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Non-animal replacement methods for veterinary vaccine potency testing: state of the science and future directions

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Non-animal replacement methods for veterinary 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. Six topics were addressed in detail by speakers and workshop participants and are reported in a series of six reports. This workshop report, the second in the series, provides recommendations for current and future use of non-animal methods and strategies for veterinary vaccine potency testing. Workshop participants recommended that future efforts to replace animal use give priority to vaccines (1) that use large numbers of animals per test and for which many serials are produced annually, (2) that involve significant animal pain and distress during procedures, (3) for which the functional protective antigen has been identified, (4) that involve foreign animal/zoonotic organisms that are dangerous to humans, and (5) that involve pathogens that can be easily spread to wildlife populations. Vaccines identified as the highest priorities were those for rabies, *Leptospira spp*., *Clostridium spp*., Erysipelas, foreign animal diseases (FAD), poultry diseases, and fish diseases. Further research on the identification, purification, and characterization of vaccine protective antigens in veterinary vaccines was also identified as a priority. Workshop participants recommended priority research, development, and validation activities to address

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critical knowledge and data gaps, including opportunities to apply new science and technology. Recommendations included (1) investigations into the relative impact of various adjuvants on antigen quantification assays, (2) investigations into extraction methods that could be used for vaccines containing adjuvants that can interfere with antigen assays, and (3) review of the current status of rabies and tetanus human vaccine *in vitro* potency methods for their potential application to the corresponding veterinary vaccines. Workshop participants recommended enhanced international harmonization and cooperation and closer collaborations between human and veterinary researchers to expedite progress. Implementation of the workshop recommendations is expected to advance alternative *in vitro* methods for veterinary vaccine potency testing that will benefit animal welfare and replace animal use while ensuring continued protection of human and animal health.

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1. Introduction

Veterinary vaccines contribute to improved human and animal health and welfare by preventing 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 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 Department of Agriculture (USDA), which regulates veterinary vaccines, and the 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), 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]. 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 replacement of animal use for potency testing of veterinary vaccines.

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 regulatory approval of a vaccine, testing is required to ensure that each subsequent production lot is pure, 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. This paper provides workshop recommendations to advance the use and development of alternative methods that can replace animals for the potency testing of veterinary vaccines. Recommendations for human vaccines are available elsewhere in these proceedings [3].

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 [5] and veterinary vaccines [6] are available in the respective papers in these proceedings.

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 human vaccines [7] and veterinary vaccines [8] are available in these proceedings.

3. Requirements for veterinary vaccine potency testing

Strict regulations and guidelines are designed to ensure that every veterinary vaccine distributed in or from the United States 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 inactivated vaccines still require animals for potency testing, significant numbers of animals are necessary.

Veterinary vaccines contribute to the health and well being of people and animals. 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 foodborne 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].

4. Prioritizing vaccine potency tests for future replacement activities

Potency testing procedures for many veterinary vaccines still require the use of animals; therefore, the development and validation of additional replacement tests could significantly benefit animal health and welfare. Workshop participants prioritized the veterinary vaccines that should be targeted for further development and validation of *in vitro* replacement tests. The criteria for prioritization included:

- Vaccines that use large numbers of animals per test and for which many serials are produced annually
- Vaccines that involve significant animal pain and distress during testing procedures
- Vaccines for which the functional protective antigen has been identified and characterized
- Vaccines that involve foreign animal/zoonotic organisms
- Vaccines that involve pathogens that can be easily spread to wildlife populations

Based on these criteria, the following vaccines were given highest priority for further development of alternative replacement methods:

- Rabies vaccines
- *Leptospira spp*. vaccines
- *Clostridium spp*. vaccines
- Erysipelas vaccines
- Vaccines for foreign animal diseases (FADs) especially those posing viral biohazards that require enhanced security and biosafety measures (e.g., foot and mouth disease [FMD] and bluetongue disease)
- Poultry vaccines
- Fish vaccines
- New vaccines that are currently undergoing prelicensing development and evaluation

Rabies, *Leptospira spp.*, and *Clostridium spp.* vaccines were identified as the highest priorities because their required potency tests use large numbers of animals and involve significant pain and distress. For example, analysis of serials released in the UK between 2007 and 2009 indicated that potency tests involving live challenge testing for *Leptospira spp*. and rabies vaccines accounted for a high proportion (>25%) of animals used in batch potency testing [14]. Vaccine challenge tests that require live viruses and bacteria that are hazardous to laboratory workers, livestock, companion animals, and wildlife were also considered high priorities (e.g., rabies and FMD vaccines). In addition, prioritization of vaccines for which the functional protective antigen has previously been identified would greatly facilitate the successful development of antigen quantification methods. Finally, new vaccines were included as high priorities in order to encourage the development of replacement alternatives early in the development cycle.

As shown in **Table 1**, several of the vaccines identified as high priorities, those that currently use animals in vaccination-challenge or toxin-neutralization testing, have alternative serology methods either in development or accepted for use by specific regulatory authorities. Therefore, validated refinement methods already exist and represent critical first steps toward the ultimate goal of identifying *in vitro* replacement methods for these high-priority vaccines.

For many veterinary vaccines, regional differences affect the availability and implementation of *in vitro* replacement assays. For example, the USDA published an *in vitro* ELISA potency test for inactivated swine erysipelas vaccine (*Erysipelothrix rhusiopathiae)*, while the European Directorate for the Quality of Medicines & HealthCare (EDQM) published a mouse-based serology test in the European Pharmacopoiea (Ph. Eur.) (**Table 2**). The EDQM has developed, validated, and approved an *in vitro* test for inactivated Newcastle disease vaccine that is not a standard requirement in the United States (**Table 2**). Clearly, improved international communication and harmonization may expand the number of veterinary vaccines for which replacement methods are available and/or accepted for use. However, regional differences in disease status, product composition, number of manufacturers, and funding may all affect priorities established in those specific regions.

Table 1. Targets for non-animal replacements: examples of veterinary vaccine potency assays using animals for immunization (serology), challenge testing, or toxin–antitoxin neutralization testing

Vaccine Product (Disease)	3Rs Alternative	Traditional Test Procedure for Which the Alternative Method is Applicable	Regulatory References	Scientific References
Inactivated Bacterins and Toxoids				
Clostridium novyi (Type B); Bovine (black disease)	Immunization (rabbits) and serology[a, b]- *In vitro* immunochemical method or neutralization in cell culture (specific details not provided in the Ph. Eur. monograph)	Rabbit immunization/Mouse toxin neutralization test	USDA SAM 207 (2007) [32]; 9 CFR 113.108; Ph. Eur. Monograph 362 [33]	EDQM 2007 [34]; Hendriksen et al. 1998 [35]
Clostridium septicum; Bovine (malignant edema)	Immunization (rabbits) and Serology[b, c]- *In vitro* Immunochemical method or neutralization in cell culture (specific details not provided in the Ph. Eur. monograph)	Rabbit immunization/mouse toxin neutralization test	Ph. Eur. Monograph 364 [36]	EDQM 1997, 2007; Hendriksen et al. 1998 [35]
Clostridium perfringens C/D; Bovine (enterotoxemia)	Immunization (rabbits) and serology[b, c]- *In vitro* immunochemical method or neutralization in cell culture (specific details not provided in the Ph. Eur. monograph)	Rabbit immunization/mouse toxin neutralization test	USDA SAM 201 (Type C, 2008) [37]; SAM 203 (Type D, 2007) [38]; 9 CFR 113.111 and 112; Ph. Eur. Monograph 363 [39]	Rosskopf-Streicher et al. 2004 [40]; EDQM 2007 [34]; Hendriksen et al. 1998 [35]
Clostridium sordellii; Bovine (big head)	-	Rabbit immunization/ mouse toxin neutralization test	USDA SAM 212 (2007) [41]; 9 CFR 113.109	-
Clostridium haemolyticum; Bovine (red water disease)	-	Immunization followed by live spore challenge in guinea pigs	USDA SAM 209 (2008) [42]; 9 CFR 113.107	-
Tetanus antitoxin products; Equine (*Clostridium tetani*)	Immunization and serology[a,,b] (guinea pigs) – *In vitro* toxin binding inhibition (TOBI), indirect ELISA[a]	Guinea pig immunization/Guinea pig toxin–antitoxin neutralization test[e]	USDA SAM 206 (2007) [43]; USDA SAM 217 (2009) [44]; Ph. Eur. Monograph 697 [45]; Council of Europe 1996 [46]	Hendriksen et al. 1994 [47]
Leptospira interrogans Serovar *canicola* bacterin Canine leptospiral (adjuvanted and non-adjuvanted)	Immunization and serology[b,d] (hamsters) (*in vitro* method to determine antibody levels, no further details provided in the Ph. Eur.)	Immunization challenge test in hamsters[f]	USDA SAM 625 (2009) [48]; 9 CFR 113.103; Ph. Eur. Monograph 447 [49]	-
Leptospira interrogans Serovar *hardjo* bacterin Bovine *Leptospira hardjo*	Immunization and serology[b,d] (guinea pigs) – micro-agglutination test	Cattle immunization challenge[g]: Immunization challenge test in hamsters	9 CFR 113.105 [50]; Ph. Eur. Monograph 1939 [51]	-
Inactivated Viral Vaccines				
Rabies vaccine (*Lyssavirus rabies)*	Immunization (mice) and serology[b]– *In vitro* rapid fluorescent focus inhibition test (RFFIT)	Immunization challenge in mice (intracerebral)[h]	USDA SAM 308 (2007) [52]; 9 CFR 113.209; Ph. Eur. Monograph 451 [53]	Cliquet et al. 1998 [54]; Kramer et al. 2009 [55], 2010 [56]; Nagarajan et al. 2006 [57]

Vaccine Product (Disease)	3Rs Alternative	Traditional Test Procedure for Which the Alternative Method is Applicable	Regulatory References	Scientific References
European foot and mouth disease vaccine; Foot-and-mouth disease virus; Bovine	-	Viral challenge of vaccinated cattle	-	Golde et al. 2005 [58]; Rodriquez and Grubman 2009 [59]

[a]Accepted by U.S. regulatory authorities.
[b]Published in the European Pharmacopoeia.
[c]The European Pharmacopoeia states that following serology, an immunochemical method or neutralization in cell cultures is considered acceptable following product specific validation.
[d]Applicable after in-house (product-specific) validation.
[e]No longer listed in the European Pharmacopoeia.
[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.
[h]Not for routine batch release (European Pharmacopoeia).

5. Veterinary vaccine potency testing: non-animal replacement alternative methods

5.1. State of the science

Current veterinary vaccines consist of (1) modified live (attenuated) virus and bacteria, (2) inactivated (killed) viruses and bacteria, (3) toxoid or bacterin toxoids, (4) peptide and subunit vaccines, and (5) genetically engineered products. The general types of potency tests employed by vaccine manufacturers include the following:

- Titration of live organisms (*in vitro* but occasionally *in vivo*)
- *In vitro* assays such as ELISAs or other quantitative methods
- Serology methods (*in vivo* to *in vitro*)
- Vaccination–challenge *in vivo* methods using either the host animal (fish, poultry) or laboratory animals (e.g., hamsters, mice) [17]

For a typical U.S. veterinary vaccine manufacturer, 37% of tests use *in vitro* titration assays, 22% use *in vitro* ELISAs, 12% use some other *in vitro* method, 8% use *in vivo* serology test, and 21% use *in vivo* vaccination–challenge methods [17]. These data exclude poultry and fish vaccine potency testing but do suggest that *in vitro* methods are being applied for most potency testing conducted on veterinary vaccines. Animal welfare concerns, increased scientific accuracy, and the financial benefits associated with *in vitro* assays provide significant incentives to veterinary vaccine manufacturers for the replacement of animals for potency testing procedures, especially if a vaccine product can be released without the potential concern for repeat *in vivo* testing [17, 60].

5.1.1. Modified live vaccines

In vitro potency testing procedures are currently used in the release of many modified live (attenuated) and genetically modified vaccines (**Table 2**) but are not widely used for the potency release of inactivated vaccines. In the United States, the USDA's Center for Veterinary Biologics (CVB) publishes many supplemental assay methods (SAMs) that provide detailed, validated protocols for the safe and effective potency testing of specific veterinary vaccines. To further facilitate the use of alternative *in vitro* methods, the CVB and other regulatory authorities provide many of the critical reagents and reference standards necessary to conduct these potency assays.

In vitro potency methods for the quantification of several modified live bacterial vaccines are currently outlined in publicly available USDA SAMs. For example, enumeration methods that quantify the colony-forming units (CFUs) of specified live organisms are described for *Brucella abortus* [48], *Erysipelothrix rhusiopathiae* [61], and avirulent *Pasteurella haemolytica* (new name *Mannheimia haemolytica*) [62] vaccines. In addition, the CVB has published an *in vitro* potency assay that uses indirect fluorescent antibody staining of inoculated cell culture to quantify bacterial titers for *Chlamydophila felis* (formerly feline *Chlamydia psittaci*) [63]. As the majority of

bacterial vaccines for veterinary use are inactivated, toxoid- or bacterin–toxoid-based, there are relatively few modified live bacterial vaccines available for veterinary use.

For live or genetically engineered viruses, virus titration is performed in cell cultures using endpoints such as plaque formation; cytopathology; and, indirectly, virus neutralization by virus-specific serological reagents. For example, *in vitro* titration assays utilizing the enumeration of plaque-forming units (PFUs) are available for feline calicivirus [64], feline rhinotracheitis virus [65], and Marek's disease vaccines [66].

For other live viral vaccines, the virus is quantified by determining its cytopathic effect in primary cell culture. These include vaccines for the following:

- Porcine transmissible gastroenteritis [67]
- Porcine rotavirus [68]
- Infectious canine hepatitis [69]
- Canine adenovirus [70]
- Canine distemper [64]
- Infectious bursal disease [71]

Finally, some modified live viral vaccines, such as those for feline panleukopenia [72] and canine parvovirus [73], quantify virus titers using direct or indirect fluorescent antibody staining of virus-inoculated cell cultures. Although these assay methods are approved by the USDA, it is often difficult to estimate which procedures are routinely used to release vaccine products because product-specific validation is required. However, it is estimated that approximately 50% of all U.S. veterinary vaccine serials are now released based on *in vitro* potency testing [26]. Examples of modified live veterinary vaccine potency assays that do not require the use of animals are provided in **Table 2**.

Other live vaccines, such as mink distemper virus vaccines [74], use an alternative in vitro system to quantify viral content by counting viral plaques that grow on the chorioallantoic membrane of inoculated chicken embryos. For live chicken embryo-adapted Chlamydophilia felis vaccine [75], embryonated chicken eggs are used as the indicator host system to determine vaccine titer (**Table 2**). In addition, a procedure is available for titrating Newcastle disease virus (NDV), infectious bronchitis virus (IBV), and combination NDV–IBV vaccines through the inoculation of embryonated chicken eggs in order to calculate the 50% egg infective dose (EID50) [76]. The majority of modified live vaccines use in vitro methods for potency release, however, some live attenuated vaccines, such as ovine ecthyma vaccine for sheep [77], still require a target animal vaccination–challenge potency test.

5.1.2. Inactivated vaccines

For many inactivated veterinary vaccines, especially bacterial vaccines, a key hurdle to the successful development of *in vitro* antigen quantification assays is the lack of protective antigen identity and the inclusion of complex adjuvants in vaccine formulations [60]. Therefore, many inactivated veterinary vaccines still require *in vivo* methods (i.e., serology or vaccination–challenge methods) for determining relative potency.

However, there are specific examples in which the protective antigen for an inactivated bacterial vaccine has been identified and used to develop a specific ELISA quantification assay based on comparison to a reference standard of antigen. These include reference standards available from CVB or product-specific standards developed by the manufacturer. Examples include *Erysipelothrix rhusiopathiae* bacterins for 65kD protein [78] and *Escherichia coli* bacterins testing for K99 Pilus [79], K88 Pilus [79], 987P Pilus [79] and P41 Pilus [79]. The development of the swine erysipelas potency test also included extensive work to develop humane endpoints [80] and an ELISA serology test [40].

For the potency determination of various *Leptospira interrogans* serovars, an *in vitro* ELISA assay is used to measure the relative potency of specific bacterins compared to a suitably qualified reference standard, such as the one available from the CVB. The *Leptospira interrogans* serovars tested in this way include *pomona* [81], *canicola* [48], *grippotyphosa* [82], and *icterohaemorrhagiae* [83].

Published *in vitro* assays are also available for selected inactivated virus vaccines. For example, the potency of an inactivated respiratory cattle vaccine containing several bovine respiratory viruses (bovine diarrhea [BVD], bovine respiratory syncytial virus [BRSV], bovine rhinotracheitis [BRV], bovine parainfluenza [PI_3]] is determined using an ELISA assay relative to a reference standard [84]. Additional *in vitro* methods have been published for feline leukemia virus GP70 antigen quantification [85] and inactivated canine coronavirus vaccines [86].

An *in vitro* ELISA antigen quantification assay for inactivated NDV vaccine has been developed and validated by the EDQM [18, 87, 88, 89]. The successful transition from an *in vivo* assay to an *in vitro* ELISA was aided by the fact that there was a strong correlation between antigen content and antibody response. The antigen-specific antibodies correlated with protection, and the European NDV vaccines were a homologous group with a single serotype and comparable oil-based adjuvants. Even with these distinct advantages, the replacement test took almost 10 years (1997–2006) to be incorporated in the EU monograph for inactivated Newcastle vaccines [18, 88, 90, 91]. Although publication in the EU monograph is encouraging, the *in vitro* assay is only optional because it is one of several approved assays for inactivated Newcastle vaccines currently included in the monograph. Accordingly, it is difficult to estimate how widely this or any other replacement assay is used by vaccine manufacturers to release vaccine products.

Table 2 provides specific examples of potency tests for inactivated veterinary vaccines that do not require the use of animals. This is not an exhaustive list, and in some cases general methods are available, often without detailed methodologies. Adding to the complexity, these references do not clearly indicate what assays are being used to release a product. Nor do they indicate that multiple methods may be available and approved for a specific vaccine by a specific regulatory agency.

The proceedings of the EDQM International Symposium on Alternatives to Animal Testing included a report provided by vaccine manufacturer Intervet International on the development of alternative veterinary vaccine potency tests [92]. According to this report, alternative *in vitro* potency tests for inactivated veterinary vaccines are described in only a few individual monographs. For example, of the inactivated mammalian veterinary vaccines released from the Intervet Boxmeer facility, 33 separate potency tests are conducted of which three utilize vaccination/challenge tests, 28 use serology, and two use *in vitro* techniques. The EU monographs provide detailed descriptions of only 13 of the 33 tests. Both of the *in vitro* tests used by Intervet are described.

For inactivated poultry vaccines, Intervet conducts 16 potency tests: three use vaccination–challenge methods, 14 use serology, and one has a serology or challenge option. Twelve of these potency tests are currently described in EU monographs with one *in vitro* alternative also described (currently not in use by Intervet) [92]. For fish vaccines, Intervet uses 11 potency tests, all of them vaccination–challenge tests. Five of the 11 are described in EU monographs. As yet, no *in vitro* alternatives are provided. Although this represents only one vaccine manufacturer's potency release of inactivated veterinary products, for which fewer *in vitro* methods currently exist, it does provide some indication of the potency tests utilized and the need for improved availability of both general and detailed *in vitro* methods.

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

The development of *in vitro* potency assays for the highest-priority vaccines that still use animals requires an understanding of the knowledge and data gaps that have delayed the introduction of such non-animal assays. Understanding the protective antigen was identified as the primary technical issue. However, for many veterinary vaccines, especially bacterial vaccine products, the protective antigen is unknown or is a complex combination of antigens [17]. Therefore, development of antigen quantification tests is technically difficult because demonstrating a dose response between an antigen and protection in the target species may not be possible.

Future efforts could focus upon cloning the genes for the protective antigens or obtaining the rights to those genes that have already been cloned during the development of reference standards. Purification methods could then be developed for the protective antigens, these antigens characterized, and appropriate assays developed and validated. Purified antigens may be made available to industry as reference standards.

Table 2. Examples of veterinary vaccine potency assays that incorporate *in vitro* non-animal alternative methods

Vaccine Product (Disease)	3Rs Alternative	References for Alternative Methods	Traditional Test Procedure for which the Alternative Method is Applicable	References for Traditional Methods
Modified Live Bacterial Vaccines				
Brucella abortus[a] (Cattle brucellosis)	*In vitro* titration method determining colony forming units (tryptose agar)	USDA SAM 600 (2009)[d]; 9CFR113.65 [93]	-	-
Erysipelothrix rhusiopathiae[a] (Swine Erysipelas)	*In vitro* titration method determining colony forming units (5% bovine blood agar)	USDA SAM 612 (2007) [61]	Vaccination Challenge test in swine	9CFR.113.67 [61]
Mannheimia haemolytica[a] (*Pasteurella haemolytica*) (Cattle respiratory disease)	*In vitro* titration method determining colony forming units (trypticase Soy Agar)	USDA SAM 905 (2009); 9CFR113.68 [62]	Vaccination Challenge test in cattle	-
Chlamydophila felis[a] (Feline respiratory disease)	Cell culture- *in vitro* titration method utilizing indirect fluorescent antibody staining (mouse fibroblasts; MEM)	USDA SAM 319 (2007); 9CFR113.71 [63]	-	-
Modified Live Viral Vaccines				
Feline Calicivirus[a] (Feline respiratory disease)	Cell culture- *in vitro* titration method utilizing plaque forming units (Crandall feline kidney cells; MEM)	USDA SAM 306 (2008); 9CFR113.314 [64]	-	-
Feline Rhinotracheitis Virus[a] Feline respiratory disease)	Cell culture- *in vitro* titration method utilizing plaque forming units (Crandall feline kidney cells; MEM)	USDA SAM 307 (2008); 9CFR113.315 [65]	-	-
Mareks Disease Virus[a] (Poultry neoplastic disease)	Cell culture- *in vitro* titration method (primary chick embryo fibroblasts; M199)	USDA SAM 406 (2005); 9CFR113.330 [66]	Vaccination Challenge test in chickens	Ph. Eur. Monograph 589 [94]
Porcine Transmissible Gastroenteritis: caused by Coronavirus TGEV[a] (Swine infectious diarrhea)	Cell culture- *in vitro* titration method utilizing cytopathic effect (swine testicular cells; MEM)	USDA SAM 114 (2005) [67]		
Porcine Rotavirus[a] (Swine infectious diarrhea)	Cell culture- *in vitro* method utilizing cytopathic effect or indirect fluorescent antibody technique (Rhesus monkey kidney cells; MEM)	USDA SAM 121 (2005) [68]	-	-
Infectious Canine Hepatitis: caused by Canine Adenovirus type 1[a] (Canine hepatitis)	Cell culture- *in vitro* method utilizing cytopathic effect (primary dog kidney cells; MEM)	USDA SAM 304 (2007); 9CFR113.305 [69]	-	-
Canine Distemper Virus[a] (Canine viral disease)	Cell culture- *in vitro* method utilizing cytopathic effect (Vero cells; MEM)	USDA SAM 323 (2007); 9CFR113.306 [64]	-	-
Infectious Bursal Disease Virus (IBDV)[a] (Poultry immuno-suppressive disease)	Cell culture- *in vitro* titration method of tissue culture adapted IBDV (primary chick embryo FB; M199/F10)	USDA SAM 408 (2007); 9CFR113.331 [71]	Immunization Challenge test in chickens	Ph. Eur. Monograph 587 [95]; Thornton 1976 [96]

Vaccine Product (Disease)	3Rs Alternative	References for Alternative Methods	Traditional Test Procedure for which the Alternative Method is Applicable	References for Traditional Methods
Feline Panleukopenia: caused by Feline Parvovirus[a] (Feline viral disease)	Cell culture- *in vitro* titration method utilizing indirect fluorescent antibody straining (Crandall feline kidney cells; MEM)	USDA SAM 305 (2007); 9CFR113.304 [69]	-	-
Canine Parvovirus[a] (Canine viral disease)		USDA SAM 316 (2007); 9CFR113.307 [73]	-	-
Mink Distemper Virus[a] (Mink viral disease)	Embryonated chicken eggs - titration of viral plaques on chorioallantoic membrane (CAM)	USDA SAM 303 (2007); 9CFR113.302 [74]	Immunization Challenge test in minks	-
Inactivated Bacterial Vaccines or Bacterins				
Erysipelothrix rhusiopathiae[a] (inactivated) (Swine Erysipelas)	Antigen quantification – *in vitro* ELISA	USDA SAM 613 (2009); 9CFR113.119 [78]	Immunization Challenge test in mice	USDA SAM 611 (2008); 9CFR113.119 [97]
Escherichia coli bacterins[a] (Multi-species gastro-intestinal)		USDA SAM 620 (K99 Pilus), 621 (K88 Pilus), 622 (987P Pilus), and 623 (F41 Pilus) (2010) [79]	-	-
Leptospira interrogans Serovar *pomona* bacterin[a] (swine, cattle, sheep, goats, canine, equine Leptospirosis)		USDA SAM 624 (2009); 9CFR113.101 [81]		USDA SAM 608 (2008) [98]
Leptospira interrogans Serovar *canicola* bacterin (inactivated, non-adjuvanted)[a,b] (swine, canine, cattle, equine Leptospirosis)		USDA SAM 625 (2009); 9CFR113.103 [48]; Ph. Eur. Monograph: 447 [49]	Immunization Challenge test in hamsters[e]	USDA SAM 609 (2008) [99]
Leptospira interrogans Serovar *grippotyphosa* bacterin[a] (equine, swine, canine, sheep, goats, cattle Leptospirosis)		USDA SAM 626 (2009); 9CFR113.104 [82]		USDA SAM 617 (2008) [100]
Leptospira interrogans Serovar *icterohaemorrhagiae* bacterin[a] (swine, canine, cattle, equine Leptospirosis)		USDA SAM 627 (2009); 9CFR113.102 [83]		USDA SAM 610 (2008) [101]
Leptospira interrogans Serovar *hardjo* bacterin[b] (cattle, sheep, goats, equine Leptospirosis)		9CFR113.105 [50]; Ph. Eur. Monograph 1939 [51]; Hendriksen 2008 [60]		-
Clostridium chauvoei; Bovine (Black Leg)	*In vitro* ELISA[b] (inactivated)	USDA Memo 800.104, 2003 [102]	Immunization followed by live spore challenge in guinea pigs	SAM 220 [103]; Ph. Eur. Monograph 361 [104]
Inactivated Viral Vaccines				
Bovine respiratory Viruses (BRV, BVD, PI_3, BRSV)[a] (Cattle respiratory disease)	Antigen quantification – *in vitro* ELISA	USDA SAM 120 (1991) [84]; 9CFR113.216 (BRV) [105]; 9CFR113.115 (BVD) [106]	-	-

Vaccine Product (Disease)	3Rs Alternative	References for Alternative Methods	Traditional Test Procedure for which the Alternative Method is Applicable	References for Traditional Methods
Feline Leukemia Virus (GP70)[a] (Feline Leukemia)		USDA SAM 321 (2007); 9CFR113.8 [85];	Immunization Challenge	Shibley et al. 1991 [107]
Canine Coronavirus[a] (Canine gastrointestinal disease)		USDA SAM 322 (2007) [86]	Immunization Challenge test in puppies	-
Newcastle Disease Virus[b] (Chicken respiratory disease)	Antigen quantification[c] – *in vitro* ELISA or Serology	Ph. Eur. Monograph: 870 [108]; Hendriksen 2007 [109]; Claassen et al. 2004 [88]	Immunization challenge in chickens[f]; Serology	-

[a]Accepted by U.S. regulatory authorities.
[b]Published in the European Pharmacopoeia.
[c]Applicable after in-house (product-specific) validation.
[d]Date is year of last SAM revision.
[e]The European Pharmacopoeia states endpoint is "signs" of disease and not lethality.
[f]Not for routine batch release in Europe.

The availability of reference standards would enable vaccine manufacturers to develop their own standards for in-house evaluations. Regulatory agencies such as the Animal and Plant Health Inspection Service (APHIS), the CVB, and the Biological Standardisation Programme (BSP) under the EDQM develop, produce, characterize, and distribute reference standards and other critical reagents. These references are provided to manufacturers to use in developing assays; comparing direct or indirect potency; or independently testing efficacy, identity, and purity.

The challenges caused by the adjuvants that are present in many veterinary vaccines present the second key technical issue identified by workshop participants. These challenges must be addressed during the development of *in vitro* replacement alternatives. Typically, *in vivo* potency tests evaluate the protective or immune response to the complete vaccine, including antigenic material (e.g., adjuvants, excipients). However, many typically use adjuvants such as mineral oil and aluminum salts, which may interfere with *in vitro* quantification methods. Therefore, these adjuvants would need to be separated from the antigen component of the vaccine before *in vitro* potency testing. Because the adjuvant is a critical component for developing the appropriate protective response for inactivated vaccines, additional *in vitro* tests may be required to ensure their quality. Regardless, when antigen quantification methods are being developed, the effect of an adjuvant on the immunogenicity of the protective antigen will also need to be investigated [18, 88, 91]. In addition, the effect of the inactivant on *in vitro* potency methods must be investigated.

A recent study showed that the method of inactivation (in this case, formaldehyde) on an oil-based adjuvanted inactivated Newcastle vaccine lowered the *in vitro* ELISA potency result but did not affect the *in vivo* potency result compared to the use of the inactivant B-propiolactone [110]. This study indicated that the *in vitro* potency results for commercial Newcastle vaccines inactivated with formaldehyde cannot be directly compared to those inactivated using B-propiolactone [110].

Validation of an *in vitro* potency assay begins when the assay is initially developed and involves establishing its relationship to efficacy in the target species. The protective antigen (protein) must be identified, purified, and shown to elicit protection in vaccinated animals. Antibodies to that protein should neutralize infectivity of the pathogen. Extensive validation continues through the assessment of the assay's precision, accuracy, and ruggedness, toward the transition to implementation and use over time [111]. Workshop participants recommended the following high-priority research, development, and validation activities.

5.2.1. Rabies vaccines

The current *in vivo* potency test for inactivated veterinary rabies vaccine comprises a multidilution vaccination–challenge test in mice, traditionally termed the National Institute of Health (NIH) test. It is known to be highly variable with a high frequency of invalid results [112, 113, 114]. Recently implemented reduction and refinement

alternatives to this test include (1) the use of a single-dilution vaccination (reduction) [53] that results in a significant reduction in animal usage to approximately 60 mice per test and (2) the incorporation of earlier humane endpoints of paresis, paralysis, and convulsions (refinement) [52].

In addition, several alternative serological methods have also been developed in which the rabies virus neutralizing antibodies are quantified from the serum of immunized animals. Two such serological methods include the rapid fluorescent focus inhibition test (RFFIT) [55, 115] and the fluorescent antibody virus neutralization test (FAVN) [54]. According to the European Pharmacopoeia, the RFFIT may be used after a correlation has been established with the mouse vaccination–challenge *in vivo* test. A recent study demonstrated good correlation between results from the RFFIT and the traditional *in vivo* challenge assay [55]. The RFFIT is also reproducible within and between laboratories, providing a potential alternative to the mouse vaccination–challenge assay [22, 56]. In fact, the European Pharmacopoeia recently published a revised draft monograph incorporating the RFFIT potency assay for inactivated rabies vaccines for veterinary use [53]. Considering these recent developments, workshop participants recommended a focused international workshop to discuss the barriers to international implementation of the RFFIT.

Several types of antigen quantification tests are currently in development for inactivated rabies veterinary vaccines, including single radial diffusion tests, antibody-binding tests, and ELISA methods [57, 116, 117]. Although the ELISA assays are reproducible, inexpensive, and quantitative, they are currently product specific, and reagents are not universally available [116]. In addition, it has yet to be demonstrated that the antigen concentration in the vaccine can be correlated with an ability to stimulate a protective immune response [118]. Furthermore, guidance and/or recommendations from global regulatory agencies are necessary to resolve how any new alternative assay (i.e., serological or antigen quantification) can be validated against the current, highly variable *in vivo* assay [22, 116].

There was broad recognition and general consensus among workshop participants that interaction between the human/veterinary regulatory agencies and vaccine manufacturers should be expanded. Such interaction would significantly increase, where appropriate, information exchange to keep all parties current on possible approaches that can be used to further the development and implementation of replacement alternatives for vaccine potency testing.

The potency release test used for human rabies vaccines is similar to that used for veterinary products. All U.S.-licensed rabies vaccines for human use define potency as the geometric mean of two valid NIH potency tests with humane endpoints defined [19]. In the EU, a similar vaccination–challenge procedure with humane endpoints is also described for human rabies vaccines [119]. The FDA has approved the replacement of several animal-based immunogenicity assays with ELISA-based potency assays for some vaccine products, but this does not include human rabies vaccines [19]. At issue is the fact that, although the neutralizing antigens are well defined, a clear correlation has not been demonstrated among the amount of antigen required to induce immune response in animals, the amount of antigen measured using alternative *in vitro* assays, and the immune response in human vaccines [19]. Consequently, serological assays may be required to serve as an intermediate step toward the successful development of an *in vitro* antigen quantification test.

Although the development of a single potency test (i.e., serological, antigen quantification) for both human and veterinary rabies vaccines is the desired goal, it may be necessary to adapt the test for both product-specific and strain-specific vaccines [116]. Because of the clear synergies between human and veterinary rabies vaccines, workshop participants recommended as a priority that manufacturers and regulatory agencies worldwide collaborate on the development and validation of a refinement or replacement assay for all rabies vaccine products.

5.2.2. Leptospira spp. vaccines

Briefly, the current *in vivo* Leptospira potency test consists of a vaccination–challenge procedure in hamsters, followed 14 days later with a lethal endpoint. The *in vivo* test is time consuming (more than five weeks) and exposes laboratory personnel to live, viable Leptospira, a zoonotic pathogen. The USDA recently developed a sandwich ELISA as an alternative *in vitro* test using rabbit polyclonal capture and a specific mouse monoclonal detecting antibody to measure the relative potency of specific bacterins compared to a qualified reference standard for several *Leptospira interrogans* serovars including *pomona* [81], *canicola* [37], *grippotyphosa* [82], and *icterohaemorrhagiae* [83]. Studies still to be completed include the testing of adjuvants and other vaccine components on assay interference [17]. The *in vivo* and *in vitro* assay methods are currently published by the USDA

in SAMs and European monographs (e.g., canine leptospiral antigen quantification method, Ph. Eur. Monograph 447; *Leptospira hardjo* antigen quantification method, Ph. Eur. Monograph 1939) [49, 51, 60, 117].

In summary, the development and validation of an *in vitro* potency assay is product- and manufacturer-specific, and manufacturers must perform the necessary studies using specific regulatory memorandums as guidance throughout this process. As a secondary priority, workshop participants recommended the continued development and implementation of ELISA antigen quantification methods, including research into the effects of adjuvants and other vaccine excipients, and the harmonization of these tests among global regulatory authorities.

5.2.3. Clostridium spp. vaccines

The typical potency test for veterinary *Clostridium spp*. vaccines is an *in vivo* rabbit/mouse toxin-neutralization test currently used, for example, for *Clostridium novyi* [32, 33, 109] and *Clostridium perfringens* [37, 38,] (**Table 1**). However, alternative methods for Clostridial toxoid potency testing have also been developed and published [34, 35, 109]. For example, European regulatory authorities have a serological potency test for *Clostridium perfringens* [39] and *Clostridium septicum* [36] vaccines that has been accepted by European regulatory authorities, although product-specific validation is still required by each vaccine manufacturer [109]. For *Clostridium chauvoei*, an alternative approach using a validated ELISA method [102, 103] and an *in vitro* replacement test for *Clostridium hemolyticum* utilizing toxin-neutralizing antibodies with the characterized protective antigen, is described [22].

Potentially, all the Clostridium protective antigens could be evaluated by antigen quantification methods, such as quantitative ELISAs, after the protective antigen has been identified by gene cloning or after rights to the protective gene have been obtained from sources that have cloned the genes for the purpose of developing reference standards. Based upon the published literature and available regulatory methods, replacement of the toxin-neutralization test for specific *Clostridium spp*. vaccines is a realistic goal but will require the global recognition of reference vaccines and the identification of the target antigens for these vaccines.

In addition to rabies vaccines, workshop participants agreed that a synergy among experts in human and veterinary tetanus vaccines could facilitate and expedite the development of a replacement potency test for both of these vaccine products. Currently, in the United States and the EU, the potency tests for human and veterinary vaccines consist of vaccination of guinea pigs and serological evaluation of antitetanus toxoid antibodies by an indirect ELISA [44] or a toxin-binding inhibition (ToBI) test [45, 47]. Efforts to develop a replacement test for either human or veterinary tetanus vaccines are impeded by the facts that toxoid vaccines are not well characterized, and potential analytical tests, including physiochemical and immunochemical tests, require much greater data generation, characterization, and validation for in-process and final product characterization [120].

A proposed blueprint for the development of an *in vitro* replacement potency test for *Clostridium tetani* included (1) the validation of currently available physiochemical and immunochemical tests, (2) parallel testing of vaccines by *in vitro* and serological methods, and (3) regulatory acceptance and implementation [120, 121]. A focused, coordinated effort by human and veterinary tetanus vaccine experts to develop a replacement implementation plan was given a high priority by all workshop participants.

5.2.4. Foreign animal disease vaccines

Vaccines for foreign animal diseases were identified as high priorities due to the biohazard imposed upon laboratory workers and the threat to livestock and wildlife. Foot and mouth disease is the most economically important viral livestock disease worldwide, infecting both domestic and wild cloven-footed animals including cattle, swine, sheep, goats, and deer [122, 123, 124]. Control of FMD has proven difficult because of the rapid replication of the virus, persistence of the virus in both infected and vaccinated animals, existence of multiple serotypes, and the lack of a globally available and effective vaccine supply [124, 125, 126].

Inactivated vaccines are commonly used but limited by the vaccines' short shelf life, the short duration of immunity, the need to include many antigens to obtain broad immunity, and biosafety concerns with production of live virus [123, 124, 1237]. Improved vaccines currently in development include (1) recombinant protein and peptide vaccines, (2) DNA vaccines, (3) empty capsid vaccines, and (4) adenoviral or fowlpox-vectored vaccines [122, 123, 124, 127]. There is also growing need for a marker FMD vaccine that would differentiate infected from vaccinated animals (DIVA). The development of such a vaccine would be significant because vaccination can interfere with disease surveillance using serological testing, and may result in a country's loss of FMD-free status and substantial economic loss [128].

As superior, functionally characterized vaccines are developed, greater opportunities to reduce, refine, or replace animal use in potency testing will undoubtedly arise. To date, the most successful vaccine strategy has been the development of a recombinant, replication-defective human adenovirus type 5 that expresses the FMD capsid sequence. Solid efficacy has been demonstrated in cattle and swine [124]. However, it is uncertain whether a single vaccine approach can successfully overcome all the shortcomings of the current inactivated vaccines. A combination of different vaccine strategies is likely to be required for effective disease control [124, 125].

Currently, the vast majority of FMD infections occur in Asia, Africa, and South America. FMD-free regions include North America, Europe, and Australia [124, 125]. Because of significant safety concerns associated with the production of large amounts of FMD virus, the United States prohibits live virus vaccine production on its mainland [124]. To achieve global disease control, vaccines with improved themostability and a longer duration of immunity are required, especially in those regions of the world without advanced infrastructures [125]. For the complete control and eradication of FMD, vaccination, surveillance, and an effective monitoring program are necessities [126].

5.2.5. Poultry vaccines

Workshop participants recommended poultry vaccines as priorities for future research and development of *in vitro* assays because of the large number of target animals currently used in vaccination–challenge and vaccination–serology testing procedures. *In vitro* potency testing of live viruses is typically performed in primary cell cultures using endpoints such as plaque formation and cytopathology. Examples of live virus poultry vaccines that use *in vitro* potency assessment include those for Marek's disease [129] and infectious bursal disease [130]. Other examples of non-animal potency testing for poultry vaccines include a procedure for titrating Newcastle disease virus (NDV) vaccine, infectious bronchitis virus (IBV) vaccine, and a combination NDV-IBV vaccine that uses embryonated chicken eggs to determine the EID_{50} [76].

As described earlier, an *in vitro* ELISA antigen quantification for inactivated NDV is validated and accepted for use in the EU [18, 87, 88. 89]. Additional antigen quantification assays have been developed for infectious bursal disease virus and IBV vaccines; however inadequate funding has prevented further validation [18, 87, 131]. Although the technology is now available, sufficient resources and efforts must still be adequately applied to validate these replacement potency assays and gain regulatory approval. Finally, as new and better characterized poultry vaccines are developed through the use of viral-vectored systems, purified recombinant proteins, or DNA vaccines, alternative *in vitro* approaches to potency testing should become available [128].

5.2.6. Fish vaccines

Fish vaccine potency tests were highlighted at the workshop because of the large number of animals used, including unvaccinated controls, in vaccination–challenge procedures [14]. The majority of fish vaccine potency release tests consist of host animal vaccination–challenge methods. Little progress has been achieved in reducing, refining and replacing the use of animals (fish) for this process [132]. Fish inactivated bacterial vaccines have been successfully used in aquaculture, but only recently has the industry developed effective viral vaccines. The number of available fish vaccines increased significantly in the 1990s [133]. Increasingly, adjuvants and immunostimulants are being used to enhance vaccine potency in fish, thereby further complicating the ability to develop refinement or replacement potency testing procedures [134].

For many fish vaccines, the correlation of serological response and protection is not well established either, impeding the development of serological potency tests [132]. However, some protective antigens have been identified for inactivated bacterial vaccines, such as those protecting from *Vibrio salmonicida* and *Vibrio anguillarum* diseases. This suggests that serology or antigen quantification methods could be developed for selected vaccine products [132]. Finally, research and development efforts are expected to expand as additional fish vaccines enter the market and more animal health companies develop vaccines for aquaculture use.

Each of the priority vaccines described above requires a significant investment of time and resources because of (1) the complexities associated with moving from an *in vivo* test method to one that does not require animals and (2) the costs associated with the significant research, development, and validation of *in vitro* vaccine potency test methods [18, 22]. Therefore, early and frequent interactions with regulators are strongly encouraged throughout this process to maximize the likelihood of a final product that will be accepted by regulatory authorities and to avoid any unnecessary delays.

6. Achieving broader acceptance and use of currently available non-animal replacement methods for veterinary vaccine potency testing

Workshop participants agreed that the primary impediment to broader acceptance and use of available non-animal replacement methods is the associated cost and time required for each vaccine manufacturer to conduct a product-specific validation of the *in vitro* potency assay for each specific vaccine. In addition, the lack of international harmonization on alternative potency methods often means that the veterinary vaccine manufacturer must perform multiple potency release tests for the same vaccine depending on its point of manufacture and use. As a starting point, workshop participants recommended that regulatory agencies harmonize the general principles for the validation of alternative potency tests. In the United States, the CVB has issued general guidelines on the validation of *in vitro* potency assays [111] and relative potency assays and reference preparations based on ELISA antigen quantification [135].

International organizations also play an important role in this harmonization process. The International Cooperation on Harmonisation of Technical Requirements for Registration of Veterinary Medicinal 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 the 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 Organization of Animal Health (OIE), which participates as an associate member in the VICH process by supporting and disseminating the outcomes at a worldwide level (http://www.vichsec.org). As VICH guidelines are developed and reviewed by members of the international animal health community, there is increased acceptance of the regulatory principles that should facilitate faster and more uniform implementation. Examples of VICH guidelines that have been adopted by APHIS include VICH GL 41: Examination of Live Veterinary Vaccines in Target Animals for Absence of Reversion to Virulence (VICH 2007 (adopted by the U.S. in 2008) and GL 44: Target Animal Safety for Veterinary Live and Inactivated Vaccines (2008) (adopted in the U.S. in 2010). In addition, a draft guideline is in development by VICH to consider a waiver for the Target Animal Batch Safety Test [26].

In addition to harmonizing general principles, there is a need to harmonize the testing procedures for individual vaccine antigens, including development of the necessary reagents. For example, reference standards such as specific antibodies, viruses, bacteria, and antigens can be accessed from the CVB by U.S. entities to aid in the development of *in vitro* potency test assay development. Broad international availability of reference standards, supported by the national and regional regulatory authorities, would greatly help to convert animal-based tests to non-animal assays. Additionally, universal reference standards could be monitored and maintained by organizations such as the OIE, USDA, World Health Organization (WHO), or EDQM.

The availability of reference standards is a key factor in the ability of vaccine manufacturers to switch to an *in vitro* replacement assay. For example, in an ELISA, the reference must be analyzed in conjunction with the sample so that a direct comparison of test vaccine to a known reference can be used to determine a relative potency. Relative potency is defined by the CVB as the potency of a product as determined by comparison with an approved reference [135]. For *in vitro* antigen potency assays, the unknown is typically compared with a working reference that was generated from the master reference. The master reference potency must have been previously correlated, directly or indirectly, to host animal immunogenicity.

As the master reference is correlated to host immunogenicity, its relative stability must be monitored over time to ensure that the reference remains stable during storage. Currently, 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 [135]. After the dating period, each reference must be requalified in the host animal immunogenicity test. To avoid the use of additional animals for requalification, workshop participants recommended that requalification be conducted in any currently acceptable potency test.

Development of new requalification tests is the responsibility of the vaccine manufacturer. This requires significant resources, especially in the development stage. Vaccine manufacturers cannot afford to dedicate these resources to products that are older and less profitable. Therefore, prioritization of veterinary vaccines for replacement testing and the potential availability of reference standards can significantly accelerate the animal test replacement process.

Workshop participants recommended that stability monitoring for both products and reference standards be considered early in the development process. They recommended that regulatory authorities work with industry stakeholders to set expectations for the stability monitoring program [17]. The stability monitoring of references typically requires that multiple previously validated tests be conducted on a 3-, 6-, or 12-month schedule (Brown 2010, personal communication). As test methods change so might the stability monitoring methods and even the reference standard itself. Consequently, regulatory agencies may require flexibility to work with the vaccine manufacturers in bridging reference standards and methodologies as industry moves toward *in vitro* replacement assays.

Regulatory guidance will also be required on the development and application of new technologies to the development of veterinary vaccines such as genetically engineered (rDNA) products, including inactivated/subunit, live (or inactivated) gene-deleted, or live vector (gene insertion) products [17].

Clearly, vaccine manufacturers must decide which products to prioritize for specific non-animal replacement potency testing. Typically, considering the output of industry resources, this decision is based upon product revenue and profitability. To aid in this process and to expedite replacement testing, sufficient resources are essential to develop and maintain reference standards specifically for industry use. In addition, broad accessibility of general procedural guidelines (as well as specific testing procedures) for individual antigens would further facilitate the international harmonization of replacement assay development and use.

7. Other issues to be addressed to facilitate the replacement of animals in veterinary vaccine potency testing

A key issue that should be addressed is the available funding for research and development of alternative methods. This research and development should be funded not just by industry stakeholders but also by government granting agencies, industry associations, and animal welfare advocacy groups. For example, the U.S. National Institutes of Health may offer funding opportunities for veterinary vaccines for those animal diseases associated with human health, such as rabies. Furthermore, academic research into test method alternatives should be promoted, and manufacturers should be encouraged, where appropriate, to present and/or publish their research findings regarding their alternative test methods.

Workshop participants also encouraged the increased availability of regulatory guidance documents in the public domain.

As indicated in Section 5, the inclusion of adjuvants in veterinary vaccines complicates the development of alternative methods because of their reported interference with antigen quantification assays. Consequently, priority should be given to developing replacement potency tests for vaccines that do not contain adjuvants. Where adjuvants are required, priority should be given to those adjuvants for which methods already exist to separate the adjuvant from the antigen. Newly developed adjuvants improve the immune response but may also be more difficult to separate from the antigen. In such instances, regulatory agencies may consider allowing manufacturers to measure potency on the bulk material, before the addition of adjuvant, or allowing antigen testing on the bulk material with an additional characterization/quantitative test on the final product. There is a clear need for further research on simpler adjuvants (and/or the methods to extract then) that may exert an effect on the animal's immune system but that do not directly interact with the antigen.

Detailed protocols for available replacement alternatives that have been reviewed and endorsed by scientific groups should be readily available in the public domain to facilitate scientific exchange and consideration. For example, detailed protocols and supporting data for validated methods, such as those that appear or are referenced in the European Pharmacopoeia monographs, should be freely available to manufacturers and the scientific community to facilitate the implementation of alternative methods.

Further incentives for industry stakeholders to develop, validate, and implement alternative methods need to be clearly conveyed and implemented by regulatory agencies. Workshop participants identified several examples of incentives that may be considered attractive to relevant vaccine manufacturers, including an expedited regulatory review time, waiving the variation fee (if applicable), and the opportunity to utilize intermittent *in vivo*/*in vitro* parallel data to expedite validation of new *in vitro* methods.

8. Discussion

This was the first international workshop in the United States that focused on the reduction, refinement, and replacement of animal use for safety and potency release testing of both human and veterinary vaccines. A key accomplishment of the workshop was bringing together experts from industry, academia, and government in the areas of safety and potency testing for both human and animal vaccines. There was broad recognition among the vaccine manufacturers and regulatory authorities and a general consensus among the participants that international workshops vastly improve information exchange not only between global regions but also between regulatory authorities (e.g., the USDA and the FDA) in the same country. This interaction may accelerate development of alternative methods once priorities are firmly established.

The presentations and subsequent breakout group sessions allowed participants to clarify the current status of *in vitro* replacement testing procedures and establish the key criteria to identify those vaccines for prioritization. A focus on inactivated vaccines for rabies, *Leptospira spp*., and *Clostridium spp*. diseases was generated from this debate. An important outcome of this workshop was the recommendation for a similar international workshop to specifically discuss the development, validation, and implementation of alternative reduction, refinement, and replacement potency testing assays for rabies vaccines for both human and veterinary use. This workshop is currently scheduled for October 11–13, 2011 in Ames, Iowa.

The workshop reflected a growing awareness of the need for alternative tests for both poultry and fish vaccines, in which the vaccines are typically tested in large numbers of target animals. Because the number of fish vaccines has grown significantly in the last 20 years, much more research and greater focus is needed to identify protective antigens for replacement testing. Finally, workshop participants recognized the uniqueness of veterinary vaccines and the need to focus on more-modern, stronger revenue-generating vaccines that can support the cost of new test method development.

This workshop also brought attention to (1) the development and use of more-complex adjuvants and (2) the use of multiple adjuvants to generate solid and sustained immunity with poorer immunogens (vaccines) and to lower vaccine antigen levels. The use of more-complex or multiple adjuvants further complicates potency replacement efforts and therefore highlights the need for much more extensive research into simpler adjuvants and/or methods to extract them from the protective antigen.

Workshop participants were encouraged by the significant number (estimated to be between 50% and 70%) of veterinary vaccines, especially the modified live viral and bacterial vaccines that now use *in vitro* potency tests. Clearly, better estimates of the number of veterinary vaccine serials released using replacement methods would be beneficial and would also focus the discussion on those vaccines for which replacement potency testing is not yet available or in use.

Accessing the information on the current state of the art of veterinary vaccine potency tests is challenging because some procedures or general guidelines are not universally available. This results in an unnecessary hindrance to the implementation of the 3Rs for vaccine product release.

The growing role of international organizations such as the VICH and the OIE is apparent. Workshop participants agreed that the harmonization of guidelines and reference standards for broad use by the vaccine community would likely increase the interaction between those organizations and the national regulatory groups. In addition, workshop participants clearly expressed the need for additional funding for these regulatory groups to allow greater availability of some of these key reagents (e.g., reference standards) to vaccine manufacturers. Although the vaccine companies must develop and validate product-specific assays, the reference standards would provide the basis for this further development and validation.

This workshop set the stage for a series of specific 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 animals for both human and veterinary vaccine potency testing. Implementation of the workshop recommendations discussed in this report is expected to advance alternative methods for veterinary vaccine potency testing that will benefit animal welfare while ensuring continued protection of human and animal health.

9. Conclusions

This veterinary vaccine session summarized the current status of *in vitro* potency testing for veterinary vaccines and identified the critical issues to further advance and implement *in vitro* replacement assays for currently used *in vivo* challenge or toxin-neutralization testing. To focus these efforts, criteria were established for vaccines that should have the highest priority for development of replacement testing methods. Based upon these criteria, the highest-priority vaccines were identified as those for rabies, *Leptospira spp*., *Clostridium spp.*, erysipelas, foreign animal diseases (e.g., FMD), poultry diseases, and fish diseases. Workshop participants also prioritized the research, development, and validation activities necessary to expedite veterinary vaccine potency testing with fewer animals.

Workshop participants recognized that there are special considerations with veterinary vaccines due to the complexity of antigenic material and the inclusion of complex adjuvants. They acknowledged that, in many cases, reduction/refinement testing may precede the introduction of *in vitro* replacement assays. This, combined with the number of veterinary vaccines and their value to the veterinary industry, suggests that the priorities identified are correct and have the highest chance of successful implementation.

There was consensus among workshop participants on the need for more universally available reagents and harmonized approaches. The successful implementation of these activities will require additional resources at both national and international levels. Finally, workshop participants agreed that the continued interaction of the global vaccine community (i.e., manufacturers, regulatory agencies, animal health organizations), both human and veterinary, could expedite the unified goal of the replacement of animals for veterinary vaccine potency testing.

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