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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 veterinary vaccine potency testing: state of the science and future directions

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Abstract

Veterinary vaccines contribute to improved human and animal health and welfare by preventing diseases and deaths caused by a wide range of infectious agents. However, testing necessary to ensure vaccine effectiveness and safety can involve large numbers of animals and significant pain and distress. 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. This workshop report is the fourth in a series of six, and addresses methods and strategies for veterinary vaccine potency testing that can avoid or lessen pain and distress, improve animal welfare, and reduce animal use. Vaccine potency tests considered to have the highest priority for further reduction and refinement were those that require an infectious agent challenge test or an *in vivo* toxin neutralization test, those that require large numbers of animals, and those that require the use of infectious agents hazardous to laboratory workers and/or animals. Vaccines identified as high priorities for improved alternative test methods were rabies, *Clostridium spp*., *Leptospira spp*., foreign animal diseases (e.g., foot and mouth disease), and poultry and fish vaccines. The workshop recommended priority research, development, and validation activities to address critical knowledge and data gaps, including opportunities to apply new science

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and technology. Recommendations to support more humane animal use included development and use of humane endpoints for all challenge tests, development of serologic assays to replace challenge tests, and development of *in vitro* toxin neutralization tests to replace *in vivo* TNTs. Workshop participants recommended approaches to reduce the number of animals required for potency testing, and recommended enhanced international harmonization and cooperation, and closer collaborations between human and veterinary researchers to expedite progress in the development and application of alternative methods. Implementation of the workshop recommendations is expected to advance new methods for veterinary vaccine potency testing that will benefit animal welfare and reduce animal use while ensuring continued protection of human and animal health.

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

1. Introduction

Veterinary vaccines contribute to improved human and animal health and welfare by preventing infection and controlling infectious agents that can cause disease and death. 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 international 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; European Commission, Joint Research Centre, IHCP), the Japanese Center for the Validation of Alternative Methods (JaCVAM), and Health Canada.

The workshop addressed the state of the science of human and veterinary vaccine potency and safety testing. Participants developed recommendations for future progress in three major areas: (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]. Workshop reports for each of the three topics for human vaccines and for each of the three topics for veterinary vaccines were prepared based on the contributions of invited experts and the general public during the various plenary presentations and dedicated breakout group sessions [3, 4, 5, 6, 7, 8]. This report addresses methods and strategies for the reduction and refinement of animal use for potency testing of veterinary 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 challenge testing. Challenge

testing is conducted to determine the amount of vaccine required to protect animals from infection with live agents, and inadequately protected and control animals often develop clinical disease or die. The second strategy is the development and use of serological methods that can eliminate the need for challenge testing. In serological methods, the amount of protective antibody produced is measured and serves as an indicator of vaccine potency. The final strategy involves the application of methods and approaches that may reduce the number of animals 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:

- Identified criteria to prioritize vaccine potency and safety tests for future alternative test method development, and identified high priorities using these criteria
- Reviewed the current state of the science of alternative methods and discussed ways to promote the implementation of available methods
- Identified knowledge and data gaps that need to be addressed
- Identified and prioritized research, development, and validation efforts needed to address these gaps in order 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, following the approval of a vaccine, testing is then required to 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. Workshop recommendations to advance the use and development of alternative methods that can replace animals for the potency testing of human [3] and veterinary vaccines [4] are available elsewhere in these proceedings.

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 veterinary vaccines are provided in this paper. Recommendations for human vaccines are available elsewhere in these proceedings [5].

The final plenary session addressed methods and approaches for reducing, refining, and replacing animal use to assess the safety of serial production lots of human and veterinary vaccines [11, 28, 29, 30]. Breakout groups then discussed the state of the science and developed recommendations for advancing alternative methods for vaccine safety testing. Workshop recommendations to advance the use and development of alternative methods for safety testing of veterinary vaccines [8] and human vaccines [7] are available in these proceedings.

3. Requirements for veterinary vaccine potency testing

Strict regulations and guidelines are designed to ensure that each veterinary vaccine product released for sale is pure, safe, potent, and effective [31]. An estimated 18,000 serials (batches) of veterinary vaccines are released annually in the United States for approximately 2000 different products that protect animals from 213 different animal diseases [12]. Given that many of the inactivated vaccines still require animals for potency testing, significant numbers of animals are necessary.

Veterinary vaccines contribute to the health and well being of animals and people. In addition to controlling and preventing diseases of companion and domestic animals, vaccines help ensure a safe and efficient global food supply. They reduce the transmission of zoonotic and food borne infections from animals to people. Vaccines also reduce the need for low-level antibiotics to control some diseases in food animals.

Due to the number of animals used annually for the release of veterinary vaccines, global regulatory agencies actively encourage the evaluation, development, and implementation of novel approaches that reduce, refine, and replace (3Rs) the use of animals in vaccine safety and potency product release testing [12, 14, 22].

Current veterinary vaccines consist of modified live (attenuated) viruses and bacteria, inactivated (killed) viruses and bacteria, toxoid or bacterin toxoids, peptide and subunit vaccines, and genetically engineered products. The general types of potency tests employed by vaccine manufacturers include (1) titration of live organisms (*in vitro* assays, but occasionally *in vivo*), (2) *in vitro* assays such as enzyme-linked immunosorbent assays (ELISAs) or other antigen quantification methods, (3) serology methods to quantify protective antibody or antitoxin, and (4) vaccination-challenge *in vivo* methods using either the host animal (e.g., fish, poultry) or laboratory animals (e.g., hamsters, mice) [17]. Reduction and refinement procedures are especially applicable to serological and vaccination–challenge potency testing methods, both of which require the use of animals.

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

Potency testing for many veterinary vaccines still requires the use of animals for challenge tests, serological assays, and some toxin neutralization tests (TNTs). For these veterinary vaccine testing situations, workshop participants recommended that the following should have the highest priority for further development and validation activities relevant to reduction and refinement of animal use:

- Potency testing that involves significant animal pain and distress, such as challenge testing and *in vivo* TNTs
- Potency testing that uses large numbers of animals, based on both the number of animals used per potency test and the number of serials tested annually
- Potency testing that requires live viruses and bacteria that are highly contagious and/or hazardous to laboratory workers, livestock, pets, and wildlife, including foreign animal diseases in countries that are free of such diseases (e.g., foot and mouth disease and bluetongue disease)
- Potency testing for which there are ongoing alternative development and validation activities
- Potency tests for new vaccines that are currently undergoing prelicensing development and evaluation

Based on these criteria, the highest priorities recommended for further development of alternative vaccine potency test methods include the following:

- Rabies vaccines
- *Leptospira spp.* bacterial vaccines
- *Clostridium spp.* bacterial vaccines for which an *in vivo* TNT is still used
- Any potency test that still requires a challenge test
- Potency tests that still require the use of foreign animal diseases, especially foot and mouth disease and bluetongue disease
- Poultry vaccines
- Fish vaccines
- New vaccines

Rabies, *Clostridium spp.,* and *Leptospira spp.* vaccines, as well as fish and poultry vaccines, were identified as the highest priorities because these potency tests require large numbers of animals, and most involve either challenge or *in vivo* toxin neutralization testing that can involve significant animal pain and distress. Workshop

participants also recommended that manufacturers seek and consider alternative assays during the development phase for new vaccines that could then be used for subsequent lot release potency and safety testing.

Many of these priority vaccines already have alternative potency test assays in various stages of development and validation and published guidances for some alternative tests are available in some countries and regions. For example, the Council of Europe publishes monographs in the European Pharmacopoeia, and the U.S. Department of Agriculture publishes regulations according to Title 9 of the U.S. Code of Federal Regulations. The USDA also publishes supplemental assay methods (SAMs) that include more detailed test method protocols. Vaccine manufacturers can use this guidance to conduct product-specific validation for each vaccine testing procedure and submit it to the appropriate regulatory authority for approval.

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

5.1. State of the science

Many veterinary vaccines still require the use of animals for challenge tests to quantify and demonstrate potency before serial release. Although death is not a required endpoint for veterinary vaccines, death often occurs when there is rapid progression from a clinically normal to an overtly ill condition. Moribund condition currently serves as the default endpoint for early termination of *in vivo* vaccine potency tests [32]. Ideally, earlier euthanasia of individual animals should be based upon standardized, earlier, and more humane endpoints.

Humane endpoints are criteria that can be used as the basis for ending a test or research procedure earlier in order to avoid further pain and distress. Ideally, humane endpoints can be used as criteria to end a procedure *before* the onset of animal pain and distress [33, 34, 35]. However, the use of earlier, more humane endpoints must be capable of achieving the specific testing or research objectives. The use of available, scientifically valid humane endpoints wherever possible is also a fundamental principle of the U.S. Public Health Service Policy on Humane Care and Use of Laboratory Animals [36], as well as existing statutory requirements in the United States [37] and the European Union (former Directive 86/609 and recently issued Directive 2010/63) [38, 39].

Specific policies have been adopted that incorporate the use humane endpoints during vaccine potency testing procedures [33, 40, 41]. The use of humane endpoints is also reflected in regulatory guidance documents and/or legislation (e.g., U.S. Code of Federal Regulations, European Directorate for the Quality of Medicines (EDQM), and associated European Pharmacopoeia) of relevant agencies in numerous regions and countries worldwide [33. 34, 35, 40, 41, 42] (**Table 1**). For example, the Center for Veterinary Biologics (CVB) Notice No. 04-09, issued in 2004 [43], provides for humane endpoints by stating that moribund animals may be humanely euthanized and considered as deaths provided that the details for humane endpoints are stated in the outline of production for the specific vaccine.

Several types of observations and measurements may serve as humane endpoints:

- Observation of clinical signs, including behavioral changes
- Measurement of changes in physiological parameters such as body temperature, body weight, heart rate, and respiratory rate
- Measurement of changes in serum or blood biomarkers, such as specific clinical chemistry or hematology parameters [25, 35, 42]

Successful implementation of clinical signs as humane endpoints requires comprehensive training of animal care staff [35, 46].

For some existing vaccines that require a challenge test, the procedure has been refined to reduce animal pain and distress by incorporating earlier and more humane endpoints. For example, earlier humane endpoints for veterinary rabies vaccines have been approved and adopted for use in the United States and internationally, thereby reducing both the duration and severity of pain and distress in test animals [47, 48, 54] (**Table 2**). Humane endpoints of paresis, paralysis, and/or convulsions were determined to sufficiently predict rabies infection and indicate that the animal would not recover [49]. Similar humane endpoints are in place for human rabies vaccine potency testing [19, 50].

Table 1. Regulations, policies, and guidance for the use of humane endpoints

Organization	Citation	Text
USDA	9 CFR 117.4(e) (1998) [40]	"Test animals that show clinical signs of illness that are due to test may be treated or humanely destroyed if the illness has progressed to a point ….when death is certain to occur without therapeutic intervention."
USDA	CVB Notice No. 04-09 (2004) [43]	Sets the provision for humane endpoints by stating that moribund animals may be humanely euthanized and considered as deaths, provided the details for humane endpoints are stated in the Outline of Production for the specific vaccine.
CCAC	CCAC Guidelines (1998) [44]	"… investigators should eliminate, mitigate, or minimize potential pain and distress whenever feasible and consistent with good scientific practice."
OLAW, NIH	Public Health Service Policy on Humane Care and Use of Laboratory Animals (2002) [36]	"… discomfort to animals will be limited to that which is unavoidable for the conduct of scientifically valuable research, and that unrelieved pain and distress will only continue for the duration necessary to accomplish the scientific objectives. Animals that would otherwise suffer severe or chronic pain and distress that cannot be relieved should be painlessly killed at the end of the procedure, or if appropriate, during the procedure"
a) Council of Europe	a) Introduction to the European Pharmacopoeia since 3rd edition (1998)	a) General statement on commitment to "alternative procedures"
b) Council of Europe (2011)	b) Vaccines for veterinary use (Ph. Eur. Monograph 062)	b) "… if it is indicated that an animal is considered to be positive, infected etc. when typical clinical signs occur then as soon as it is clear that result will not be affected the animal in question shall either be euthanised or given suitable treatment to prevent unnecessary suffering."
	European Pharmacopoeia 7th Edition [41]	
European Union 2010	Directive 2010/63/EU [39]	"The methods selected should avoid, as far as possible, death as an end-point due to the severe suffering experienced during the period before death. Where possible, it should be substituted by more humane end- points using clinical signs that determine the impending death, thereby allowing the animal to be killed without any further suffering."
ICLAS	Demers et al. 2006 [45]	"Death or severe pain and distress should be avoided as end points. The earliest possible end point should be used that is consistent with the scientific objectives. Studies should be designed to minimize any pain or distress likely to be experienced by the animals, while meeting the scientific objectives. The duration of studies involving pain and distress should be kept to a minimum. Pilot studies should be encouraged as a means of determining morbidity, time course of effects, and frequency of observations required to set an earlier end point …. Animals should be monitored by means of behavioral, physiological, and/or clinical signs at an appropriate frequency to permit timely termination of the experiment once the end point has been reached."

USDA – United States Department of Agriculture
CFR – Code of Federal Regulations
CVB – Center for Veterinary Biologics
CCAC – Canadian Council on Animal Care
OLAW, NIH – Office of Laboratory Animal Welfare, National Institutes of Health
ICLAS – International Council for Laboratory Animal Science

Earlier humane endpoints for other vaccine-challenge tests have also been described and endorsed by regulatory authorities. For example, post-challenge earlier humane endpoints for the swine vaccine for *Erysipelas rhusiopathiae* include pathognomonic, diamond-shaped erythematous skin lesions, elevated temperature, and

arthritis [22, 51, 52]. For challenge testing of chicken fowlpox vaccine, earlier humane endpoints include pox lesions, warty eruptions, and scabs on the comb and wattles [22, 53] (**Table 2**).

Table 2. Veterinary 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
Inactivated rabies virus vaccine (*Lyssavirus rabies)*	Convulsions, paralysis, paresis[a,b]	Cussler et al. 1998 [49]; Bruckner et al. 2003 [54]; Wunderli et al. 2006 [55]; 9 CFR 117.4e [40]; 9 CFR 113.209; USDA SAM 308 (2007) [47]; Ph. Eur. Monograph 451 [48]	Moribund condition or death previously used as study endpoints
Inactivated swine erysipelas vaccine (*Erysipelothrix rhusiopathiae*)	Pathognomonic, diamond-shaped erythematous skin lesions, elevated temperature, arthritis[a]	Johannes et al. 2003 [52]; Srinivas 2011 [22]; 9 CFR 113.67 [56]	Moribund condition or death previously used as study endpoints
Fowlpox virus vaccine	Pox lesions, warty eruptions/scabs on combs and wattles[a]	Srinivas 2011 [22]; 9 CFR 113.326 [57]	Moribund condition or death previously used as study endpoints

[a] Accepted by U.S. regulatory authorities.
[b] Published in the European Pharmacopoeia.

However, humane endpoints for many commonly used veterinary vaccines, including some *Clostridium* and *Leptospira spp.* vaccines, have not been identified. The potency test for many *Clostridium spp.* vaccines produced in the United States consists of vaccination-challenge in laboratory animals or, more commonly, vaccination of laboratory animals followed by titration of serum in a toxin–antitoxin neutralization test using mice. Although the potency tests for all of these *Clostridium spp*. disease vaccines incorporate humane euthanasia as described in 9 CFR 117.4 [40], earlier more humane endpoints have yet to be developed for challenge tests and toxin–antitoxin neutralization tests. Applicable examples include potency testing of *Clostridium chauvoei* [58] and *Clostridium haemolyticum* [59] using vaccination–challenge testing in guinea pigs, and potency testing of *Clostridium botulinum* using a vaccination–challenge test in mink [60]. For *Clostridium perfringens* Type C [61], *Clostridium perfringens* Type D [62], *Clostridium novyi* Type B [63], and *Clostridium sordellii* [64], rabbits are vaccinated, and serum is titrated using a toxin–antitoxin neutralization test in mice.

For many *Leptospira spp*. vaccines, hamsters are used to assess potency by vaccination–challenge methods [65, 66]. Humane endpoints have yet to be defined for these vaccines, including *Leptospira interrogans* serovars; pomona [67], canicola [68], icterohaemorrhagiae [69], and grippotyphosa [70]. Animals that become moribund are humanely euthanized as outlined in 9 CFR 117.4 [40].

In summary, progress has been made in identifying earlier humane endpoints for some vaccines that still involve challenge testing with live agents. However, workshop participants concluded that it is imperative for concerted efforts to be made to identify humane endpoints for all vaccines that still require challenge testing and for which there are no earlier humane endpoints, especially for *Clostridium* and *Leptospira spp.* vaccines. These efforts should include systematic collection and evaluation of data to identify appropriate clinical or other objective parameters that are predictive of current test endpoints, and that can then be used as earlier humane endpoints to reduce pain and distress in test animals.

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

Workshop participants agreed that earlier humane endpoints should be investigated for use for all challenge testing situations. Key priorities included the need for a thorough understanding of disease progression during a

challenge test, including the identification of earlier humane endpoints through routine systematic collection and evaluation of all clinical signs.

Although humane endpoints have been identified and incorporated into regulatory guidelines for some products, many potency tests still use procedures in which the endpoint is moribund condition rather than early clinical signs consistent with disease pathogenesis (9 CFR 117.4) [40]. Identifying earlier endpoints for rabies vaccine testing than the current obvious clinical signs of paresis, paralysis, and convulsions is also desirable. However, this will require better understanding of measurable and/or other subtle observable changes that precede and predict these more obvious clinical signs.

Workshop participants identified key knowledge gaps that need to be addressed to advance the validation and implementation of humane endpoints:

- Improved understanding of measurable and/or observable changes that lead to overt clinical signs and moribund condition
- Improved understanding and investigation of potentially useful objective quantitative endpoints
- Understanding of the causes that lead to inconsistent patterns of moribund condition
- Identification of earlier endpoints for diseases with rapid progression from normal clinical state to death
- Confirmation that the use of humane endpoints for specific challenge tests do not lead to a higher rate of inconclusive test outcomes
- Increased availability of data collected to identify humane endpoints, and public access to such data
- Improved understanding of early clinical signs in fish that are predictive of eventual moribund condition or death

Workshop participants recommended increasing awareness within the vaccine industry about the concept of humane endpoints and providing greater access across the industry to all relevant information about useful endpoints as well as the process for identifying humane endpoints. An existing example that could be distributed is a video demonstrating the assessment and use of paresis, paralysis, and convulsions as humane endpoints for rabies vaccines [54].

Workshop participants recommended the following priority activities to advance the validation and implementation of humane endpoints:

- Improved guidance on and training in recognition of early clinical signs
- Identification of earlier humane endpoints for all vaccine potency challenge testing, with a priority for *Leptospira spp.* challenge testing
- Identification of earlier humane endpoints for control groups in challenge testing, in which clinical signs may be earlier and more overt in vaccinates that have partial but inadequate protection
- Systematic collection and evaluation of clinical and other objective data during prelicensing efficacy tests, which may identify earlier humane endpoints applicable for use in subsequent lot release testing
- Enhanced, more innovative methods for observing animals

To better understand disease progression during challenge testing and to expedite future data collection, workshop participants recommended that clinical data for control animals should be initially analyzed to identify earlier clinical endpoints. Control data should also be evaluated to determine if there is a threshold percentage of controls that exhibit the specific humane endpoints that is sufficiently predictive that the remainder of the controls will succumb. If so, this threshold could be used as the basis for early termination of all remaining control animals. Workshop participants suggested that earlier humane endpoints for control animals might be especially applicable to poultry and fish vaccines. Clinical observations from prelicensing vaccination efficacy studies should be maintained, summarized, and reviewed to identify earlier humane endpoints for post-licensing serial release potency testing. Humane endpoints are more likely to be identified for challenge testing in which animals take longer to develop and succumb to disease (e.g., *Leptospira spp.*).

Workshop participants recommended further research to develop enhanced, innovative methods for observing animals more frequently to detect the onset of clinical signs identified as humane endpoints. Adaptation of video or real-time computer monitoring techniques may be useful for this purpose. Workshop participants also recommended that the frequency of animal observations should be increased to that necessary to minimize spontaneous deaths, with observations made at least twice daily.

Finally, workshop participants recommended that broader implementation of humane endpoints can be supported by (1) disseminating information on the clinical signs associated with each disease and (2) sharing information between manufacturers and regulators regarding valid and acceptable humane endpoints. Manufacturers should be

encouraged to identify and implement humane endpoints for all challenge testing. Nevertheless, the need for more frequent observation of animals and advanced clinical surveillance and monitoring equipment could add additional expense. Manufacturers will also need to work with appropriate regulatory authorities to modify documentation such as Outlines of Production in accordance with established procedures.

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

6.1. State of the science

For some vaccines, the traditional vaccine potency challenge test has been replaced by a serological method. Serological methods measure the amount of protective antibody produced in vaccinated animals, which is compared to the reference antibody value known to provide protection in the challenge test. A validated laboratory assay quantifies the amount of protective antibody produced in response to the vaccination dose. Serological testing thereby provides for significant refinement compared to challenge testing by avoiding the pain and distress resulting from infections in unprotected vaccinates and control animals.

Serological methods have been developed and validated for many veterinary vaccines, such as inactivated *Erysipelothrix rhusiopathiae* vaccine [71] (**Table 3**). However, for some vaccines, the serum collected from vaccinated animals may still require additional animals in a test to determine the presence of protective antibodies to toxin. This test, known as the *in vivo* Toxin Neutralization Test (TNT), involves combining the protective antibody collected form vaccinated animals with known amounts of toxin produced by the causative agent (e.g., *Clostridium spp.* vaccines). This mixture is administered to laboratory animals to assess whether the level of protective antibody fully neutralizes the toxin. If sufficient protective antibody is present to fully neutralize the toxin, the test animals are not exposed to any free toxin and survive. However, if there is residual free toxin, the test animals develop clinical signs or die. Validated *in vitro* methods to quantify antibodies for some toxins are now available. Examples include an indirect ELISA for inactivated erysipelas vaccines [71], an indirect ELISA [72] and a toxin-binding inhibition (ToBI) test for tetanus [73], and the *in vitro* RFFIT (rapid fluorescent focus inhibition test) for rabies vaccines (**Table 3**). Toxin neutralization tests using cell cultures are available for potency testing for *Clostridium perfringens* C/D [74], *Clostridium septicum* [75], and *Clostridium novyi* (Type B) vaccines [76, 77] (**Table 3**).

Serological methods for potency testing of *Leptospira spp.* vaccines are available for dogs [78] and cattle [79] (**Table 3**). Canine leptospirosis vaccine (inactivated, nonadjuvanted) is a preparation of inactivated whole organisms and/or antigenic extract(s) of one or more *Leptospira interrogans* serovars (e.g., canicola, icterohaemorrhagiae, or any other epidemiologically appropriate serovar). Bovine leptospirosis vaccine (inactivated) is a preparation of inactivated whole organisms and/or antigenic extract(s) of one or more suitable strains of *L. borgpetersenii* (serovar hardjo) or *L. interrogans* (multiple serovars). For each of the serovars for which protection is claimed, the antibody response is measured in vaccinated, disease-free guinea pigs. Serum is tested using any suitable validated method, such as a micro-agglutination test, to measure the antibodies in each sample [79] (**Table 3**).

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

Workshop participants identified knowledge gaps and research necessary to develop and validate serological methods that could be used instead of challenge tests and *in vivo* TNT:

- Identification of functional protective antigens and corresponding antibodies
- Availability of reagents and standards to conduct specific antibody quantification assays and stability testing procedures
- Improved substrates to detect enzymatic activity of toxins using specific cell lines
- Research into new immune-based methodologies

The functional antigen and protective antibody must be identified in order to develop and standardize an *in vitro* assay to quantify the antibody response. The goal is to identify a serological threshold of protective antibody above which the vaccine is considered adequately potent. The threshold should correlate with *in vivo* protection.

Serological testing usually involves a relative potency test in which the antibody response in the test serial is compared to that of a standard reference. The requirement for a standard reference varies by authority and the

specific approved license. A number of issues associated with a standard reference must be addressed, including preparation, quantification, validation, stability, and requalification. Qualification of the standard reference requires an *in vivo* host animal immunogenicity test or preparation of a serial adjusted to be equivalent to the reference that is then used in the host animal efficacy study. Typically, the frozen reference can be stored for up to five years. Under refrigeration conditions, the reference can be stored for up to two years [97]. However, assays must be developed, validated, and implemented that can adequately monitor the stability of the reference during storage.

Table 3. Examples of veterinary 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	References For Traditional Methods
Inactivated rabies vaccine (*Lyssavirus rabies*)	Immunization (mice) and serology[a]: *In vitro* RFFIT (rapid fluorescent focus inhibition test)	Kramer et al 2010 [80]; Kramer et al. 2009 [81]; Cliquet et al. 1998 [82]; Nagarajan et al. 2006 [83]; USDA SAM 315 (1986) [84]; Ph. Eur. Monograph 451 [85]	Immunization challenge in mice (intracerebral)[b]	9 CFR 113.209 [47]
Inactivated swine erysipelas vaccine (*Erysipelothrix rhusiopathiae*)	Immunization (mice) and serology: *In vitro* antibody quantification[a,c]– ELISA	Rosskopf-Streicher et al. 2001 [86]; Beckmann and Cussler 1994 [87]; USDA SAM 613 (2009) [88]; Ph. Eur. Monograph 064 [71]	Mouse lethal challenge test	9 CFR 113.119; USDA SAM 611 (2008) [89]
Clostridium novyi (Type B); Bovine (Black disease)	Immunization (rabbits) and serology[a d]: *In vitro* immunochemical method or neutralization in cell cultures (specific details not provided in Ph. Eur. monograph)	Hendriksen et al. 1998 [90]; EDQM 1997, 2007 [91]; Ph. Eur. Monograph 362 [76]	Rabbit immunization/mouse toxin neutralization test	9 CFR 113.108; USDA SAM 207 (2007) [63]
Clostridium septicum; Bovine (malignant edema)	Immunization (rabbits) and serology[a d]: *In vitro* immunochemical method or neutralization in cell cultures (specific details not provided in Ph. Eur. monograph)	Hendriksen et al. 1998 [90]; EDQM 1997, 2007 [91]; Ph. Eur. Monograph 364 [75]	Rabbit immunization/mouse toxin neutralization test	-
Clostridium perfringens C/D; Bovine (Enterotoxemia)	Immunization (rabbits) and serology[a d]: *In vitro* immunochemical method or neutralization in cell cultures (specific details not provided in Ph. Eur. monograph)	Rosskopf-Streicher et al. 2004 [92]; Hendriksen et al. 1998 [90]; EDQM 1997, 2007 [91]; Ph. Eur. Monograph 363 [76]	Rabbit immunization/mouse TNT	9 CFR 113.111 and 112; USDA SAM 201 (Type C, 2008) [61]; SAM 203 (Type D, 2007) [62]
Tetanus Antitoxin Products (equine); (*Clostridium tetani*)	Immunization (guinea pigs) and serology[a,c]: *In vitro* toxin-binding inhibition (TOBI), indirect ELISA[c]	Hendriksen et al. 1994 [93]; USDA SAM 217 (2009) [72]; Ph. Eur. Monograph 697 [73]; Council of Europe (1996) [94]	Guinea pig immunization/guinea pig toxin–antitoxin neutralization test	9 CFR 113.114; USDA SAM 206 (2007) [95]

Vaccine Product (Disease)	3R Alternative	References For Alternative Methods	Traditional Test Procedure for Which the Alternative Method is Applicable	References For Traditional Methods
Leptospira interrogans Serovar *canicola* bacterin Canine leptospiral (inactivated, adjuvanted and non-adjuvanted)	Immunization (hamsters) and serology[a c]: *in vitro* method to determine antibodies (a validated serological method is permitted, no further details provided in the Ph. Eur.)	Ph. Eur. Monograph 447 [78]	Immunization challenge test in hamsters[f]	9 CFR 113.103; USDA SAM 609 (2008) [68]
Leptospira interrogans Serovar *hardjo* bacterin E Bovine *Leptospira hardjo*	Immunization (guinea pigs) and serology[a c]: micro-agglutination test	Ph. Eur. Monograph 1939 [79]	Cattle immunization challenge[g]: Immunization challenge test in hamsters	9 CFR 113.105 [96]

[a] Published in the European Pharmacopoeia.
[b] Not for routine batch release (Ph. Eur.).
[c] Accepted by U.S. regulatory authorities
[d] The European Pharmacopoeia states that following serology, an immunochemical method or neutralization in cell cultures is considered acceptable following product-specific validation.
[e] Applicable after in-house (product specific) validation.
[f] The European Pharmacopoeia states endpoint is "signs" of the disease and not lethality.
[g] The European Pharmacopoeia states that cattle are used for prelicensing while serology in guinea pigs is conducted for routine batch release testing.

Protective antigens and reference standards must be identified and characterized. In addition, antibodies and other reagents necessary to conduct the serological test must be available for use. This is also true for replacement of the TNT, where there is a need for improved specific cell lines to detect the enzymatic activity of toxins. As immunological research advances, it may be possible to incorporate new methodologies based on functionality of antibodies or other immune responses, including the use of macrophages and dendritic cell lines.

Workshop participants recommended the following priority activities to advance the development and implementation of serological methods:

- Studies and actions necessary for vaccine manufacturers to implement the use of the serological assay for rabies vaccines
- Research necessary to develop ELISAs or cell-based assays for *Clostridium spp*. vaccines that currently use *in vivo* TNTs
- Development of serological methods for *Leptospira spp*. vaccines
- Identification of protective antibodies for fish vaccines
- Promotion of product-specific validation by manufacturers for available serological methods for those vaccines that currently use the challenge test

6.2.1. Rabies vaccines

Further development, validation, and implementation of serological tests for human and veterinary rabies vaccines were the highest priorities identified by workshop participants due to the large number of mice used in the test and the high variability of the current *in vivo* potency test (i.e., the mouse rabies challenge test). Over the past 10 years, extensive research has been directed toward replacing the rabies challenge test with a serological method. However, there have been significant difficulties in correlating the serological test to the mouse challenge test due to variability in the challenge test [54, 55, 85, 98].

Two serological methods have been developed for inactivated veterinary rabies vaccines: the rapid fluorescent focus inhibition test (RFFIT) [81, 84, 99] and the fluorescent antibody virus neutralization (FAVN) test [82]. A recent study demonstrated good correlation between results from the RFFIT and the challenge test [81]. In a validation study [80], the RFFIT was found to have good reproducibility within and between laboratories, thus providing a potential alternative to the mouse vaccination–challenge assay [22]. Considering these recent

developments, workshop participants recommended a focused international workshop to discuss the barriers and actions necessary to achieve international implementation of the RFFIT. Note: In January 2011, the European Pharmacopoeia published a draft monograph incorporating the RFFIT as a new alternative serological potency assay for inactivated veterinary rabies vaccines [85].

For human rabies vaccines, the *in vivo* potency release test is similar to that used for veterinary products. Potency is defined as the geometric mean of the results of two valid mouse potency challenge tests with humane endpoints [19, 50]. Although ELISA-based assays have replaced several animal-based immunogenicity assays for human vaccines, this is not yet true for human rabies vaccines. Although the neutralizing antigens for rabies are well defined, a clear correlation has not been demonstrated between the amount of antigen required to induce an immune response in animals, the amount of antigen measured using an *in vitro* assay, and the protective immune response in humans [19].

While developing a single potency test (serological or antigen quantification) for both human and veterinary rabies vaccines would be ideal, it will be necessary to adapt any potency test for both product-specific and virus strain-specific vaccines [54]. Due to the clear overlap between human and veterinary rabies vaccines, workshop participants encouraged future collaboration between human and veterinary vaccine manufacturers and regulatory agencies during the development of alternatives for rabies vaccine potency testing.

6.2.2. Clostridium spp. vaccines: replacing in vivo toxin neutralization tests

Traditional potency testing for veterinary *Clostridium spp.* vaccines includes an *in vivo* rabbit immunization study followed by quantitative assessment of toxin-neutralizing antibodies in serum from the rabbits using a toxin neutralization test. While *in vitro* TNTs have been developed for some *Clostridium* spp. vaccines, many of these vaccines still require the use of mice to demonstrate toxin neutralization *in vivo* (e.g., *C. novyi* [63] and *C. perfringens* [61] (**Table 3**)). The possibility of using *in vitro* TNTs to test *Clostridium spp.* vaccine potency is now supported by general guidance published for *C. novyi* [76], *C. perfringens* [74], and *C. septicum* [75] and by a validated ELISA to measure toxin neutralization for *C. chauvoei* [100, 101].

Global implementation is still needed for many serological methods that are now used only regionally for *Clostridium spp.* vaccines. This may require broader access to detailed test method protocols and increased availability of standard references and reagents from sources in the Europe (EDQM) and the United States (USDA). Based upon the current scientific literature and published regulatory methods, the replacement of the mouse TNT for other specific *Clostridium spp.* vaccines, as well as the global application of existing *in vitro* serological methods, appears achievable with appropriate development and validation efforts.

Vaccine manufacturers are frustrated by the need to use different potency testing methods depending upon the location of vaccine usage and sale. Animal use (as well as time and costs) would be significantly lower if there were global application of existing or newly validated alternative methods, especially for the *Clostridium spp.* and rabies veterinary vaccines.

6.2.3. Leptospira spp. vaccines (specifically Leptospira hardjo and Leptospira bratislava)

The current *in vivo Leptospira* potency test consists of hamster vaccination followed by challenge ten days later. The time-intensive *in vivo* test takes more than five weeks to complete and exposes personnel to live *Leptospira* bacteria serovars, many of which are zoonotic. The USDA recently developed an ELISA to compare the relative potency of specific bacterins to a qualified reference standard for *L. interrogans* serovars (pomona [102], canicola [103], grippotyphosa [104], and icterohaemorrhagiae [105]). Additional studies to be completed by the USDA include testing for interference by adjuvants and other vaccine components [17]. Therefore, *in vivo* vaccination–challenge methods are still in use for many *Leptospira spp.* vaccines, including the following *L. interrogans* serovars; pomona [67], canicola [68], grippotyphosa [70], and icterohaemorrhagiae [69]. Successful completion of these studies, along with product-specific validation, will be required to replace the vaccination–challenge test with the recently developed serological assays.

Both *in vivo* and *in vitro* methods are published in the relevant USDA SAMs and the European monographs (e.g., the canine leptospiral serology and antigen quantification method [78] and the bovine leptospiral serology method [79, 106, 107]. Vaccines for *Leptospira hardjo* and *L. bratislava* were also identified as candidates for conversion to serological methods due to progress made in research performed to date in addition to the extensive use of these vaccines.

6.2.4. Fish vaccines

This workshop emphasized the importance of fish vaccine potency tests because of the large number of fish used in vaccination–challenge procedures, including the large numbers of unvaccinated controls [14]. The majority of fish vaccine potency release tests consist of a host animal vaccination–challenge method; and little progress has been achieved in reducing, refining, or replacing the use of fish in this process [108]. Fish inactivated bacterial vaccines have been used successfully in aquaculture, but only recently have a significant number of effective viral vaccines for fish been developed [109]. Increasingly, adjuvants and immunostimulants are being used to enhance vaccine potency in fish, thereby further complicating the successful development of alternative fish vaccine potency tests [110]. For many fish vaccines, the correlation of serological response with protection has not been well established, thereby impeding the development of serological potency tests [108]. However, some protective antigens have been identified for inactivated bacterial vaccines such as those protecting from *Vibrio salmonicida* and *Vibrio anguillarum* diseases, suggesting that serology or antigen quantification methods could be developed for selected vaccines [108]. Workshop participants agreed that the following are needed: (1) further research to identify the antibodies involved in protective immunity, (2) development of the assays and reagents to measure these antibodies, and (3) validation of the antibody assays used in serological assessment of protective immunity. Expanded research and development efforts are expected as additional fish vaccines enter the market and more animal health companies develop vaccines for aquaculture use.

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

7.1. State of the science

Reducing the number of animals used in challenge and serological assays is considered a high priority for all regulatory agencies, vaccine manufacturers, and other interested stakeholders. The number of animals used per test has been reduced over time as improvements have decreased variability in assays, which can lead to using fewer animals to achieve the same statistical power [107, 111, 112].

In addition, where it can be scientifically justified, limiting the number of dose groups can reduce the number of animals used. For example, single-dose potency testing can be used in place of multidose testing for inactivated veterinary rabies vaccines under certain testing circumstances [48] (**Table 4**). However, a single-dose potency test only shows that the vaccine under test meets the minimum requirement for potency. It is not a quantitative estimate of potency [19, 46]. A single-dilution test is best used when production is consistent, quality control systems are excellent, and the amount of the test serial antigen is well over the minimum qualified standard. For a multidilution test, the test response curve is compared to a standard curve, and the median effective dose is determined by relative potency. For human rabies vaccines, a multidose approach uses approximately 200 mice per test, compared to 60 mice per test in the single-dilution test [19].

Another reduction alternative is using fewer animals per group in multidilution tests. For example, a recent report indicated that statistically valid assays could be obtained using the mouse rabies challenge test when 9 mice instead of 18 were used for each dose for potency assessment of human rabies vaccine [113].

Table 4. Veterinary vaccine potency assays that incorporate reduction alternative methods

Vaccine Product (Disease)	3R Alternative	References	Traditional Test Procedure for Which the Alternative Method is applicable
Rabies vaccine (*Lyssavirus rabies*)	Single-dilution assay[a]	Bruckner et al. 2003 [54]; 9 CFR 113.209 [47]; de Moura et al. 2009 [113]; Ph. Eur. Monograph 451 [48]	Multiple-dilution assays

[a] Published in the European Pharmacopoeia.

Another example of reducing animal use for testing of a biological product is the modified tuberculin-PPD test recently published by the World Organization for Animal Health (OIE) [26, 114]. Employing an experimental design different from that of the current standard test, the modified test involves the injection of each guinea pig with test and reference serials, thus allowing each animal to act as its own control. The modified test, which decreases the number of animals used by more than 50%, reduces the amount of individual animal variability while maintaining statistical power [26, 114].

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

Workshop participants agreed that further reduction of animal numbers required for vaccination–challenge and serological tests will require better understanding of the causes of variability and the basis for incomplete or inconclusive results that necessitate repeat testing. In addition, for many *in vivo* potency tests, the number of unvaccinated controls may be reduced, thereby reducing the number of animals experiencing pain and distress. Significant reduction can be achieved in fish vaccines, which can use 50 to 100 control animals per test.

The recommended approaches to potential reduction of group sizes involves a systematic investigation into the causes of excessive variation and repeat testing, followed by a sustained effort to reduce or eliminate the sources contributing to variation and incomplete test results. As the factors affecting experimental variability are reduced or eliminated, the minimum number of animals required to maintain the necessary statistical power should be reassessed.

To reduce the number of control animals used in vaccination–challenge potency tests, archival control data should be reviewed to determine if control size might be reduced while maintaining statistical power. Workshop participants agreed that flexibility should be incorporated into the regulatory process so that the reduction of animals can be applied on a case-by-case basis, especially for minor-use situations.

Additionally, increased emphasis should be placed on evaluating the use of single-dilution assays to replace multidilution methods for both vaccination–challenge and serology assays. This again would require a retrospective review of existing data as well as more-detailed procedures and technical training to ensure valid test results. As discussed earlier, the current *in vivo* potency test for inactivated veterinary rabies vaccine is a multidilution vaccination–challenge test in mice (NIH test) known to be highly variable with a high frequency of invalid results [55, 115, 116]. Reduction variations include the use of a single-dilution vaccination regimen [48] or a reduction in the number of animals used per dilution [113]. Regulatory authorities and vaccine companies should assess whether these reduction variations will meet their specific needs for potency testing and individual vaccine product release.

8. Reduction and refinement: potential application of human vaccine potency testing alternatives to veterinary vaccines

There are three general areas in which the application of recent progress with human vaccine reduction and refinement alternatives might be applied to veterinary vaccines and vice versa. First, knowledge could be shared by researchers and vaccine manufacturers for human and veterinary zoonotic vaccines in which the mechanism of pathogenesis is similar across species. For example, synergy between experts in human and veterinary tetanus vaccines could facilitate and expedite the development of an alternative potency test for both human and veterinary tetanus vaccines. One successful crossover is potency testing of human and veterinary vaccines, which involves vaccinating guinea pigs and then quantifying anti-tetanus toxoid antibodies in serum by indirect ELISA [72] or by a ToBI test [73, 93]. Workshop participants encouraged a collaborative effort between human and veterinary tetanus vaccine experts to expedite global implementation and use of the serological methods to further refine animal use.

As previously discussed, further development, validation, and implementation of a serological test for both human and veterinary rabies vaccines is a high priority due to the variability of the *in vivo* potency test and the large numbers of mice used in the mouse challenge test. Workshop participants recommended formation of a working group of experts in both human and veterinary rabies vaccines to focus on such efforts.

Secondly, interaction was also proposed between human and veterinary vaccine experts to share knowledge of human pathogens that have genetic, phenotypic, and structural characteristics similar to those of animal pathogens (e.g., *Bordetella pertussis* [whooping cough] in humans and *Bordetella bronchiseptica* [kennel cough] in dogs). This knowledge exchange could provide valuable insights to support advancement of alternative potency testing methods.

Finally, the workshop participants encouraged human disease vaccine specialists to share information on how master references or standard references for vaccines are used, validated, stored, dated, qualified, requalified, and monitored. This information should include policies and procedures for distribution and cost sharing. Reference monitoring requirements are extensive and difficult to meet, thereby delaying *in vitro* test method development. Therefore, increased interaction between human and veterinary vaccine experts may be valuable. Workshop participants also suggested further discussion among regulatory authorities of ways to identify and simplify requirements for extension of stability and shelf life of references. They also recommended increased cooperation between human and veterinary vaccine stakeholders to produce, organize, monitor, and supply references and reagents in order to facilitate the development of alternative potency tests.

9. Achieving broader acceptance and use of currently available reduction and refinement methods for potency testing of veterinary vaccines

Workshop participants concluded that broader acceptance and use of reduction and refinement methods could result from improved information disseminated in global regulatory guidance documents to multiple stakeholder groups, countries, and regions. Such efforts should include (1) agreement on potency testing among national and international regulatory authorities and organizations and (2) continued harmonization efforts to avoid multiple potency testing of the same serial marketed in different countries. In addition, general principles and procedures for the validation of alternative vaccine test methods should be standardized and harmonized internationally, including those necessary to define humane endpoints.

As a starting point, workshop participants recommended that regulatory agencies harmonize the general principles for validation of alternative potency tests. For example, the U.S. Animal and Plant Health Inspection Service (APHIS) CVB has issued general guidelines for the validation of (1) *in vitro* potency assays [117] and (2) relative potency assays and reference preparations based on ELISA antigen quantification [97]. Guidance documents are needed to support the refinement of animal use, especially for serological ELISAs for *Clostridium spp.* vaccines.

Workshop participants recognized that international organizations can serve an important role in achieving global harmonization and implementation of alternative methods. For example, the International Cooperation on Harmonisation of Technical Requirements for Registration of Veterinary Products (VICH) is a trilateral program of collaboration among the regulatory authorities and animal health industries of the European Union, Japan, and the United States. The VICH aims to harmonize technical requirements for registration of veterinary medicinal products by establishing and implementing specific guidelines after extensive input and review from national regulatory authorities.

The VICH was established under the auspices of the World Organisation for Animal Health (OIE), which participates as an associate member in the VICH process by supporting and disseminating the outcomes at the worldwide level (http://www.vichsec.org/). Because VICH guidelines are developed by the international scientific community, there is broad-based review and acceptance, which should expedite implementation. Examples of VICH guidelines include VICH guidelines GL 41: Examination of Live Veterinary Vaccines in Target Animals for Absence of Reversion to Virulence [118] (adopted by the U.S. in 2008) and GL 44: Target Animal Safety for Veterinary Live and Inactivated Vaccines [119] (adopted by the U.S. in 2010). In addition, a draft guideline is in development to harmonize data requirements for waiving the target animal batch safety test for inactivated veterinary vaccines [26].

Harmonizing testing procedures for individual vaccines and ensuring ready availability of necessary reagents on a global basis would help support the wider use of alternative methods for vaccine testing. Organizations such the USDA (CVB and APHIS) and the Biological Standardisation Programme (BSP) under the EDQM develop, produce, characterize, and distribute selected references and reagents. These references and reagents are available to manufacturers for their use in developing assays; for direct or relative potency comparisons; or for independent efficacy, identity, and purity testing.

The availability of reference standards is a key factor in the ability of vaccine manufacturers to transition to an alternative potency assay. As the master reference is correlated to host immunogenicity, its relative stability must be monitored over time to assure that the reference remains stable during storage. For example, in the United States, a frozen master reference is allowed a maximum dating of five years or, if stored under refrigeration, a maximum

dating of two years [97]. After the dating period, each reference must be requalified in the host animal immunogenicity test. To reduce, refine, and replace the use of animals for requalification, workshop participants recommended that requalification should be conducted in any currently acceptable potency test.

Development of new requalification tests for reference standards is the responsibility of vaccine manufacturers. However, this can require significant resources, and vaccine manufacturers are less likely to commit resources to products that are older and less profitable. Therefore, the ready availability of reference standards that are requalified using new alternative tests could accelerate reduction, refinement, and replacement. Workshop participants emphasized that broad international availability of reference standards supported by regional and international authorities would be ideal and that universal reference standards could be monitored and maintained by organizations such as the OIE, USDA, WHO (World Health Organization), or EDQM.

10. Discussion

This was the first international workshop in the United States to focus on the reduction, refinement, and replacement of animal use for potency and safety testing for human and veterinary vaccines. A key aspect of the workshop was bringing together experts from industry, academia, and government in the areas of potency and safety testing for both human and animal vaccines. The commonalities in testing objectives and challenges, together with the exchange of knowledge and experience between scientists working in either human or animal vaccines, provided an obvious strategic synergy during the discussions.

International participation in the workshop also contributed to improved sharing of scientific and regulatory perspectives, ideas, and progress from many different countries and regions. Despite different approaches, terminology, and processes, all stakeholders have the same goal regardless of their geographic location: to produce and ensure safe and effective vaccine products for people and animals. There was also a unified commitment from industry participants, government officials, and all other stakeholders to encourage and support potency and safety testing methods that reduce or avoid the need for animals and to provide for the most humane use of animals where they must still be used.

Plenary session presentations and subsequent breakout groups allowed participants to clarify the current status of alternative methods for vaccine potency and safety testing and to discuss and identify priorities for future efforts to reduce, refine, and replace animal use for vaccine potency testing. While the ultimate goal is to avoid the need to use animals for vaccine potency and safety testing, for many vaccines this will require significant research, development, and validation efforts. This session, therefore, focused on identifying priorities and ways to both further reduce pain and distress and reduce the number of animals required for individual tests for studies that still require the use of animals.

Workshop participants agreed that every effort should be made to minimize or avoid pain and distress. They agreed that death is not a scientifically required endpoint for veterinary vaccine potency testing and that euthanasia of moribund animals should always be incorporated in test procedures. More importantly, workshop participants recognized that earlier humane endpoints have now been developed and validated for several vaccination–challenge procedures. Accordingly, workshop participants identified as an urgent priority the identification and implementation of earlier, more humane endpoints for all other challenge tests, as well as for *in vivo* TNTs. The systematic collection and identification of potential clinical signs and other objective parameters predictive of eventual moribund condition, or other earlier indicators of the lack of vaccine protection can accomplish this. To minimize or avoid spontaneous deaths, the effective implementation of humane endpoints may require monitoring more frequently.

Serological potency methods that quantify protective antibodies in vaccinated animals can avoid the significant pain and distress involved in vaccination–challenge procedures. Serological methods are currently used for several potency tests, including those for tetanus, erysipelas, and some clostridial diseases. However, there are still regional differences in the availability and implementation of some assays. Increased availability of reagents and availability of validation study results would likely aid in broader use of such methods.

Workshop participants identified alternative methods for potency testing of rabies vaccines as one of the highest priorities for further efforts to develop, validate, and implement alternative methods. The recent development and validation of a serological test for veterinary rabies vaccines were highlighted. Workshop participants recommended

that a workshop should be convened to focus on advancing and implementing vaccine serological and antigen quantification methods for both human and veterinary rabies vaccines.

Workshop participants recognized that *in vitro* serological test methods have been developed for many *Clostridium spp.* and some *Leptospiral spp.* vaccines and recommended extension of this technology to other related vaccines. Workshop participants recommended increased involvement and participation by vaccine manufacturers in efforts to identify how to expand the use of such methods in order to further reduce and refine animal use.

Workshop participants identified control groups in challenge tests as an immediate priority target for both reduction and refinement strategies. Retrospective reviews of archival clinical data could identify earlier humane endpoints specific to the infected controls and also gauge the potential for reducing the number of control animals necessary to maintain statistical significance.

Continued basic and applied research is critical to better understanding of how more-complex vaccines confer immunity. This applies especially to vaccines that may confer immunity through both humoral antibody and cell-mediated responses. Additional basic research will be needed to address fish vaccines potency determinants and to generate a better understanding of how these vaccines confer protection. This is important as aquaculture vaccines are becoming more widely used with the growing global implementation of fish farming. Workshop participants noted that further research is needed to identify and understand the role of antibodies in fish protective immunity and to develop assays and reagents to measure these antibodies in order to support serological assessment of protective immunity. Additional research is also needed to develop cell-based or other *in vitro* methods that can replace *in vivo* TNTs.

Workshop participants identified some serological vaccine potency tests that have been adopted in some regions of the world but that are not yet universally implemented. Participants recommended that international harmonization and greater global availability of reagents could facilitate increased use.

Workshop participants recommended development of global regulatory guidance documents to gain broader acceptance and use of alternative testing methods. This should include international agreement on a high-level roadmap and continued harmonization efforts to avoid multiple potency testing on the same serial marketed in different countries. General principals and procedures for validation of alternative vaccine test methods should be standardized and harmonized internationally.

Lastly, there is a critical need for the development and broad availability of reagents and reference standards such as antibodies, viruses, bacteria, and antigens to accelerate transition to reduction and refinement testing. The availability of reference standards is a key factor in the ability of vaccine manufacturers to switch to an alternative potency assay.

11. Conclusions

This workshop session provided a comprehensive review of the state of the science and availability of alternative methods that can further reduce and refine animal use for veterinary vaccine potency testing where animals must still be used. Participants identified critical research and development needs and priorities to further advance and implement reduction and refinement methods, and identified potency testing that should have the highest priority for reduction and refinement efforts.

Implementation of earlier human endpoints, *in vitro* serological methods, *in vitro* TNTs, and various reduction strategies identified at this workshop can be expected to have a significant near-term impact on improving the welfare of animals and reducing animal use for vaccine potency testing. Furthermore, increased targeted efforts to conduct the recommended high priority research and development activities can also be expected to have a similar impact. To facilitate future progress, this workshop set the stage for a series of future workshops that will focus on alternatives for specific priority vaccines. This and future workshops are expected to help achieve international regulatory consensus on test methods and practical implementation of scientifically sound and valid alternative methods.

International experts and leaders from industry, government, academia, and other stakeholder groups participated enthusiastically in this workshop, and provided evidence of a strong global commitment to reducing, refining, and replacing animals in veterinary vaccine potency testing. Continued cooperation and collaboration will undoubtedly accelerate development and use of alternative methods. Finally, implementation of the workshop recommendations

can be expected to advance alternative methods for vaccine potency testing that will not only benefit animal welfare and reduce animal use but also support the continued protection of people and animals.

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