immunochemical tests.

## Existing obstacles in test development & implementation in lot-release testing

Although the range of 3R alternatives that has been developed is impressive (Table 3), there are still some scientific hurdles to be overcome, particularly when correlates of protection are difficult to assess in animal models and *in vitro* immunological models or serology, as is the case for vaccines against herpes infections and pandemic influenza [39,40]. Answering the scientific questions given above will be a matter of time. There is an incentive, both at the level of vaccines manufacturers and the regulatory authorities, to move forward as was shown during a recent conference organized by EDQM in Dubrovnik [41]. However, implementation of the consistency approach, as well as the other test modifications described in this article, is not only dependent on scientific progress, but also of overcoming other barriers, such as test guideline harmonization or successful validation.

**Table 3. Summary of 3R alternatives in lot-release potency testing**

| Product | Classical animal model | 3R alternative |
|---|---|---|
| Tetanus immunoglobulin | Toxin neutralization test | ELISA or ToBI test |
| Vaccines for human & veterinary use | Lethal challenge procedure | **Use of humane end point** |
| Diphtheria and tetanus toxoid vaccines | Lethal/clinical challenge | **Serology** |
| Diphtheria and tetanus toxoid vaccines | Multidose testing | **Single dose testing** |
| Hepatitis A vaccine | Serology | **Antigen quantification** |
| Hepatitis B vaccine | Serology | **Antigen quantification** |
| Inactivated polio vaccine | Serology | Antigen quantification |
| HPV vaccine (rDNA) | Serology | **Antigen quantification** |
| Whole cell pertussis vaccine | Lethal challenge | Serology |
| Rabies vaccine | Lethal challenge | Antigen quantification |
| Erysipelas vaccine | Lethal challenge | **Serology** |
| Newcastle disease vaccine | Lethal challenge | **Antigen quantification** |
| Leptospira hardjo vaccine | Lethal challenge | Antigen quantification |

3R alternatives in bold have been accepted by the European Pharmacopoeia

Author Proof

In various parts of the world different sets of test guidelines are being used, such as the monographs of the *Ph.Eur.* in the Member States of the Council of Europe, the Codes of Federal Regulations in the USA and the procedures described in the Technical Report Series of the WHO in most 3rd World Countries. Owing to differences in test guidelines, additional testing might be needed to license a vaccine lot in different parts of the world. Progress in test harmonization has been achieved by the activities of the International Conference on Harmonization (ICH) and the International Cooperation on Harmonization of Technical Requirements for Registration of Veterinary Medicinal Products (VICH). However, potency testing has not been on the agenda yet.

Another obstacle is validation. The aim of a validation study is to demonstrate, usually in a multilaboratory trial, the relevance and reliability of the new test method [42]. Owing to their complex structure, validation studies are time consuming, difficult to organize and expensive. Furthermore, a validation study might be complicated by the poor reproducibility of the traditional animal test, making the demonstration of a high correlation almost impossible. This is, for instance, the case in the validation of *in vitro* antigenicity tests as an alternative to the rabies vaccine NIH test [8]. Nevertheless, validation studies, preferably in close collaboration with regulatory bodies, should be supported as several of these studies have been performed successfully, as described in the section on serological models.

Demonstration of relevance requires concurrent demonstration of the whole range of potencies in a validation study, including high potent and subpotent products, as well as concurrency with clinical data. The problem with traditional vaccines, particularly human traditional vaccines, is that information on clinical data is not available. Additionally, in some cases, subpotent products are not available and have to be produced artificially for the purpose of validation, such as by heating or inactivation. However, these samples are not representative for what could happen in reality [3]. Another strategy is to use expired products with demonstrated low potencies.

Finally, in case of lot-release testing, variation of test performance requires revision of the product registration dossier, which is a time consuming and costly exercise, particularly when the product is a component in various combined vaccines. This is not an incentive for manufacturers to introduce 3R methods. It might be a stimulus for vaccine producers to be rewarded when revisions include a 3R spin-off.

## Expert commentary

For vaccine research and development and preclinical testing, the opportunities for complete replacement of *in vivo* potency models by *in vitro* alternatives is limited although reductions in numbers of animals needed have been achieved by using functional *in vitro* immunological tests for screening of potential vaccine antigens. Progress in this area will continue, although the feasibility of mimicking the complex immune response in a test tube will be limited, at least for the time being. Nevertheless, the implementation of new and robust vaccine production technologies such as in rDNA vaccine and glycol-conjugate vaccine production, together with strict monitoring of the vaccine production process and the availability of new (*in vitro*) functional immunoassays has resulted in much more defined products and consistent production procedures. This has ultimately allowed for lot-release testing that is mainly based on characterizing the vaccine lot by using analytical test tools, such as physicochemical and immunochemical method.

Furthermore, quality control of traditional vaccines, such as toxoids, is now within reach of the consistency approach. Production procedures are more robust than they were in the past, quality systems such as GMP and QA have been implemented in manufacturing facilities and in-process testing is performed on every critical step in the production process. Above all, we now better understand the limitations of current animal models to measure vaccine characteristics relevant for predicting efficacy in the target species. Considerable progress is being made in the development and optimization of analytical tests that can be used for demonstrating consistency. Nevertheless, several fundamental questions still need to be answered, particularly with regard to antigen–adjuvant interactions in the case of combined vaccines. Together with other hurdles to be taken, such as validation and guideline harmonization, it is expected that the implementation of the consistency approach in vaccine quality control as a generic strategy to replace the use of animals will take considerable time. Considering the public concern about animal use, it is recommended to continue 3R activities in replacing potency tests for individual vaccine products. The list of achievements is impressive, taking into account that many more 3R models are about to be validated. However, ultimately, it is the consistency approach that will enable an almost total replacement of animal use in lot-release potency testing.

## Five-year view

Innovative developments in vaccine production and testing, and increasing public concern about laboratory animal use will move vaccine research and development, preclinical testing and regulatory-required quality control to an increased use of *in vitro* and analytical methods, and, ultimately, to a reduction and refinement in animal use. However, progress is tedious, time consuming and dependent of improved *in vitro* imaging of complex immunological processes. This will be particularly true for vaccine research and development and preclinical testing with its fundamental need for models that provide functional information of selected vaccine candidates. Significant achievements in terms of animal use are more likely to occur in regulatory required vaccine quality control. Replacing the current principle of final lot testing by an approach based on demonstration of consistency, particularly in the area of the traditional vaccines would allow for the use of *in vitro* physicochemical and immunochemical methods instead of animal models. In this strategy laboratory animals will only be needed for the characterization of the first few lots produced.

### Financial & competing interests disclosure

*The author has no relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending, or royalties.*

*No writing assistance was utilized in the production of this manuscript.*

Author Proof

## Key issues

- Laboratory animal use has been instrumental to the development of vaccines currently available and, consequently, has contributed significantly to improvements in public health.
- Animals are needed for vaccine research and development and preclinical testing and, particularly, for lot-release testing of traditional (inactivated/detoxified) vaccines.
- Concern about animal use, recognition of the limitations of animal models, and safety and cost considerations have stimulated the interest in models or test strategies that allow for a replacement, reduction or refinement (3Rs) in laboratory animal use.
- In vaccine research and development and preclinical testing, 3R progress will be particularly valuable in improving *in vitro* methodologies to study specific immune responses and in prescreening of vaccine (antigen) candidates.
- In regulatory-required potency testing, substantial progress has been achieved in acceptance and implementation of 3R methods, such as the use of serological models and *in vitro* methods.
- Validation and lack of harmonization in test guidelines might slow down acceptance and implementation of 3R methods.
- A significant step in an almost complete replacement of laboratory animal use could be by implementation of the consistency approach as a new paradigm for lot-release testing in vaccine quality control.
- Consistency testing combines implementation of quality systems, such as good manufacturing practice and quality assurance, in the manufacturer's laboratory with in-progress testing and the availability of innovative analytical tools.
- Validation and lack of harmonization in test guidelines might slow down acceptance and implementation of 3R methods, and having these issues higher on the political agenda might be a step forward.

Author Proof

## References


1 Parish HJ. *A History of Immunization*. E and S Livingstone Ltd, Edinburgh/ London, UK (1965).

2 Weisser K, Hechler U. *Animal Welfare Aspects in the Quality Control of Immunobiologicals. A Critical Evaluation of Animal Tests in Pharmacopoeial monographs*. FRAME, London/Nottingham, UK (1997).

3 Hendriksen C, Spieser J-M, Akkermans A *et al.* Validation of alternative methods for potency testing of vaccines. *ATLA* 26, 747–761 (1998).

4 Ehrlich P. Die Wehrtbemessung des Diphtherieheilserum und deren theoretische Grundlage (Assessing the potency of the diphtheria serum and its theoretical foundations). *Klin. Jahrbuch* VI, 299 (1897).

5 Prigge R. Experimentelle Untersuchungen über die Wirksamkeit von Diphtherie Impfstoffen (Experimental investigations of the activity of diphtheria vaccines). *Deutsch. Med. Wochenschr.* 39, 1478–1482 (1937).

6 Aldhous P, Coghlan A, Copley J. Let the people speak. *New Scientist* 22, 26–31 (1999).

7 Russell WMS, Burch RL. *Principles of Humane Experimental Technique*. Methuen, London, UK (1959).

8 Bruckner L, Cussler K, Halder M *et al.* Three Rs approaches in the quality control of inactivated rabies vaccines. The report and recommendations of ECVAM workshop 48. *ATLA* 31(4), 429–454 (2003).

9 van Straaten-van de Kapelle I, van der Gun JW, Marsman FR, Hendriksen CFM, Van de Donk HJM. Collaborative study on test systems to assess toxicity of whole cell pertussis vaccine. *Biologicals* 25, 41–57 (1997).

10 Relyveld E, Bengounia A, Huet M, Kreeftenberg JG. Antibody response of pregnant women to two different adsorbed tetanus toxoids. *Vaccine* 9 (5), 369–372 (1991).

11 Kreeftenberg JG. Report of an informal meeting about alternative methods for the potency control of the diphtheria and tetanus components in vaccines. *Dev. Biol. Stand*. 65, 261–266 (1986).

12 Stainer DW, Jakus CM, Sparkus JD. Reduction in animal usage for potency testing of diphtheria and tetanus toxoids. *Develop. Biol. Stand.* 65, 241–244 (1986).

13 Knight PA, Roberts PAG. Studies on the minimal number of animals required to achieve assurance of satisfactory potency in diphtheria and tetanus vaccines. *Develop. Biol. Stand*. 65, 245–255 (1986).

14 Hendriksen CFM, Gun van der JW, Marsman FR, Kreeftenberg JG. The effects of reductions in the numbers of animals used for the potency assay of the diphtheria and tetanus components of adsorbed vaccines by the methods of the European Pharmacopoeia. *J. Biol. Stand*. 15, 353–362 (1987).

15 Winsnes R, Hendriksen C, Sesardic D, Akkermans A, Daas A. Serological assays as alternatives to the *Ph.Eur.* challenge test for batch release of tetanus vaccines for human use. *Dev. Biol. Stand.* 101, 277–288 (1999).

16 Mussett MV, Sheffield F. A collaborative investigation of a potency assay for adsorbed tetanus vaccines based on the protection of mice from titanic paralysis. *J. Biol. Stand.* 4, 141–148 (1976).

17 Knight PA. The evaluation of the response of guinea pigs to diphtheria toxoids. *J. Biol. Stand.* 2, 69–78 (1974).

18 Wunderli PS, Dreesen DW, Miller TJ, Baer GM. The rabies peripheral challenge test: more accurate determination of vaccine potency. *Vaccine* 24(49–50), 7115–7123 (2006).

19 Hartinger J, Foltz T, Cussler K. Clinical end points during rabies vaccine control tests. *ALTEX* 18(1), 37–40 (2001).

20 Johannes S, Hartinger J, Hendriksen CF, Morton DB, Cussler K. Humane end points in the efficacy testing of swine erysipelas vaccines. *ALTEX* 20(1), 11–15 (2003).

21 Hendriksen CFM, Steen B, Visser J, Cussler K, Morton D, Streijger F. The evaluation of humane end points in pertussis vaccine potency testing. In: *Humane End points in Animal Experiments for Biomedical Research*. Hendriksen CFM, Morton DB (Eds). The Royal Society Medicine Press, London, UK 106–113 (1999).

22 Anon. Vaccines for veterinary use. *Pharmeuropa* 13, 363–369 (2002).

23 Winsnes R, Hendriksen C. Collaborative study for the validation of serological methods for potency testing of tetanus toxoid vaccines for human use. *Pharmeuropa Spec. Issue Bio.* 2001(2), 1–92

(2000).

24 Winsnes R, Sesardic D, Daas A, Behr-Gross ME. Collaborative study for the validation of serological methods for potency testing of diphtheria toxoid vaccines-part 1. *Pharmeuropa Bio.* (2), 35–68 (2004).

25 Winsnes R, Sesardic D, Daas A, Behr-Gross ME. Collaborative study for the validation of serological methods for potency testing of diphtheria toxoid vaccines-part 2. *Pharmeuropa Bio.* (1), 73–88 (2006).

26 Ph.Eur. *Assay of Diphtheria Vaccine (Absorbed), General Method, 2.7.6. Ph. Eur. (6th Edition)*. Council of Europe, Strasbourg, France (2008).

27 Ph.Eur. *Assay of Tetanus Vaccine (Absorbed), General Method 2.7.8. Ph. Eur. (6th Edition)*. Council of Europe, Strasbourg, France (2008).

28 van der Ark A, van Straaten-van de Kapelle I, Olander R-M *et al.* The pertussis serological potency test collaborative study to evaluate replacement of the mouse protection test. *Biologicals* 28, 105–118 (2000).

29 Uhlrich S. Multiplex systems for serology. Presented at: *the International Conference of the European Directorate for the Quality of Medicines & HealthCare, Council of Europe 'Alternatives to Animal Testing: New Approaches in the Development and Control of Biologicals'*. Dubrovnik, Croatia, 23–24 April 2008 (111–114).

30 Descamps J, Mary A, Rommel E, Anhoury ML, De Neys R, Duchêne M. Release potency tests of hepatitis vaccines. *Develop. Biol. Stand.* 101, 289–294 (1999).

31 Landys Chovel Cuervo M, Reyes Huerta N. Validation of an *in vitro* potency test for the Cuban Hepatitis B vaccine. *Dev. Biol.* 111, 305–312 (2002).

32 Rooijakkers EJ, Uittenboogaard JP, Groen J, Osterhaus AD. Rabies vaccine potency control: comparison of ELISA systems for antigenicity testing. *J. Virol. Methods* 58(1–2), 111–119 (1996).

33 McCullough KC, Hendriksen CFM, Seebeck T. *In vitro* methods in vaccinology. In: *Veterinary Vaccinology*. Pastoret P-P, Blancou J, Vannier P, Verschueren C (Eds). Elsevier, Amsterdam, The Netherlands 70–112 (1997).

34 Piersma SJ, Leenaars MP, Guzylack-Piriou L, Summerfield A, Hendriksen CF, McCullough KC. An *in vitro* immune response model to determine tetanus toxoid antigen (vaccine) specific immunogenicity: selection of sensitive assay criteria. *Vaccine* 24(16), 3076–3083 (2006).

35 Bergamin F, Vincent IE, Summerfield A, McCullough KC. Essential role of antigen-presenting role of cell-derived BAFF for antibody responses. *Eur. J. Immunol.* 37(11), 3122–3130 (2007)

36 Hendriksen CFM, Arciniega JL, Bruckner L *et al.* The consistency approach for the quality control of vaccines. *Biologicals* 36(1), 73–77 (2008).

37 Hendriksen C. Reduction of numbers of animals used in the quality control of biologicals. *ATLA* 32(Suppl. 2), 53–59 (2004).

38 Metz B, Brunel F, Chamberlin C *et al.* The potential of physicochemical and immunochemical assays to replace animal tests in the quality control of toxoid vaccines. *ATLA* 35, 323–331 (2007).

39 Eichelberger M, Golding H, Hess M *et al.* FDA/NIH/WHO public workshop on immune correlates of protection against influenza A viruses in support of pandemic vaccine development, Bethesda, Maryland, US, December 10–11, 2007. *Vaccine* 26(34), 4299–4303 (2008).

40 Morrison LA. Vaccines against genital herpes: progress and limitations. *Drugs* 62(8), 1119–1129 (2002).

41 *The International Conference of the European Directorate for the Quality of Medicines & HealthCare, Council of Europe. 'Alternatives to Animal Testing: New Approaches in the Development and Control of Biologicals'*. Dubrovnik, Croatia, 23–24 April 2008.

42 Balls M, Karcher W. The validation of alternative test methods. *ATLA* 23, 884–886 (1995).

43 Metz B, Hendriksen CF, Jiskoot W, Kersten GF. Reduction of animal use in human vaccine quality control. *Vaccine* 20, 2411–2430 (2002).

### *Websites*

101 VaxDesign Corporation: introducing the MIMIC™ System www.vaxdesign.com/mimic-technology (Accessed 1 November, 2008)

102 World Health Organization. Recommendations for Diphtheria, Tetanus, Pertussis and Combined Vaccines. WHO Technical Report Series, No.927, Annex5: (Amendments 2003), 10pp. Geneva, Switzerland. who.int/biologicals/publications/trs/areas/vaccines/dtp/ANNEX%205%20DTPP138–147.pdf (Accessed 9 September, 2008)

## Affiliation

• Coenraad FM Hendriksen
Netherlands Vaccine Institute (NVI),
PO 11, 3720AL Bilthoven,
The Netherlands
Tel.: + 31 302 742 503
coenraad.hendriksen@nvi-vaccin.nl

Author Proof