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

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Abstract

NICEATM and ICCVAM convened an international workshop to review the state of the science of human and veterinary vaccine potency and safety testing methods, and to identify opportunities to advance new and improved methods that can further reduce, refine, and replace animal use. This report addresses methods and strategies identified by workshop participants for replacement of animals used for potency testing of human vaccines. Vaccines considered to have the highest priority for future efforts were (1) vaccines for which antigen quantification methods are already developed but not validated, (2) vaccines/components that require the largest number of animals, (3) vaccines that require an *in vivo* challenge test, and (4) vaccines with *in vivo* tests that are highly variable and cause a significant number of invalid tests. Vaccine potency tests identified as the highest priorities for replacement were those for diphtheria and tetanus, pertussis (whole cell and acellular), rabies, anthrax, polio vaccine (inactivated) and complex combination vaccines based on DT or DTwP/aP. Research into understanding the precise mechanism of protection afforded by vaccines and the identification of clinically relevant immunological markers are needed to facilitate the successful implementation of *in vitro* testing alternatives. This report also identifies several priority human vaccines and associated research objectives that are necessary to successfully implement *in vitro* vaccine potency testing alternatives.

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1. Introduction

Vaccines contribute to improved animal and human health and welfare by preventing and controlling infectious diseases. However, testing necessary to ensure vaccine effectiveness and safety can involve large numbers of animals and significant pain and distress. Accordingly, the U.S. Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) and the U.S. National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM) 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 human 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. Workshop recommendations to advance the use and development of alternative methods that can replace animals for the potency testing of human vaccines are provided in this paper, while the report on replacement of animals for veterinary vaccines is available elsewhere in these proceedings [4].

The third plenary session addressed (1) potency testing methods that refine procedures to avoid or lessen pain and distress by incorporating earlier humane endpoints or by using antibody quantification tests instead of challenge tests and (2) methods and approaches that reduce the number of animals required for each test [21, 22, 23, 24, 25, 26, 27]. Breakout groups then discussed the state of the science and developed recommendations for future progress. Workshop recommendations to advance the use and development of alternative methods that can reduce and refine animal use for potency testing of human vaccines [5] and veterinary vaccines [6] are available in the respective publications 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 the respective publications in these proceedings.

3. Requirements for human vaccine potency testing

Human vaccines save lives, prevent disease and morbidity, and represent a critical tool for successful and cost-saving health interventions [10]. For example, for each U.S. birth cohort that receives a series of seven vaccines that protect against infectious diseases including diphtheria, tetanus, pertussis, measles, mumps, rubella, and polio, an estimated 14 million disease episodes and 33,000 premature deaths are prevented. These vaccinations are estimated to save 43 billion dollars in medical and societal costs [10, 31]. Because of the number of animals used annually for the release of human vaccines, many 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 [11, 19].

The U.S. Public Health Service Act [32] and certain sections of the U.S. Food, Drug and Cosmetic Act [33] give the FDA the authority to regulate human vaccines. Section 351 of the Public Health Service Act states that the approval of a biologics license is based on the demonstration of product safety, purity, and potency and assurance that the facility for manufacture, processing, and packaging meets the standards to ensure that any product released for distribution is safe, pure, and potent. The regulatory definitions of *safety*, *purity*, and *potency* are detailed in Title 21 of the U.S. Code of Federal Regulations, and testing methodology and validation must be included in the biologics license application (BLA) [34]. Safety and potency testing may be performed on the final bulk sample or final container sample and may consist of either *in vivo* or *in vitro* tests or both. Post-licensing changes to potency or safety tests require a supplement to the license with adequate rationale and data to support the alternative method (potency or safety) or to support the demonstration of lack of need for a particular test (safety).

Current human vaccines consist of:

- Live viruses
- Modified live (attenuated) viruses and bacteria

- Inactivated (killed) viruses and bacteria
- Toxoids (chemically inactivated toxins)
- Polysaccharides

The general types of potency tests employed by vaccine manufacturers include (1) titration of live organisms (*in vitro*, but occasionally *in vivo*); (2) *in vitro* assays such as enzyme-linked immunosorbent assays (ELISAs) or other antigen quantification methods; (3) *in vivo* methods involving immunization of small laboratory animals (e.g., guinea pigs, rats, mice) followed by challenge with toxin/virus/bacteria or titration of immune sera to measure the antibody response [19]. *In vitro* potency testing is used for live, attenuated vaccines but is not commonly used for inactivated vaccines [35].

The Center for Biologics Evaluation and Research (CBER) has an active research program that evaluates, develops, and integrates novel scientific technologies for use in product regulation. The CBER program includes the development and analysis of approaches that reduce, refine, and replace the use of animals. CBER encourages the development of these alternative methods for vaccine safety and potency testing with appropriate relevance, supporting data, and test method validation. Reduction and refinement approaches can be applied to all serological or vaccination–challenge methods that use animals to determine the potency of a human vaccine.

4. Prioritizing vaccine potency tests for future research, development, and validation activities

The current potency testing methods for several human vaccines still utilize animals (**Table 1**), and the development of alternative assays for these products would significantly reduce the number of animals used for testing and benefit animal welfare. To identify human vaccines that should have priority for further development and validation of *in vitro* replacement tests, criteria for prioritization were established at this workshop. Workshop participants agreed on the following vaccine prioritization criteria for development of *in vitro* potency tests:

- Vaccines for which antigen quantification methods are already developed but not validated for routine use
- Vaccines/components that require the largest number of animals
- Vaccines that require an *in vivo* challenge test
- Vaccines with *in vivo* tests that are highly variable and cause a significant number of invalid tests
- Vaccines for which the relationship between antigen quantity and potency are well understood
- Combined vaccines (recognizing that their complexity may make successful replacement more difficult than single-component vaccines)
- Vaccines that have a well-defined and understood mode of action or known target
- Vaccines for which the manufacturing process is well understood and consistent

Based upon the key criteria described above, workshop participants identified the following vaccines as the highest priorities for additional research, development, and validation efforts:

- Diphtheria and tetanus toxoid vaccines
- Whole cell and acellular pertussis vaccines
- Rabies vaccines
- Anthrax vaccines
- Complex combination vaccines
- Inactivated polio vaccines

Diphtheria and tetanus toxoids were considered the highest priorities because the current potency tests require large numbers of animals. Rabies and anthrax vaccines were identified as priorities because the tests also require large numbers of animals and may involve significant pain and distress. In addition, the vaccine-challenge test for these vaccines requires the use of live virus, toxin, or bacteria that pose significant safety risks for laboratory technicians. The current *in vivo* potency test for inactivated rabies vaccines is also known to be highly variable [19] and produces a significant number of invalid tests.

Finally, the use of antigen quantification methods for those vaccines for which the protective antigen is already known was considered to be a high priority for validation and further development. As shown in **Table 1**, serological methods are currently in development or have been approved for use by specific regulatory authorities for many of these high-priority vaccines. The availability of these refinement methods indicates that they should therefore aid in the development of replacement methods based on *in vitro* antigen quantification [36].

Table 1. Examples of human vaccine potency tests that reduce or refine the use of animals

Vaccine Product	3Rs Alternative	References For Alternative Methods	Traditional Test Procedure for which the Alternative Method is Applicable	References for Traditional Methods
Bacterins and Toxoids				
Tetanus toxoid vaccine and tetanus component in combined vaccines (*Clostridium tetani)*	Single-dilution immunization and serology[b, c] –*in vitro* toxin-binding inhibition (ToBI[b, c]), indirect ELISA[b, c], Humane Endpoint - toxin-injected hind leg paresis[b, c]	Ph. Eur. 2.7.8. Assay of tetanus vaccine (adsorbed) [37]; WHO TRS 927, Annex 5, 2003 [39]	Guinea pig or mouse lethal challenge test	U.S. Minimum Requirements, 1952 [38];
Diphtheria toxoid vaccine and diphtheria component in combined vaccines (*Corynebacterium diphtheriae)*	Single-dilution immunization and Serology[b, c] – ELISA or Vero Cell Assay[b, c] ; Humane endpoint - erythema score following intradermal challenge in guinea pigs[b, c]	Ph. Eur. 2.7.6 Assay of diphtheria vaccine (adsorbed) [40]; WHO TRS 927, Annex 5, 2003 [39]	Guinea pig lethal challenge test	U.S. Minimum Requirements, 1947 [41];
Acellular pertussis vaccine: whooping cough (*Bordetella pertussis)*	Immunization (mice) and serology[a, b, c] ELISA	Ph. Eur. Monograph 1356 [42] and 1595 [43]; Japanese Minimum Requirements for Biological Products, 2006 [44]; WHO TRS 878, Annex 2, 1998 [45]	Mouse serology test[c, d]	
Viral Vaccines				
Rabies vaccine; (*Lyssavirus rabies*)	Immunization (mice) and Serology[b, c], Humane Endpoints - convulsions, paralysis, paresis[a, b, c]	Ph. Eur. Monograph 216 [46]; WHO TRS 941, Annex 2, 2007 [47]	Mouse multiple-dilution lethal challenge test	Seligmann 1973 [48]

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

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

Currently, some human vaccines do not require the use of animals because *in vitro* methods have been developed that quantify the presence of the protective antigen. These so-called *antigen quantification methods* are based on binding of key protective antigens in the vaccine batch to specific antibodies using *in vitro* immunoassays. At the present time, these methods represent the most promising *in vitro* approaches for the replacement of animals used in vaccine potency testing. It is important to note, however, that *in vitro* antigen quantification methods only measure antigen quantity and do not necessarily reflect biological activity. Another limitation of these methods is the requirement for adjuvant removal from the product before testing. Therefore, it is necessary to also demonstrate that the process of adjuvant removal does not affect recovery and/or integrity of the antigen, which could interfere with its detection in the assay [35].

5.1. State of the science

To date, *in vitro* tests have been successfully implemented (regulatory acceptance) for only a few products. These include hepatitis A/B vaccines, inactivated polio vaccine, polysaccharide and polysaccharide conjugate vaccines, and human papillomavirus vaccine (**Table 2**). For the recombinant hepatitis B vaccine, two types of *in vitro* immunoassays have been developed as alternatives to the mouse immunogenicity test. The first is based on the

direct determination of the hepatitis B surface antigen (HbsAg), while the other is based on the neutralization of HbsAg and subsequent detection of remaining anti-HbsAg antibodies in an ELISA [35, 49, 50].

Table 2. Examples of human vaccine potency tests that replace the use of animals

Vaccine Product	Available *In Vitro* Alternative Method	References for Alternative Methods	Traditional Test Procedure for which the Alternative Method is Applicable
Viral vaccines			
Hepatitis A vaccine (hepatitis A virus)	Antigen quantification[a, b]	Ph. Eur. Monograph 1935 [51] and general method 2.7.1 [52]; WHO TRS 858, 1995 [53]	Mouse serology[c]
Hepatitis B vaccine (hepatitis B virus)	Antigen quantification[a, b]	Ph. Eur. Monograph 1056 [54] and ch 2.7.15 [55]; WHO TRS 889, 1999 [56]	Mouse serology[c]
Inactivated polio vaccine (poliovirus)	Antigen quantification[a, b]	Ph. Eur. Monograph 214 [57] and general method 2.7.1 [52]; WHO TRS 910, 2002 [58]	Rat serology[d]
Human papillomavirus vaccine	Antigen quantification[a, b]	WHO (in press)	Mouse serology
Polysaccharide and polysaccharide conjugate vaccines	NA[e]	-	Antigen quantification[a, b, f]

[a]Published in the European Pharmacopoeia.
[b]WHO Technical Report Series.
[c]No mouse test in European Pharmacopoeia.
[d]Not for routine lot release (Ph. Eur.).
[e]Traditional method is antigen quantification
[f]Accepted by U.S. regulatory authorities

Perhaps the most critical need for a valid alternative for potency testing is in the area of rabies vaccines. The traditional method for inactivated rabies vaccine potency testing is a multiple-dilution vaccination challenge test in mice. This test, commonly referred to as the NIH test, because of the origin of its development (the National Institutes of Health), is based on the relative proportion of animals protected from rabies in groups receiving the test vaccine compared to those immunized with a reference vaccine. The need for an alternative test is critical because the traditional potency test requires large numbers of animals that experience unrelieved pain and distress. Approximately half of the test animals die or develop acute signs of rabies (e.g., convulsions, paresis, paralysis). The inherent variability of this assay contributes to a high number of invalid assays, which leads to further use of animals for repeat testing [59]. The potential exposure of laboratory technicians to biohazardous materials during testing is also an important consideration.

In recent years, national and international regulatory agencies have introduced one or more 3Rs initiatives for rabies vaccine potency testing that can be seen as significant improvements to the welfare of test animals. These include (1) a single-dilution assay, which reduces the number of dilutions (and therefore animals) for the test (reduction); (2) routine use of humane endpoints to allow earlier study termination (refinement); (3) a serological assay that obviates the need for *in vivo* challenge with rabies virus (refinement).

There are antigen quantification methods available that may one day eliminate the need for an *in vivo* potency test (replacement), leading to a significant reduction in the use of animals for testing. Several types of *in vitro* ELISA procedures for rabies vaccines have been developed during the past few years, taking advantage of advances in defining the neutralizing epitopes [59, 60, 61]. While these assays would confer distinct advantages over *in vivo* test methods (e.g., no animals required, faster, less costly), limitations that have impeded their development and implementation include the following: (1) they are not universally applicable to all vaccine strains, (2) they cannot be used in combination with adjuvants, and (3) they do not correlate well with the *in vivo* challenge test [35, 59]. Clear correlations have yet to be demonstrated among the amount of antigen required to induce a protective immune response in animals, the amount of antigen measured using alternative *in vitro* assays, and the immune response in human vaccines [19]. Therefore, it is difficult to correlate potency defined by protection in animals versus potency defined by alternative methods [19, 35]. However, further studies targeted toward the potential usefulness of ELISA

procedures for rabies vaccine potency testing are ongoing [19]. These initiatives represent critical first steps toward developing a scientifically valid alternative replacement method for potency testing of rabies vaccines. It is of note that the European Pharmacopoeia has now issued a draft proposal for comments for the use of antibody quantification for lot release of rabies vaccines for veterinary use, subject to product-specific validation [62].

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

With the identification of the highest-priority vaccines for replacement potency assay development, the knowledge and data gaps that have previously prevented use of non-animal potency assays must be addressed. A thorough understanding of the pathogenesis of an infectious disease and associated protective mechanisms is necessary to accurately identify the specific antigen or key epitope responsible for eliciting a protective immune response. Once a clinically relevant antigen/epitope is identified, further studies are required to fully understand its role in protection and how the antigen in the vaccine may be affected by the vaccine manufacturing process (e.g., epitope modification due to detoxification and absorption to adjuvant) and how this process may alter how the vaccine is presented to the immune system and how it is processed in the target species. Studies can then be conducted to determine the structure and stability of antigen so that immunological markers can ultimately be identified to measure the immune response in the target species.

For some vaccine antigens, the mechanism of protection is well understood, and the antigens are well characterized. This is the case for diphtheria and tetanus antigens. Despite the complexity of the detoxification and production processes involved in manufacturing these toxoid vaccines, methods are available for *in vitro* quantification of the antigen in the final product [36, 63]. However, the amount of antigen detected using an *in vitro* method and the biological activity (*in vivo* potency) may depend on additional factors: for example, in certain combination vaccines containing tetanus toxoid as an active ingredient and Hib polysaccharide conjugated to tetanus toxoid (e.g. DTwP + Hib-TT [63] and an experimental DTaP-IPV + Hib- vaccine [64]) tetanus potency measured using the *in vivo* method was increased relative to the potency of a vaccine which did not contain the Hib-TT component due to the presence of the carrier protein. In such cases the amount of tetanus toxoid detected by an *in vitro* binding assay may not be different because the *in vitr*o assay cannot detect the conjugated tetanus toxoid [62]. In such circumstances this may be an additional challenge to address during replacement of an *in vivo* test with an *in vitro* detection method.

The availability of selected animal disease models can facilitate identification of immunological markers, which will then aid in developing *in vitro* tests. For example, vaccines containing recombinant *Papillomavirus* virus-like particles (VLPs) have been used to control disease in cows, dogs, and rabbits. These successes provided the basis for subsequent licensure of a bivalent and quadravalent human papilloma virus (HPV) vaccine to control cervical cancer in humans [66].

Ready access to relevant information plays a key role in the development, validation, and subsequent implementation of *in vitro* vaccine potency test methods. Ideally, manufacturers and regulatory authorities should frequently interact and share information in order to avoid duplicating efforts in defining procedures used to validate alternative *in vitro* tests. However, proprietary issues may limit the dissemination of information to outside stakeholders. Increased engagement by academic researchers could provide a means through which information is publicized through various vehicles such as journal publications, workshops, and scientific/professional society meetings.

Any strategy for the development of alternative replacement methods must be based on a step-by-step approach. Ideally, the definitive potency assay (*in vivo* or *in vitro*) should be developed before licensing to eliminate the need for parallel *in vivo* testing for licensing or complex comparability protocols.

Finally, there is a clear need to differentiate between tests limited to measure the amount of antigen and additional tests for demonstrating manufacturing consistency, as a single test only assessing antigen quantity is unlikely to provide all the required information. Changes in the structural properties of the product that affect its efficacy may not be detected by some tests. Therefore, an additional priority would be to determine the ability of *in vitro* antigen quantification test in predicting product molecular stability and/or biological activity.

Significant time and resources are required for each of the above priority vaccines because of (1) the complexities associated with moving from an *in vivo* test method to one that does not use animals and (2) the significant costs associated with the research, development, and validation of *in vitro* vaccine potency test methods [18, 20].

Therefore, workshop participants strongly encourage early and frequent interactions with regulators throughout this process to maximize the likelihood of a final design 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 human vaccine potency testing

Workshop participants agreed that broader access to all relevant information describing successfully implemented alternative potency testing methods would have an obvious impact on expanding their use and acceptance. For example, open online access to the United States Pharmacopeia (USP) and European Pharmacopoeia (Ph. Eur.) monographs would greatly facilitate the dissemination of methods and procedures that have been implemented for use by regulators in various regions. Manufacturers are also encouraged to frequently consult with the relevant regulatory authorities regarding their planned study design and how best to incorporate alternative methods into vaccine potency testing (i.e., product-specific validation). All workshop participants recognized the need for harmonization during the vaccine development phase and noted that any guidelines that would standardize an approach, or a roadmap on how to transition from *in vivo* to *in vitro* testing, would be helpful. Collaborative interlaboratory studies involving both national control authorities and supranational organizations, such as the European Directorate for the Quality of Medicines (EDQM; Ph. Eur.) and WHO, could accomplish this.

Workshop participants recognized that the significant costs and the lack of available funding associated with additional research, development, and validation efforts of *in vitro* vaccine potency test methods are the primary barriers to progress. More specifically, they agreed that concerns associated with rabies vaccine antigen quantification methods need to be addressed. This includes increasing the availability of reference standards and antisera for the various rabies strains, as well as maintaining a consistent source of antisera (or, preferably, a set of nonproprietary monoclonal antibodies). Workshop participants identified the following issues that would aid in facilitating the broader acceptance of alternative methods:

- Establishment of readily available and/or nonproprietary reference standards
- Sharing of resources, including key reagents and assay procedures
- Educational outreach in bioethics and training in the conduct of 3Rs assays
- International harmonization of requirements for acceptable test methods

7. Replacement methods: extrapolating veterinary vaccine potency testing to human vaccines and vice versa

Significant differences exist between human and veterinary vaccine production and formulation, most notably the presence of complex adjuvants or adjuvant combinations in veterinary vaccines. Extrapolating results from *in vitro* methods developed for a veterinary vaccine to the similar human vaccine is often not possible. For example, human influenza vaccines are often not adjuvanted, while veterinary influenza vaccines (e.g., avian, swine) may contain mineral oil or other more complex adjuvants. Use of adjuvants may also result in significantly lower antigen content in veterinary vaccines compared to that present in nonadjuvanted human vaccines. Veterinary vaccines are often not as well characterized as the corresponding human vaccines and are typically composed of complex mixtures that include antigens derived from the whole cell, cell lysates, or cell culture supernatants. Each of these variables could influence both safety and potency of the vaccine product.

Table 3 provides a list of veterinary vaccines for which antigen quantification methods have been accepted for use by some regulatory authorities.

Taking into account these significant differences between human and veterinary vaccines, workshop participants discussed ways to extrapolate alternative potency testing results from veterinary to human vaccines. Increased interaction and communication between global regulatory agencies, research institutions, and vaccine manufacturers was recognized as the critical first step in this process. One suggestion was to develop an interagency task force to deal with specific issues relevant to both human and veterinary medicine. For example, the increased complexity of adjuvants in certain veterinary vaccines has resulted in a better understanding of adjuvant/antigen interactions, which may be useful to human vaccine manufacturers as additional adjuvants are considered for approval in their products. Conversely, antigen quantification methods that are currently available for human rabies vaccines for in-process control (WHO technical guidelines) may be useful to veterinary rabies vaccine manufacturers.

Table 3. Examples of veterinary vaccine potency assays that incorporate *in vitro* non-animal 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] ; 9 CFR 113.65 [67]	-	-
Erysipelothrix rhusiopathiae[a] (swine erysipelas)	*In vitro* titration method determining colony-forming units (5% bovine blood agar)	USDA SAM 612 (2007) [68]	Vaccination challenge test in swine	9 CFR 113.67 [68]
Mannheimia haemolytica[a] (*Pasteurella haemolytica*) (cattle respiratory disease)	*In vitro* titration method determining colony-forming units (trypticase soy agar)	USDA SAM 905 (2009); 9 CFR 113.68 [69]	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); 9 CFR 113.71 [70]	-	-
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); 9 CFR 113.314 [71]	-	-
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); 9 CFR 113.315 [72]	-	-
Mareks disease virus[a] (poultry neoplastic disease)	Cell culture– *in vitro* titration method (primary chick embryo fibroblasts; M199)	USDA SAM 406 (2005); 9 CFR 113.330 [73]	Vaccination challenge test in chickens	Ph. Eur. Monograph 589 [74]
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) [75]	-	-
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) [76]	-	-
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); 9 CFR 113.305 [77]	-	-
Canine distemper virus[a] (canine viral disease)	Cell culture– *in vitro* method utilizing cytopathic effect (Vero cells; MEM)	USDA SAM 323 (2007); 9 CFR 113.306 [78]	-	-
Infectious bursal disease virus (IBDV)[a] (poultry immunosuppressive disease)	Cell culture– *in vitro* titration method of tissue culture adapted IBDV (primary chick embryo FB; M199/F10)	USDA SAM 408 (2007); 9 CFR 113.331 [79]	Immunization challenge test in chickens	Ph. Eur. Monograph 587 [80]; Thornton 1976 [81]

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); 9 CFR 113.304 [82]	-	-
Canine parvovirus[a] (canine viral disease)		USDA SAM 316 (2007); 9 CFR 113.307 [83]	-	-
Mink distemper virus[a] (mink viral disease)	Embryonated chicken eggs – titration of viral plaques on chorioallantoic membrane (CAM)	USDA SAM 303 (2007); 9 CFR 113.302 [84]	Immunization challenge test in mink	-
Inactivated Bacterial Vaccines or Bacterins				
Erysipelothrix rhusiopathiae[a] (inactivated) (swine Erysipelas)	Antigen quantification – *in vitro* ELISA	USDA SAM 613 (2009); 9 CFR 113.119 [85]	Immunization challenge test in mice	USDA SAM 611 (2008); 9 CFR 113.119 [86]
Escherichia coli bacterins[a] (multispecies gastro-intestinal)		USDA SAM 620 (K99 Pilus), 621 (K88 Pilus), 622 (987P Pilus), and 623 (F41 Pilus) (2010) [87]	-	-
Leptospira interrogans Serovar *pomona* bacterin[a] (swine, cattle, sheep, goats, canine, equine leptospirosis)		USDA SAM 624 (2009); 9 CFR 113.101 [88]	Immunization challenge test in hamsters[e]	USDA SAM 608 (2008) [89]
Leptospira interrogans Serovar *canicola* bacterin (inactivated, nonadjuvanted)[a, b] (swine, canine, cattle, equine leptospirosis)		USDA SAM 625 (2009); 9 CFR 113.103 [90]; Ph. Eur. Monograph 447 [91]		USDA SAM 609 (2008) [92]
Leptospira interrogans Serovar *grippotyphosa* bacterin[a] (equine, swine, canine, sheep, goats, cattle leptospirosis)		USDA SAM 626 (2009); 9 CFR 113.104 [93]		USDA SAM 617 (2008) [94]
Leptospira interrogans Serovar *icterohaemorrhagiae* bacterin[a] (swine, canine, cattle, equine leptospirosis)		USDA SAM 627 (2009); 9 CFR 113.102 [95]		USDA SAM 610 (2008) [96]
Leptospira interrogans Serovar *hardjo* bacterin[b] (cattle, sheep, goats, equine leptospirosis)		9 CFR 113.105 [97]; Ph. Eur. Monograph 1939 [98]; Hendriksen 2008 [99]		-
Clostridium chauvoei; bovine (black leg)	*In vitro* ELISA[b] (inactivated)	USDA Memo 800.104, 2003 [100]	Immunization followed by live spore challenge in guinea pigs	USDA SAM 220 [101]; Ph. Eur. Monograph 361 [102]

Vaccine Product (Disease)	3Rs Alternative	References for Alternative Methods	Traditional Test Procedure for Which the Alternative Method is Applicable	References for Traditional Methods
Inactivated Viral Vaccines				
Bovine respiratory viruses (BRV, BVD, PI_3, BRSV)[a] (cattle respiratory disease)	Antigen quantification – *in vitro* ELISA	USDA SAM 120 (1991); 9 CFR 113.216 (BRV); 9 CFR 113.115 (BVD) [103]	-	-
Feline leukemia virus (GP70)[a] (feline leukemia)		USDA SAM 321 (2007); 9 CFR 113.8 [104]	Immunization challenge	Shibley et al. 1991 [105]
Canine coronavirus[a] (canine gastrointestinal disease)		USDA SAM 322 (2007) [106]	Immunization challenge test in puppies	-
Newcastle disease virus[b] (chicken respiratory disease)	Antigen quantification[c] – *in vitro* ELISA or serology	Ph. Eur. Monograph 870 [107]; Hendriksen 2007 [108]; Claassen et al. 2004 [109]	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.

8. Other issues to be addressed to facilitate the replacement of animals in human vaccine potency testing

Workshop participants agreed on the need for global harmonization, which could be accomplished by convening meetings and workshops with the various regulatory agencies and international research organizations to discuss alternative *in vitro* vaccine potency testing strategies. The development and implementation of an online, worldwide database of available alternative methods was viewed as a potential significant advancement. Workshop participants also recognized the importance of standardizing lot-release criteria and encouraging mutual recognition of test results among regulatory authorities. This would avoid duplicative testing that results in more animals being used and extends the time before products can be released to the public. Participants also recognized the need for stepwise advancement in which specific vaccines are prioritized and administrative procedures, such as a reduction in the frequency of official control testing, may precede technical strategies based on refinement and replacement alternatives. Incentives by regulatory authorities to encourage the implementation of new validated 3Rs approaches for potency testing of human vaccines were also viewed as important considerations. Finally, it was considered critical for regulators to communicate what is required of a quality control production system to allow development of new products without the need for *in vivo* testing.

9. Conclusions

This was the first international workshop held 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 potency and safety testing for both human and animal vaccines. Several other recent symposia have sought to further the science of the 3Rs as they apply to the use of animals for quality control of biologicals. These symposia addressed various aspects of promoting and facilitating the use of non-animal methods for the quality control of either human or veterinary biologicals, as well as increasing the understanding and implementation of the

consistency approach to the manufacturing of human and veterinary biologics. Several workshops have been convened over the past several years that were all dedicated to finding new ways to advance the development and implementation of alternative methods for the quality control of human or veterinary biologicals [110, 111, 112].

This workshop highlighted the need for basic scientific research, as well as product-specific validation efforts, to advance the development, validation, and implementation of alternative methods to replace animals used in potency testing of human vaccines. These specific recommendations were considered necessary to address scientific knowledge gaps that exist in our understanding of disease processes and/or product-specific information as they relate to vaccine function. There was strong agreement among the workshop participants that a thorough in-depth understanding of the precise mechanism of protection afforded by a vaccine in the target population is a key first step in moving from *in vivo* to *in vitro* potency testing.

Recurring themes that surfaced during the discussions included (1) the need for more open access to methods and information such as pharmacopoeial monographs; (2) increased sharing of information among regulators, manufacturers, and the scientific community; (3) international harmonization of requirements on test methods and specifications (e.g., ICH Q2 R Development and validation of potency assays); (4) recognition that the regulatory authorities should encourage the use of 3Rs approaches by various means; and (5) the need for widely available validated reference standards, antisera, and reagents. Although specific detailed recommendations from each workshop/symposium differed, these same general themes of the need for specific scientific research, the need for international harmonization of regulations and specifications, the need for sharing of information, the responsibility that regulators have for encouraging the use of alternative methods, and the need for validated reagents were evident in the output from each workshop/symposium. By building on and reaffirming these important conclusions and recommendations for 3Rs advancement the outcome of this workshop will advance alternative methods for vaccine potency testing that will reduce, refine, and replace the use of animals in testing, while maintaining and promoting scientific quality and the protection of human health, animal health, and the environment.

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