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NICEATM-ICCVAM[#] International Workshop on Alternative Methods to Reduce, Refine, and Replace the Use of Animals in Vaccine Potency and Safety Testing: State of the Science and Future Directions
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Improving animal welfare and reducing animal use for human vaccine potency testing: state of the science and future directions

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Abstract

NICEATM and ICCVAM convened an international workshop to review the state of the science of human and veterinary vaccine potency and safety testing methods and to identify opportunities to advance new and improved methods that can further reduce, refine, and replace animal use. Topics were addressed in detail by speakers and workshop participants and are reported in a series of six reports. This workshop report, the third in the series, addresses methods and strategies for human vaccine potency testing that can refine animal use to lessen pain and distress, improve animal welfare, and reduce animal use. Workshop participants agreed that the following potency tests for human vaccines should have the highest priority for development of reduction and/or refinement methods: (1) potency tests for vaccines that are most commonly used, (2) potency tests that require the largest number of animals, (3) potency tests that cause severe animal pain and distress, (4) potency tests where the knowledge base of each antigen is advanced, and (5) potency tests for which alternative methods already exist or are in development. Based on these criteria, the highest-priority human vaccines were identified as diphtheria and tetanus vaccines, pertussis vaccines (whole cell and acellular), rabies vaccine, anthrax vaccine, and complex combination vaccines (containing diphtheria, tetanus, and pertussis together with other antigens such as IPV, Hib, and HepB). For successful implementation of reduction and refinement

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alternatives, further research is required into the development and broader use of humane endpoints, serological potency methods, and approaches that would reduce the number of animals used in currently approved potency assays. Because the workshop focused on both human and veterinary vaccines, workshop participants also recommended that human vaccine potency testing methods for rabies and tetanus be reviewed for their potential application to the corresponding veterinary vaccines. Participants agreed that achieving broader acceptance and use of alternative methods, requires that the general principles and procedures for the validation of alternative methods for vaccines be standardized/harmonized internationally. The research, development, validation, and harmonization activities recommended at this workshop are expected to lead to new reduction and/or refinement of animal use in human vaccine potency testing methods and more widespread adoption of existing methods that can reduce animal use and improve animal welfare while ensuring the continued safety and efficacy of human vaccines.

Keywords: human vaccines; vaccine potency testing; vaccine safety testing; refinement alternatives; reduction alternatives; ICCVAM

1. Introduction

Vaccines contribute to improved human and animal health and welfare by preventing diseases and deaths caused by infectious agents. However, the testing necessary to ensure vaccine effectiveness and safety can involve large numbers of animals and significant pain and distress. In the United States, the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) and the National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM) promote the scientific validation and regulatory acceptance of test methods that accurately assess the safety of chemicals and products while reducing, refining (less or no pain and distress), and replacing animal use. Accordingly, NICEATM and ICCVAM recently identified vaccine potency and safety testing as one of their four highest priorities [1].

ICCVAM is an interagency committee of Federal agencies that is charged by law with evaluating new, revised, and alternative test methods with regulatory applicability. ICCVAM members represent 15 U.S. Federal regulatory and research agencies that require, use, generate, or disseminate safety testing data. These include the U.S. Department of Agriculture (USDA), which regulates veterinary vaccines, and the U.S. Food and Drug Administration (FDA), which regulates human vaccines. ICCVAM is a permanent interagency committee of the National Institute of Environmental Health Sciences (NIEHS) under NICEATM. NICEATM administers ICCVAM, provides scientific and operational support for ICCVAM-related activities, and conducts validation studies on promising new safety testing methods. NICEATM and ICCVAM serve a critical public health role in translating research advances from the bench into standardized safety testing methods that can be used in regulatory practice to prevent disease and injury.

To promote and advance the development and use of scientifically valid alternative methods for human and veterinary vaccine testing, NICEATM and ICCVAM organized the International Workshop on Alternative Methods to Reduce, Refine, and Replace the Use of Animals in Vaccine Potency and Safety Testing: State of the Science and Future Directions. The workshop was held at the National Institutes of Health in Bethesda, Maryland, on September 14–16, 2010. It was organized in conjunction with the European Centre for the Validation of Alternative Methods (ECVAM), the Japanese Center for the Validation of Alternative Methods (JaCVAM), and Health Canada.

The workshop addressed the state of the science and developed recommendations for future progress in three major areas for human and veterinary vaccines: (1) *in vitro* replacement methods for potency testing; (2) reduction and refinement methods for potency testing; and (3) reduction, refinement, and replacement methods for vaccine safety testing [2]. Reports were prepared for each of the three topics for human vaccines and for each of the three topics for veterinary vaccines [3, 4, 5, 6, 7, 8]. This report addresses methods and strategies for the reduction and refinement of animal use for potency testing of human vaccines.

Three major strategies for reduction and refinement are discussed. The first is the application of earlier humane endpoints to reduce the duration and severity of pain and distress that can occur during lethal challenge testing. Challenge testing is conducted to determine the amount of vaccine that will protect animals from infection with live agents (virus or bacteria) or challenge with toxin. Inadequately protected animals often develop clinical disease or

die. The second strategy is the development and use of serological methods that can eliminate the need for direct challenge testing. In serological methods, the amount of protective antibody produced is measured and serves as an indicator of vaccine potency. The procedure is significantly less severe than challenge methods. The final strategy involves the application of methods and approaches that may reduce the number of animals used in each test.

2. Goals and organization of the workshop

The goals of the international workshop were to (1) identify and promote the implementation of currently available and accepted alternative methods that can reduce, refine, and replace the use of animals in human and veterinary vaccine potency and safety testing; (2) review the state of the science of alternative methods and identify knowledge and data gaps that need to be addressed; and (3) identify and prioritize research, development, and validation efforts needed to address these gaps in order to advance alternative methods that will also ensure continued protection of human and animal health.

The workshop was organized with four plenary sessions and three breakout group sessions. In the breakout sessions, workshop participants were asked to do the following:

- Identify criteria for prioritization of vaccines for future alternative test method development and prioritize vaccines using the identified criteria
- Review the current state of the science of alternative methods and discuss ways to promote the implementation of available methods
- Identify knowledge and data gaps that need to be addressed
- Identify and prioritize research, development, and validation efforts needed to address these gaps to advance alternative methods while ensuring continued protection of human and animal health

The workshop opened with a plenary session in which expert scientists and regulatory authorities from the United States, Europe, Japan, and Canada outlined the importance of vaccines to human and animal health [9, 10] and described national and international regulatory testing requirements for human and veterinary vaccines [2, 11, 12, 13, 14, 15, 16]. Authorities emphasized that, after licensing of a vaccine, testing must ensure that each subsequent production lot is safe and sufficiently potent to generate a protective immune response in people or animals [11, 12].

The second plenary session addressed methods that have been accepted and methods that are in development that do not require the use of animals for assessing the potency of vaccines [17, 18, 19, 20]. This was followed by breakout sessions to discuss the state of the science and recommendations for future progress for *in vitro* potency tests for human and veterinary vaccines [3, 4].

The third plenary session addressed (1) potency testing methods that refine procedures to avoid or lessen pain and distress by incorporating earlier humane endpoints or by using antibody quantification tests instead of challenge tests and (2) methods and approaches that reduce the number of animals required for each test [21, 22, 23, 24, 25, 26, 27]. Breakout groups then discussed the state of the science and developed recommendations for future progress. Workshop recommendations to advance the use and development of alternative methods that can reduce and refine animal use for potency testing of human vaccines are provided in this paper. Recommendations for veterinary vaccines are available elsewhere in these proceedings [6].

The final plenary session addressed methods and approaches for reducing, refining, and replacing animal use for assessing the safety of production lots of human and veterinary vaccines [11, 28, 29, 30]. Breakout groups for human and veterinary vaccines then discussed the state of the science and developed recommendations for advancing alternative methods for vaccine safety testing [7, 8].

3. Requirements for human vaccine potency testing

Human vaccines save lives, prevent disease and morbidity, and represent a critical tool for successful and cost-saving health interventions. For example, for each U.S. birth cohort that receives a series of vaccines against various diseases (e.g., diphtheria, tetanus, pertussis, measles, mumps, rubella, polio), an estimated 14 million disease episodes and 33,000 premature deaths are prevented [10]. These vaccinations are estimated to save 43 billion dollars in medical and societal costs [10, 31].

Many variables complicate animal testing (e.g., experimental variation, costs, animal numbers, pain, distress). The complex nature of animal testing has prompted many regulatory agencies to actively encourage the evaluation,

development, and implementation of novel approaches that reduce, refine, and replace (3Rs) the use of animals in testing the potency and safety of vaccines for product release [19, 32].

The U.S. Public Health Service Act [33] and certain sections of the U.S. Food, Drug and Cosmetic Act [34] give the FDA the authority to regulate vaccines. Section 351 of the Public Health Service Act states that the approval of a biologics license is based on the demonstration of product safety, purity, and potency and assurance that the facility for manufacture, processing, and packaging meets the standards to ensure that product released for distribution is safe, pure, and potent. The regulatory definitions of *safety*, *purity*, and *potency* are detailed in Title 21 of the U.S. Code of Federal Regulations (Section 600.3) [35]. Testing methodology and validation must be included in the biologics license application. Safety and potency testing may be performed on the final bulk sample or final container sample and may consist of either *in vivo* or *in vitro* tests or both. To change a potency or safety test after licensing requires a supplement to the license with rationale and data to support the alternative (potency and safety) or demonstration of lack of need (safety).

The Center for Biologics Evaluation and Research (CBER) has an active research program that evaluates, develops, and integrates novel scientific technologies for use in product regulation, including the development and analysis of approaches that reduce, refine, and replace the use of animals. CBER encourages the development of these alternative methods for vaccine potency and safety testing with appropriate relevance, supporting data, and test method validation.

4. Prioritizing vaccine potency tests for future refinement and reduction efforts

Potency testing methods for several human vaccines still test animals (**Table 1**). Therefore, the development and subsequent validation of alternative reduction and refinement assays for any human vaccine have the potential to significantly reduce animal numbers and the associated pain and distress during testing. To prioritize human vaccines for further development and validation of alternative tests, workshop participants established the following criteria:

- Vaccines that are most commonly used
- Vaccines for which existing methods require the largest number of animals per test
- Vaccines for which current potency tests cause severe animal pain and distress
- Vaccines for which the knowledge base of each antigen (including antigenic properties, antigenic potency, important, epitopes, etc.) is advanced, allowing determination of those antigens that are most amenable to the development of alternative tests
- Vaccines for which alternative tests are in development or already exist (e.g., *in vitro* assays using Vero cells for diphtheria vaccine)

Workshop participants identified the following vaccines as the highest priorities for additional research, development, and validation efforts:

- Diphtheria and tetanus toxoids (due to [1] requirement for toxin challenge; [2] use of large numbers of animals; and [3] the fact that neutralizing antibody production is the primary correlate of protection, allowing serological methods to be developed)
- Whole cell pertussis vaccines (due to the severity of the challenge test)
- Rabies vaccines (due to the severity and inherent variability of the challenge test)
- Anthrax vaccines (due to the severity of the challenge test)
- Combination vaccines (due to the large numbers of animals used to test each vaccine and the possibility of developing a single serological assay for multiple components in the same vaccine, including diphtheria, tetanus, and acellular pertussis antigens)

The rationale for the selection of the vaccines listed above was based primarily on the number of animals currently used to document potency, the number of lots produced each year, and the severity of pain and distress in the current challenge tests.

Table 1: Examples of human vaccine potency assays that incorporate immunization and *in vitro* antibody quantification (serology) alternative methods

Vaccine Product (Disease)	3R Alternative	References For Alternative Methods	Traditional Test Procedure for which the Alternative Method is Applicable	Traditional Test Reference
Tetanus toxoid vaccine and tetanus component in combined vaccines (*Clostridium tetani)*	Single-dilution immunization and serology[b, c] –*in vitro* toxin-binding inhibition (ToBI[b, c]), indirect ELISA[b, c]	Dobbelaer et al. 1997 [36]; Ph. Eur. 2.7.8. Assay of tetanus vaccine (adsorbed) [37]; WHO TRS 927, 2003 [38]	Guinea pig or mouse lethal challenge test	U.S. Minimum Requirements, 1952 [39]; Ph. Eur. 2.7.8 [37]; WHO TRS 927, 2003 [38]
Diphtheria toxoid vaccine and diphtheria component in combined vaccines (*Corynebacterium diphtheriae)*	Single-dilution immunization and Serology– ELISA or Vero Cell Assay[b, c]	Ph. Eur. 2.7.6 Assay of diphtheria vaccine (adsorbed) [40]; WHO TRS 927, 2003 [38]	Guinea pig lethal challenge test	U.S. Minimum Requirements, 1947 [41]; Ph. Eur. 2.7.6 [40]; WHO TRS 927, 2003 [38]
Acellular pertussis component in combined vaccines Whooping cough (*Bordetella pertussis)*	Immunization (mice) and serology[a, b, c] ELISA	Ph. Eur. Monograph 1356 [42] and 1595 [43]; Arciniega et al. 1998 [44]; Japanese Minimum Requirements for Biological Products, 2006 [46]; WHO TRS 878, 1998 [47]	Multiple-dilution mouse serology[c]	Ph. Eur. 2.7.7 [45]
Rabies vaccine; (*Lyssavirus rabies)*	Immunization (mice) and Serology[b, c]	Ph. Eur. Monograph 216 [48]; WHO TRS 941, 2007 [49]	Mouse multiple-dilution lethal challenge test	Seligmann 1973 [50]

[a]Accepted by U.S. regulatory authorities.
[b]Published in the European Pharmacopoeia.
[c]WHO Technical Report Series number and year of publication.

5. Human vaccine potency testing: using humane endpoints to refine animal use

5.1. State of the science

Assays to measure the potency of vaccines are frequently based on an immunization-challenge procedure in laboratory animals. While replacing animal use is the ultimate objective for developing alternative vaccine potency tests, an immediate transition from an *in vivo* test to a non-animal test method is technically difficult and, where possible, time consuming. However, interim success toward improving animal welfare in vaccine potency testing may be accomplished by developing refinement alternatives that reduce the pain and distress that may be experienced by the animals. For example, humane endpoints are criteria that can be used as the basis for ending a test procedure early in order to avoid further pain and distress. Ideally, humane endpoints may be used to end a procedure before the onset of animal pain and distress [37, 51, 52]. However, the use of earlier, more humane endpoints must allow the specific testing objectives to be met. With the development of earlier, more humane criteria for detecting infection and/or toxicity it is possible to minimize the extent of animal pain and distress during *in vivo* immunization–challenge procedures before they are humanely euthanized.

The use of humane endpoints to reduce animal pain and distress is reflected in the regulatory guidance documents and/or legislation of relevant agencies in numerous regions and countries worldwide. Examples include the European Directorate for the Quality of Medicines & HealthCare (EDQM) and the associated European

Pharmacopoeia (Ph. Eur.) and the U.S. Code of Federal Regulations [51, 52, 53, 54, 55, 56, 57]. The adoption of scientifically valid humane endpoints whenever possible is also a fundamental principle of the U.S. Public Health Service Policy on Humane Care and Use of Laboratory Animals [58], as well as existing statutory requirements in the United States [58] and the EU [48]. Specific legislation has been adopted to use humane endpoints during vaccine potency testing procedures [55, 56].

The use of humane endpoints during *in vivo* testing of biological products is required in the United States for veterinary vaccines [56] and for both human and veterinary vaccines in Europe [59, 60]. Although there have been significant advances in the identification of early humane endpoints in human vaccine potency testing, more work remains in identifying and validating humane endpoints for specific disease processes. For example, the use of humane endpoints during the potency testing of human inactivated rabies, tetanus, and diphtheria vaccines (**Table 2)** has been widely accepted by regulatory authorities around the world, including the EDQM and the WHO [61, 38, 49]. Clinical signs such as a clear reduction in body weight, or slow and circular movements followed by cramps and paralysis, are considered the first neurological symptoms of rabies and now serve as reliable indicators for humane endpoints [62]. Additional examples of approved humane endpoints include, paresis of the injected hind limb of mice and erythema observed following intradermal challenge in guinea pigs for tetanus and diphtheria vaccine potency tests, respectively.

Hendriksen and colleagues (1999) [63] evaluated potential humane endpoints during the potency assay of whole cell pertussis vaccine. A loss of muscle coordination and a decrease in body temperature were identified as useful humane endpoints. However, these early endpoints have yet to achieve broad regulatory acceptance, and research continues toward furthering the refinement alternatives for whole cell pertussis vaccine potency testing.

Table 2: Examples of human vaccine potency assays that incorporate earlier humane endpoints for challenge testing

Vaccine Product (Disease)	Humane Endpoints	References	Traditional Test Procedure for Which the Alternative Method is Applicable	Traditional Test Reference
Inactivated rabies virus vaccine (*Lyssavirus rabies)*	Convulsions, paralysis, paresis[a, b, c]	Ph. Eur. Monograph 216 [48]; WHO TRS 941, 2007 [49]	Mouse multiple-dilution lethal challenge test	Seligmann 1973 [50]
Tetanus toxoid vaccine (*Clostridium tetani)* and tetanus component in combined vaccines	Toxin-injected hind leg paresis[a, b]	Ph. Eur. 2.7.8. Assay of tetanus vaccine (adsorbed) [37]; WHO TRS 927, 2003 [38]	Guinea pig or mouse lethal challenge test	Ph. Eur. 2.7.8. Assay of tetanus vaccine (adsorbed) [37]; WHO TRS 927, 2003 [38]
Diphtheria component in combined vaccines (*Corynebacterium diphtheriae)*	Erythema score following intradermal challenge in guinea pigs[a, b]	Ph. Eur. 2.7.6 Assay of diphtheria vaccine (adsorbed) [40]; WHO TRS 927, Annex 5, 2003 [38]	Guinea pig lethal challenge test	Ph. Eur. 2.7.6 Assay of diphtheria vaccine (adsorbed) [40]; WHO TRS 927, 2003 [38]

[a]Published in the European Pharmacopoeia.
[b]WHO Technical Report Series number and year of publication.
[c] Accepted by U.S. regulatory authorities.

5.2. Knowledge gaps and priority research, development, and validation activities

Workshop participants discussed current knowledge and data gaps. They focused on the research needed to fill these gaps to further advance the development, validation, and implementation of potency tests that incorporate humane endpoints.

Some current vaccine potency tests involve an initial immunization followed by either (1) direct challenge with an infective organism or toxin *in vivo* or (2) an *in vivo* toxin neutralization assay in a second group of animals using antisera collected from the immunized animals. In both tests, animals are subjected to considerable pain and distress before they are considered moribund or die. The development of methods that can detect earlier signs of infection,

or toxicity markers that accurately predict death, would reduce the extent to which animals are in pain before they can be humanely euthanized. These signs may include the following: (1) paralysis or dehydration; (2) erythema; (3) pathophysiological parameters such as body temperature, body weight, or tachycardia; and (4) behavioral parameters such as stereotypic behavior, self-mutilation, or aggression [25]. Workshop participants agreed that there was no logical or scientific rationale for requiring death as an endpoint in vaccine challenge potency testing. However, they emphasized the importance of ensuring that any earlier signs are accurately predictive of death, given the potential impact on potency assessment. Among the most important data and knowledge gaps identified by the participants were:

- Identifying humane endpoints for vaccines for which suitable serology methods do not exist or would not help predict potency because protection is not predominately antibody mediated
- Recognizing clinical signs at the institutional level, noting the need for training and standardization of classical signs of humane endpoints

Workshop participants called for comprehensive training and routine systematic collection and evaluation of all clinical signs that occur during a challenge test. They recognized the need for detailed institutional protocols and guidance documents instead of a strict general protocol as the best ways to facilitate identification and validation of humane endpoints. Also, identifying and validating humane endpoints during the development/validation of serological methods was considered the most efficient and expeditious means toward implementation of humane endpoints.

The workshop participants also discussed practical issues. These included (1) ensuring that observation intervals are consistent and sufficiently narrow to allow identification of clinical signs; (2) recognizing when early termination of the test is not practical due to a rapid progression of pathology; and (3) considering new technologies for monitoring activity, body temperature, body weight, etc., remotely (sensory cages) to avoid stress from human contact. However, participants also recognized the limitations associated with using new technologies, such as expense of equipment, strict monitoring/calibration requirements, and training requirements.

6. Human vaccine potency testing: using serological methods to refine animal use

6.1. State of the science

As shown in **Table 1**, many of the high-priority vaccines that currently use animals in challenge testing already have serological methods available or in development. In these test methods, the amount of a specific antibody in the blood of immunized animals is used as surrogate for demonstrating protection against challenge by the relevant pathogen or toxin. Clearly, these vaccines should be targeted for further validation efforts because they are most likely to lead to successful implementation of refinement alternatives. Some of the earliest and most widely accepted examples of using serological methods to measure a specific antibody response in immunized animals include the enzyme-linked immunosorbent assay (ELISA) and toxin-binding inhibition (ToBI) test assay in animals immunized with tetanus toxoid [64].

An interlaboratory validation study to evaluate the suitability of *in vitro* serological assay systems for the assessment of the potency of tetanus toxoid in single and multicomponent veterinary vaccines was reported in 1994 [65]. The study evaluated the ELISA; the ToBI; and the passive hemagglutination (HA) test, an agglutination assay in which red blood cells are used to absorb soluble antigen on their surface and then agglutinate in the presence of antiserum specific for the absorbed antigen. It was concluded that the ELISA and the ToBi assay were both suitably validated methods, while the HA test required further standardization before use. The Ph. Eur. and the World Health Organization (WHO) have accepted the ELISA and the ToBI assay for use with serological assays for potency measurements of both human and veterinary tetanus toxoid vaccines.

When the mechanism that protects against a virulent organism or toxin is based on humoral immunity, the use of serological approaches offers certain advantages. Some regulatory agencies accept serological approaches for the measurement of potency for a number of vaccines, including those for diphtheria, acellular pertussis, and rabies (for veterinary use) (**Table 1**), as well as a number of clostridial and leptospiral (for veterinary use) species [66]. Such approaches may be particularly beneficial (in terms of animal welfare) for testing of combined vaccines. In addition to the refinement of the procedure that is obtained when serology is used instead of challenge, there is the possibility

of measuring the antigen response to more than one component in the vaccine in the same group of animals. This may ultimately remove the requirement to perform a separate potency test for each vaccine component, thereby significantly reducing the number of animals required for potency testing of some products.

6.2. Knowledge gaps and priority research, development, and validation activities

Workshop participants identified several knowledge and data gaps associated with the development, validation, and implementation of serological methods, including the following:

- To promote broader use of alternative methods, a comprehensive review and assessment of validated refinements that are approved by some authorities for toxoids should be completed and made widely available (recognizing that the proprietary nature of these methods may make this difficult).
- Further research and validation is necessary to allow broader use of the Vero cell assay and ELISA for measuring responses to diphtheria toxoid.
- Further research is necessary to allow broader use of the ELISA and ToBI for measuring antibodies to tetanus toxoid. Participants noted that the forthcoming WHO manual for DTP testing would provide additional guidance.
- Regulatory authorities encourage alternatives, but it is the manufacturers' responsibility to demonstrate a valid method for their specific product. The effort, time, and expense of this testing was recognized as a major hurdle.

Krämer and colleagues [67] demonstrated the wider transferability and reliability of using mean neutralizing antibody titers (as determined by the rapid fluorescent focus inhibition test [RFFIT]) as a potency assay for inactivated rabies vaccines for veterinary use. Based on this study, the authors suggested that the RFFIT could be used for inactivated rabies vaccine potency testing. While it is now recognized by the WHO [49] as a valid alternative test method, workshop participants emphasized that further research and validation efforts are necessary for the RFFIT to gain broader acceptance for human rabies vaccine potency testing.

Participants also recommended continued research and validation of the immunogenicity test to measure antibody response to anthrax vaccine. This test could provide a substitute for the current active protection potency test. Current research focuses on determining the suitability of either an ELISA [68] or an *in vitro* toxin neutralization assay (TNA) to measure the antibody response in immunized mice [24]. These assays have the following advantages over the current immunization–challenge assay that uses guinea pigs:

- These assays avoid challenge with virulent *B. anthracis*
- They are less costly, requiring fewer, less expensive animals than the current test
- They are more humane and cause less animal pain and distress

Estimates of the quantity of antibodies to the protective antigen (PA) obtained by ELISA and TNA are correlated, but they are not interchangeable because a precise prediction of TNA estimates from ELISA estimates could not be obtained. In addition, antibody estimates obtained by TNA are typically lower than those obtained by ELISA. The basis for this difference is not known but may be related to the fact that neutralizing antibodies represent only a fraction of the antibodies that bind PA. Further research will be needed to address these important issues [24] and experience with similar studies performed with Diphtheria vaccines may be relevant [69, 70, 71].

7. Human vaccine potency testing: strategies to reduce animal use

7.1. State of the science

The number of animals used in potency determinations can be reduced in several ways. Two obvious and immediate ways would be to simply reduce the number of animals used per dose of the vaccine and/or to reduce the number of doses used. Each would require a determination that adequate statistical power is maintained.

The EDQM (Ph. Eur.) and the WHO recommend that, after sufficient experience with the mouse rabies potency challenge test (NIH test), a manufacturer can use a single-dilution challenge assay for inactivated veterinary rabies vaccine potency testing [48, 49]. Single-dilution testing for human rabies vaccines is still at the experimental stage.

Additional attempts have been made to reduce the number of mice used at each dilution in the mouse rabies potency challenge test for inactivated human rabies vaccine. The Brazilian National Control Laboratory conducted retrospective evaluation of potency test results and concluded that the results from reducing the number of mice per

dilution from 18 to 9 in the mouse rabies potency challenge test did not adversely impact the results of the test [72]. Neither the single-dilution assay nor reduction in the number of mice per dilution has gained widespread regulatory acceptance to date. Similar reduction schemes for potency testing of diphtheria and tetanus vaccines are recommended by the WHO and the EDQM (Ph. Eur.) [37, 38, 40].

The use of serological methods for vaccine potency testing may also lead to a significant reduction in animal use in addition to the refinement benefits that this approach can provide. Combination vaccines are particularly important because of their increasingly expanded use, a trend that is expected to continue as a means to reduce the number of vaccinations a child receives [10]. These vaccines present the unique challenge of the need for potency estimates for each of the active components. For example, potency testing for the DTaP combination vaccine typically requires separate tests for each of the major components (diphtheria, tetanus, and acellular pertussis). Therefore, combining the individual potency procedures into one serological test for multivalent vaccines would have an immediate and significant impact on reducing animal use.

7.2. Knowledge gaps and priority research, development, and validation activities

Workshop participants identified several key knowledge and data gaps associated with the development, validation, and implementation of vaccine potency reduction methods including:

- Knowledge of the causes of variability of potency estimates derived from active protection (particular note was made of the highly variable mouse potency challenge test for inactivated rabies vaccine) and serological assays is needed
- Knowledge of the causes of invalid assays for some vaccines that often lead to a requirement for repeat testing is required

Although there would often be product-specific activities, which may not have broad applicability, workshop participants also suggested a number of activities to address these knowledge gaps:

- Identifying the sources of variation in the current methods and ways to reduce or eliminate these sources of variation
- Recalculating the minimum number of animals required to maintain statistical power and test validity
- Encouraging those countries that use the full (3-dilution) test for diphtheria and tetanus potency testing to reduce the number of vaccine dilutions used
- Investigating the use of homologous (product-specific) reference preparations to reduce variability/improve precision

8. Achieving broader acceptance and use of currently available reduction and refinement methods for human vaccines

An early, critical step in obtaining broader acceptance and use of reduction and/or refinement approaches in vaccine potency testing is to harmonize acceptance criteria for vaccines and obtain recognition of test results among international regulatory authorities. This could lead to the elimination of any requirement for an importing national control laboratory (NCL) to test vaccines before release for sale (note; US regulations do not require FDA to test vaccines before release for sale). Elimination of this requirement could significantly reduce animal use. Similarly, clear guidance on the requirements and general principles and procedures for validation of alternative methods should be standardized and harmonized internationally.

Manufacturers must be made aware that alternative potency testing methods are acceptable to the regulatory authorities in the United States if they are scientifically justified and validated. In this case, early and frequent communication will ensure that efforts are not lost or directed toward obtaining data that regulators do not consider valuable. For example, due to the differences in the current EU and U.S. methods for measuring diphtheria toxoid potency, the Vero cell assay for diphtheria toxoid requires additional validation to ensure that it meets all regional testing requirements. The use of Vero cells for potency testing is not currently approved in the United States but would be considered if the assay was appropriately validated with demonstration of equivalence to the current NIH test. However, it was noted that the current NIH test uses far fewer animals than the WHO/Ph. Eur. multiple-dilution assays. Use of the serological assay for diphtheria vaccine would have much lower impact in this case. Workshop

participants questioned the need for this additional testing and validation and noted that validation of refinement alternatives for potency testing of other vaccines remains a higher priority.

Additional activities that could further advance acceptance and broader use of alternative methods include the following:

- Encouraging broader access to information that describes vaccine potency testing methods that have been successfully implemented to reduce, refine, and replace the use of animals (e.g., open online access to U.S. Pharmacopeia [USP] and Ph. Eur. monographs, WHO manual of laboratory methods [now in press])
- Recognizing that, although international harmonization of methods and procedures for validation is considered important, product-specific validation may still be necessary
- Recognition of who is the driving force for decisions on changes to required testing, understanding that regulators can facilitate the change, but this needs to be a joint effort between manufacturers and regulators

9. Reduction and refinement methods: extrapolating veterinary vaccine potency testing to human vaccines and vice versa

In April 2004, the USDA Center for Veterinary Biologics (CVB) issued notice No. 04-09 (9 CFR 117.4e). The following wording may be added to all codified potency tests that are conducted by administering viable virus, bacteria, or a bacterial toxin to animals in a dose that is expected to be lethal:

> Moribund animals exhibiting clinical signs consistent with the expected disease pathogenesis that are unable to rise or move under their own power may be humanely euthanized and considered as deaths as outlined in 9 CFR 117.4. In the case of the rabies animals exhibiting paresis, paralysis, and/or convulsions may be humanely euthanized and considered as deaths as outlined in 9 CFR 117.4.

Workshop participants recommended that the U.S. FDA-CBER provide similar guidance on moribund euthanasia. This guidance could have a significant and immediate impact on refining animal use for human vaccine potency challenge testing.

Ideally, harmonized and globally accepted guidance on humane endpoints should be developed that could apply to potency testing for both human and veterinary vaccines, recognizing that specific humane endpoints must be tailored for each vaccine. Because the same approaches should be taken for veterinary and human vaccines, there is an obvious need for consultation between these groups. For example, although serological approaches to veterinary toxoid vaccines are similar to those for human vaccines, the license-specific tests might differ. Therefore, continued interaction between groups is necessary to ensure that every benefit is realized.

10. Other issues to be addressed to facilitate the reduction and refinement of animals in human vaccine potency testing

Participants also discussed the advantages offered by the consistency approach to manufacturing control. They agreed that the confidence gained internationally with a product that has a proven consistency record over time should lead to a reduced demand for additional animal testing by NCLs. The consistency approach implies the use of a set of parameters (e.g., antigen content, antigen integrity, purity, etc.) to constitute a product profile that can replace current release tests. The product profile is established to satisfy 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 the licensing stage between manufacturer and regulator [73].

11. Discussion

The International Workshop on Alternative Methods to Reduce, Refine, and Replace the Use of Animals in Vaccine Potency and Safety Testing: State of the Science and Future Directions was the first workshop in the United States focused on alternative methods to bring together regulators, manufacturers, and experts involved in both human and veterinary vaccine development and licensing. Participants shared a goal of reviewing the current state of the science and identifying opportunities for future progress in implementation of 3R alternatives in vaccine potency and safety testing. Several other recent symposia and workshops have been convened in an attempt to

further the science of the 3Rs, for example, when using animals for quality control of vaccines. These symposia addressed various aspects of (1) promoting and facilitating the use of non-animal methods for the quality control of either human or veterinary vaccines and (2) increasing the understanding and implementation of the consistency approach to the manufacturing of human and veterinary vaccines.

The following international symposia were dedicated to finding new ways to advance the development and implementation of alternative methods for the quality control of human or veterinary vaccines:

- Alternatives to Animal Testing: New Approaches in the Development and Control of Biologicals; Dubrovnik, Croatia (April 2008)
- The Consistency Approach for Quality Control of Vaccines – A Strategy to Improve Quality Control and Implement the 3Rs; Brussels, Belgium (January 2010)
- Practical Alternatives to Reduce Animal Testing in Quality Control of Veterinary Biologicals in the Americas; Buenos Aires, Argentina (February 2010)

In the current workshop, participants highlighted the need for basic scientific research, product-specific research and validation efforts, and regulatory harmonization to advance the development, validation, and implementation of alternative methods to reduce and refine (less pain and distress) the use of animals in lot release potency testing for human vaccines. Specific recommendations were considered necessary to address scientific knowledge gaps that must be filled in order to advance the development of reduction and refinement alternatives. An increase in the basic understanding of antigens and antigenic properties as it pertains to vaccines would facilitate development of serological methods. Better defining the processes and/or product specific information associated with current potency tests (e.g., Vero Cell assays for Diphtheria toxoid; the RFFIT for rabies vaccine) could improve the likelihood that refinement alternatives are developed. Identifying the specific signs of disease progression for each vaccine target would facilitate defining earlier more humane endpoints that could routinely be used to terminate studies.

More open access should be provided to methods and information that are currently restricted (e.g., pharmacopoeial monographs). Greater accessibility would increase the likelihood of implementing available alternatives. This could also be achieved by increasing interactions and dissemination of information between regulators, manufacturers, and the scientific community. This workshop was also successful in setting the stage for a series of future workshops on the identified priority vaccines. Based upon the general scientific literature and the presentations at the workshop, there is broad international consensus to reduce, refine and replace the use of animal for both human and veterinary vaccine potency testing. Implementation of the workshop recommendations is expected to advance alternative methods for vaccine potency testing that will benefit animal welfare while ensuring continued safety and efficacy of vaccines for human and veterinary use.

12. Conclusions

The human vaccine reduction and refinement alternatives session successfully summarized the current status of potency testing of human vaccines and identified the critical vaccines for which priority should be given to develop alternative tests for currently used *in vivo* challenge or toxin neutralization tests. This will allow prioritization of the necessary research, development, and validation work to expedite potency testing with earlier, more humane endpoints and using fewer animals. It was recognized that increasing our knowledge base of each antigen and building on current knowledge of existing serological methods is the most expeditious way to advance the use of serological methods in vaccine potency testing. Workshop participants agreed that there is no logical or scientific rationale for requiring death as an endpoint during the conduct of a vaccine challenge potency test. Participants also provided valuable recommendations for advancing the identification, validation, and implementation of the use of humane endpoints in vaccine potency testing. Increasing our understanding of the causes of variability in potency tests will also facilitate reduction in the number of animals required to document potency while maintaining the statistical power of the assay. Finally, the workshop participants recognized that international harmonization, with the continued interaction of the global vaccine community, human and veterinary, is the key to advancing the use of 3R alternatives in vaccine potency testing.

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References

[1] Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM). The NICEATM-ICCVAM five-year plan (2008-2012): a plan to advance alternative test methods of high scientific quality to protect and advance the health of people, animals, and the environment. National Institute of Environmental Health Sciences, 2008. NIH Publication No. 08-6410. http://iccvam.niehs.nih.gov/docs/5yearplan.htm

[2] Stokes WS, Kulpa-Eddy J, McFarland RM. Introduction and summary of the international workshop on alternative methods to reduce, refine, and replace the use of animals in vaccine potency and safety testing: state of the science and future directions. Proc Vaccinol 2011;5:1-15.

[3] McFarland R, Verthelyi D, Casey W, Arciniega J, Isbrucker R, Schmitt M, Finn T, Descamps J, Horiuchi Y, Sesardic D, Stickings P, Johnson NW, Lipscomb E, Allen D. Non-animal replacement methods for human vaccine potency testing: state-of-the-science and future directions. Proc Vaccinol 2011;5:16-32.

[4] Kulpa-Eddy J, Srinivas G, Halder M, Hill R, Brown K, Roth J, Draayer H, Galvin J, Claassen I, Gifford G, Woodland R, Doelling V, Jones B, Stokes WS. Non-animal replacement methods for veterinary vaccine potency testing: state-of-the-science and future directions. Proc Vaccinol 2011;5:60-83.

[5] Casey W, Schmitt M, McFarland R, Isbrucker R, Levis R, Arciniega J, Descamps J, Finn T, Hendriksen C, Horiuchi Y, Keller J, Kojima H, Sesardic D, Stickings P, Johnson NW, Lipscomb E, Allen D. Improving animal welfare and reducing and refining animal use for human vaccine potency testing: state-of-the-science and future directions. Proc Vaccinol 2011;5:33-46.

[6] Stokes WS, Brown K, Kulpa-Eddy J, Srinivas G, Halder M, Draayer H, Galvin J, Claassen I, Gifford G, Woodland R, Doelling V, Jones B. Improving animal welfare and reducing animal use for veterinary vaccine potency testing: state-of-the-science and future directions. Proc Vaccinol 2011;5:84-105.

[7] Isbrucker R, Levis R, Casey W, McFarland R, Schmitt M, Arciniega J, Descamps J, Finn T, Hendriksen C, Horiuchi Y, Keller J, Kojima H, Sesardic D, Stickings P, Johnson NW, Allen D. Alternative methods and strategies to reduce, refine, and replace animal use for human vaccine post-licensing safety testing: state of the science and future directions. Proc Vaccinol 2011;5:47-59.

[8] Kulpa-Eddy J, Srinivas G, Halder M, Hill R, Brown K, Draayer H, Galvin J, Claassen I, Gifford G, Woodland R, Doelling V, Jones B, Stokes WS. Alternative methods and strategies to reduce, refine, and replace animal use for veterinary vaccine post-licensing safety testing: state of the science and future directions. Proc Vaccinol 2011;5:106-119.

[9] Roth J. Veterinary vaccines and their importance to animal health and public health. Proc Vaccinol 2011;5:127-136.

[10] Schuchat A. Human vaccines and their importance to public health. Proc Vaccinol 2011;5:120-126.

[11] Finn T. U.S. FDA Requirements for human vaccine safety and potency testing. Proc Vaccinol 2011;5:137-140.

[12] Hill RE. USDA requirements for veterinary vaccine safety and potency testing. Proc Vaccinol 2011;5:141-145.

[13] Isbrucker R, Sontakke S, Smith D. Health Canada's human vaccine lot release program: impact on the 3**Rs**. Proc Vaccinol 2011;5:147-150.

[14] Woodland R. European regulatory requirements for veterinary vaccine safety and potency testing and recent progress toward reducing animal use. Proc Vaccinol 2011;5:151-155.

[15] Horiuchi Y, Ochiai M, Kataoka M, Yamamoto A, Yuen C-T, Asokanathan C, Corbel M, Kurata T, Xing D. Strategic approaches for developing alternative tests for safety and potency of vaccines. Proc Vaccinol 2011;5:156-163.

[16] Shin J, Lei D, Conrad C, Knezevic I, Wood D. International Regulatory Requirements for Vaccine Safety and Potency Testing: A WHO perspective. Proc Vaccinol 2011;5:164-170.

[17] Draayer H. Overview of currently approved veterinary vaccine potency testing methods and methods in development that do not require animal use. Proc Vaccinol 2011;5:171-174.

[18] Claassen I. Case study of development, validation, and acceptance of a non-animal method for assessing veterinary vaccine potency. Proc Vaccinol 2011;5:175-183.

[19] Levis R. Overview of the current status of human vaccine potency testing methods that replace animals. Presented at: International Workshop on Alternative Methods to Reduce, Refine, and Replace the Use of Animals in Vaccine Potency and Safety Testing: State of the Science and Future Directions. NICEAM. Bethesda, MD. September 2010. Available at: http://iccvam.niehs.nih.gov/meetings/BiologicsWksp-2010/BiologicsWksp-present.htm

[20] Descamps J, et al. A Case study of development, validation, and acceptance of a non-animal method for assessing human vaccine potency. Proc Vaccinol 2011;5:184-191.

[21] Keller JE. Overview of currently approved serological methods with a focus on diphtheria and tetanus toxoid potency testing. Proc Vaccinol 2011;5:192-199.
[22] Srinivas G. Refinement alternatives for veterinary vaccine potency testing: overview of currently approved serological methods. Presented at: International Workshop on Alternative Methods to Reduce, Refine, and Replace the Use of Animals in Vaccine Potency and Safety Testing: State of the Science and Future Directions. NICEAM. Bethesda, MD. September 2010. Available at: http://iccvam.niehs.nih.gov/meetings/BiologicsWksp-2010/BiologicsWksp-present.htm
[23] Stickings P, Rigsby P, Coombes L, Hockley J, Tierney R, Sesardic D. Animal refinement and reduction: alternative approaches for potency testing of diphtheria and tetanus vaccines. Proc Vaccinol 2011;5:200-212.
[24] Arciniega JL, Domínguez-Castillo RI. Development and validation of serological methods for human vaccine potency testing: case study of an anthrax vaccine. Proc Vaccinol 2011;5:213-220.
[25] Hendriksen CFM. Humane endpoints in vaccine potency testing. Proc Vaccinol 2011;5:221-226.
[26] Kulpa-Eddy J, Srinivas G. Approaches to reducing animal numbers in vaccine potency testing. Proc Vaccinol 2011;5:227-231.
[27] Kulpa-Eddy J, Dusek D. Application of the consistency approach in the United States to reduce animal use in vaccine potency testing. Proc Vaccinol 2011;5:232-235.
[28] Gifford G, Agrawal P, Hutchings D, Yarosh O. Veterinary vaccine post-licensing safety testing: overview of current regulatory requirements and accepted alternatives. Proc Vaccinol 2011;5:236-247.
[29] Arciniega JL, Corvette L, Hsu H, Lynn F, Romani T, Dobbelaer R. Target alternative vaccine safety testing strategies for Pertussis toxin. Proc Vaccinol 2011;5:248-260.
[30] Rubin SA. Toward replacement of the monkey neurovirulence test in vaccine safety testing. Proc Vaccinol 2011;5:261-265.
[31] Zhou F, Santoli J, Messonnier ML, Yusuf HR, Shefer A, Chu SY, Rodewald L, Harpaz R. Economic evaluation of the 7-vaccine routine childhood immunization schedule in the United States, 2001. Arch Pediatr Adolesc Med 2005;159:1136-1144.
[32] De Mattia F, Chapsal J-M, Descamps J, Halder M, Jarrett N, Kross I, Mortiaux F, Ponsar C, Redhead K, McKelvie J, Hendriksen C. The consistency approach for quality control of vaccines – a strategy to improve quality control and implement 3Rs. Biologicals 2011;39:59-65.
[33] Public Health Service Act. 1944 (as amended). 42 U.S.C. 6A. Available at: http://www.FDA.gov/RegulatoryInformation/Legislation/ucm148717.htm.
[34] Federal Food, Drug, and Cosmetic Act. 1938 (as amended). 21 U.S.C. 301 et seq. Available at: http://www.fda.gov/RegulatoryInformation/Legislation/FederalFoodDrugandCosmeticActFDCAct/default.htm.
[35] United States Department of Agriculture, Center for Veterinary Biologics, Code of Federal Regulations, Title 21, Food and Drugs. Part 600, Biological Products: General. Section 600.3. 2010.
[36] Dobbelaer R, Knight P, Lyng J. Use and validation of a single vaccine dilution assay for testing the potency of diphtheria, tetanus and combined vaccines. In: WHO Manual of laboratory methods: For testing of vaccines used in the WHO Expanded Programme on Immunization. Geneva: World Health Organization, 1997:162-74.
[37] Council of Europe. *2.7.8 Assay of Tetanus vaccine (adsorbed). European Pharmacopoeia. 1996.* Strasbourg, France: Council of Europe.
[38] World Health Organization. Recommendations for diphtheria, tetanus, pertussis and combined vaccines (Amendments 2003). In: WHO Expert Committee on Biological Standardization. Fifty-fourth report. Geneva, World Health Organization, 2005, (WHO Technical Report Series No. 927).
[39] United States Minimum Requirements: Tetanus toxoid, 1952.
[40] European Pharmacopoeia. Monograph 20706. Assay of diphtheria vaccine (adsorbed), Ph.Eur. 6th Edition. Strasbourg, France: European Department for the Quality of Medicines within the Council of Europe; 2008.Ph Eur 2.7.6
[41] United States Minimum Requirements: Diphtheria, 1947.
[42] European Pharmacopoeia. Monograph 1356. Pertussis Vaccine Acellular Component, Absorbed, Ph.Eur. 6th Edition. Strasbourg, France: European Department for the Quality of Medicines within the Council of Europe; 2008.
[43] European Pharmacopoeia. Monograph 1595. Pertussis Vaccine Acellular, Copurified, Absorbed, Ph.Eur. 6th Edition. Strasbourg, France: European Department for the Quality of Medicines within the Council of Europe; 2008.
[44] Arciniega JL, Corbel M, Dellepiane N, Dobbelaer R, Griffiths E, Heron I, et al. WHO guidelines for the production and control of the acellular pertussis component of monovalent or combined vaccines. Biologicals 1998; 26(3):195-204.
[45] European Pharmacopoeia. Monograph 20707. Assay of pertussis vaccine, Ph.Eur. 6th Edition. Strasbourg, France: European Department for the Quality of Medicines within the Council of Europe; 2008.Ph Eur 2.7.7
[46] Japanese Minimum Requirements for Biological Products. National Institute of Infectious Diseases, Japan 2006.
[47] World Health Organization. Guidelines for the production and control of the acellular pertussis component of monovalent or combined vaccines. In: WHO Expert Committee on Biological Standardization. Forty-seventh report. Geneva, World Health Organization, 1998, (WHO Technical Report Series No. 878).
[48] European Pharmacopoeia.. Monograph 216. Rabies vaccine for human use prepared in cell cultures Rabies vaccine for human use prepared in cell cultures, Ph.Eur. 6th Edition. Strasbourg, France: European Department for the Quality of Medicines within the Council of Europe; 2008.
[49] World Health Organization. Recommendations for inactivated rabies vaccine for human use produced in cell substrates and embryonated eggs. In: WHO Expert Committee on Biological Standardization. Fifty-sixth report. Geneva, World Health Organization, 2007, (WHO Technical Report Series No. 941).
[50] Seligmann EB. The NIH test for potency. In: Laboratory techniques in rabies (Eds Kaplan MM and Koprowski H) 3rd edn. WHO, Geneva, 1973:279-286.

[51] Stokes, WS. Reducing unrelieved pain and distress in laboratory animals using humane endpoints. ILAR Journal 2000;41(2):59-61.
[52] Stokes, WS. Humane endpoints for laboratory animals used in regulatory testing. ILAR Journal 2002;43(Suppl):S31-S38.
[53] Castle P. The European Pharmacopoeia and humane endpoints. In: Hendriksen CFM, Morton DB, editors. Humane Endpoints in Animal Experiments for Biomedical Research. London, Royal Society of Medicine Press, 1999:15-19.
[54] Hendriksen C, Steen B. Refinement of vaccine potency testing with the use of humane en3dpoints. ILAR Journal 2000;41(2):105-113.
[55] European Pharmacopoeia. Monograph 01/2008:0062. Vaccines for Veterinary Use, Ph.Eur. 6th ed. Strasbourg, France: European Department for the Quality of Medicines within the Council of Europe; 2008.
[56] United States Department of Agriculture, Center for Veterinary Biologics, Code of Federal Regulations, Title 9, Animals and Animal Products. Part 117- Standard Requirements. Section 117.4e. Test Animals. http://law.justia.com/us/cfr/title09/9-1.0.1.5.54.html#9:1.0.1.5.54.0.77.4
[57] European Pharmacopoeia. Monograph 0153. Vaccines for human use, Ph.Eur. 6th Edition. Strasbourg, France: European Department for the Quality of Medicines within the Council of Europe; 2008.
[58] United States Animal Welfare Act. Transportation, sale, and handling of certain animals. Title 7. U.S. Code, Chapter 54 § 2131-2159.
[59] Council of Europe. European Convention for the Protection of Vertebrate Animals Used for Experimental and Scientific Purposes. 1986, European Treaty Series. Council of Europe. European Pharmacopoeia 3rd Edition. 1997.
[60] Hendriksen CFM. Towards eliminating the use of animals for regulatory required vaccine quality control. ALTEX 2006;23:187-190.
[61] Hendriksen CFM. Refinement, reduction, and replacement of animal use for regulatory testing: current best scientific practices for the evaluation of safety and potency of biologicals. ILAR Journal 2002;43: Supplement S43-S48.
[62] Hartinger J, Folz T, Cussler K. Clinical endpoints during rabies vaccine control tests. ALTEX 2000;18:37-40.
[63] Hendriksen CFM, Steen B, Visser J, Cussler K, Morton D, Streijger F. The evaluation of humane endpoints in pertussis vaccine potency testing. In: Hendriksen CFM, Morton DB, eds. Humane endpoints in animal experiments for biomedical research. London: The Royal Society Medicine Press, 1999, p. 106–13.
[64] Collaborative study for the validation of serological methods for potency testing of tetanus toxoid vaccines for human use – part 1. Pharmeuropa Bio 2001;(2):1-92.
[65] Hendriksen C, Woltjes J, Akkermans AM, van der Gun JW, Marsman FR, Verschure MH, Veldman K. Interlaboratory validation of the in vitro serological assay systems to assess the potency of tetanus toxoid in vaccines for veterinary use. Biologicals 1994;22:257-268.
[66] Hendriksen C. Replacement, reduction and refinement alternatives to animal use in vaccine potency measurement. Expert Rev. Vaccines 2008;8(3):313-322.
[67] Kramer B, Schildger H, Behrensdorf-Nicol HA, Hanschmann KM, Duchow K. The rapid fluorescent focus inhibition test is a suitable method for batch potency testing of inactivated rabies vaccines. Biologicals 2009;37(2):119-126.
[68] Pombo M, Berthold I, Gingrich E, Jaramillo M, Leef M, Sirota L, Hsu H, Arciniega J. Validation of an anti-PA-ELISA for the potency testing of anthrax vaccine in mice. Biologicals 2004;32:157-163.
[69] Sesardic D, Winsnes R, Rigsby P, Behr-Gross M-E. Collaborative study for validation of serological methods for potency testing of diphtheria vaccines. Extended study: Correlation of serology with in vivo toxin neutralization. Pharmaeuropa Bio 2003-2:69-75.
[70] Winsnes R, Sesardic D, Daas A, Behr-Gross M-E. Collaborative study for the validation of serological methods for potency testing of diphtheria toxoid vaccines – Part 1.
[71] Winsnes R, Sesardic D, Dass A, Behr-Gross M-E (2004). Collaborative study for the validation of serological methods for potency testing of diphtheria toxoid vaccines. Phase I and II. Pharmeuropa BIO, 2003-2, 35-68.
[72] de Moura WC, de Araujo HP, Cabello PH, Romijn PC, Leite JPG. Potency evaluation of rabies vaccine for human use: the impact of the reduction in the number of animals per dilution. J Virol Methods 2009;158:84-92.
[73] Hendriksen C, Arciniega JL, Bruckner L, Chevalier M, Coppens E, Descamps J, Duchêne M, Dusek DM, Halder M, Kreeftenberg H, Maes A, Redhead K, Ravetkar SD, Spieser JM, Swam H. The consistency approach for the quality control of vaccines. Biologicals 2008;36:73-77.