

2011

Alternative methods and strategies to reduce, refine, and replace animal use for veterinary vaccine post-licensing safety testing: state of the science and future directions

Jodie Kulpa-Eddy

United States Department of Agriculture, jqn0@cdc.gov

Geetha Srinivas

United States Department of Agriculture

Marlies Halder

Institute for Health and Consumer Protection, marlies.halder@jrc.it

Karen Brown

Pair O'Docs Enterprises

Hans Draayer

Pfizer Animal Health, hans.draayer@pfizer.com

See next page for additional authors

Follow this and additional works at: <http://digitalcommons.unl.edu/usdaarsfacpub>

Kulpa-Eddy, Jodie; Srinivas, Geetha; Halder, Marlies; Brown, Karen; Draayer, Hans; Galvin, Jeffrey; Claassen, Ivo; Gifford, Glen; Woodland, Ralph; Doelling, Vivian; Jones, Brett; and Stokes, William S., "Alternative methods and strategies to reduce, refine, and replace animal use for veterinary vaccine post-licensing safety testing: state of the science and future directions" (2011). *Publications from USDA-ARS / UNL Faculty*. 1106.

<http://digitalcommons.unl.edu/usdaarsfacpub/1106>

This Article is brought to you for free and open access by the U.S. Department of Agriculture: Agricultural Research Service, Lincoln, Nebraska at DigitalCommons@University of Nebraska - Lincoln. It has been accepted for inclusion in Publications from USDA-ARS / UNL Faculty by an authorized administrator of DigitalCommons@University of Nebraska - Lincoln.

Authors

Jodie Kulpa-Eddy, Geetha Srinivas, Marlies Halder, Karen Brown, Hans Draayer, Jeffrey Galvin, Ivo Claassen, Glen Gifford, Ralph Woodland, Vivian Doelling, Brett Jones, and William S. Stokes



ELSEVIER

Available online at www.sciencedirect.com

SciVerse ScienceDirect

Procedia in Vaccinology 5 (2011) 106 – 119

**Procedia in
Vaccinology**

www.elsevier.com/locate/procedia

NICEATM-ICCVAM[#] International Workshop on Alternative Methods to Reduce, Refine, and Replace the Use of Animals in Vaccine Potency and Safety Testing:
State of the Science and Future Directions
Bethesda, Maryland, USA, 14-16 September 2010

Alternative methods and strategies to reduce, refine, and replace animal use for veterinary vaccine post-licensing safety testing: state of the science and future directions

Jodie Kulpa-Eddy^a, Geetha Srinivas^b, Marlies Halder^c, Karen Brown^d, Hans Draayer^e, Jeffrey Galvin^e, Ivo Claassen^f, Glen Gifford^g, Ralph Woodland^h, Vivian Doellingⁱ, Brett Jonesⁱ, William S Stokes^{i*}

^aUnited States Department of Agriculture, Animal and Plant Health Inspection Service, Veterinary Services, Riverdale, Maryland, USA; ^bUnited States Department of Agriculture, Animal and Plant Health Inspection Service, Center for Veterinary Biologics, Ames, Iowa, USA; ^cEuropean Commission Joint Research Centre, Institute for Health and Consumer Protection, European Centre for the Validation of Alternative Methods, Ispra, Italy; ^dPair O'Docs Enterprises, Parkville, Missouri, USA; ^ePfizer Animal Health, Lincoln, Nebraska, USA; ^fCentral Veterinary Institute, Lelystad, The Netherlands; ^gCanadian Food Inspection Agency, Canadian Centre for Veterinary Biologics, Ottawa, Ontario, Canada; ^hVeterinary Medicines Directorate, Surrey, United Kingdom; ⁱIntegrated Laboratory Systems, Inc., Research Triangle Park, North Carolina, USA; ⁱNational Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods, Division of the National Toxicology Program, National Institute of Environmental Health Sciences, Research Triangle Park, North Carolina, USA

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 last in the series, addresses methods and strategies for veterinary vaccine post-licensing safety testing that can reduce, refine, and replace animal use (the 3Rs). It also provides recommendations for priority research and other activities necessary to advance the development and/or implementation of 3Rs methods for veterinary vaccine post-licensing safety testing. Workshop participants gave priority for future efforts to vaccines that (1) use large numbers of animals per test, (2) produce large numbers of serials annually, (3) use additional animals for safety testing. They also prioritized poultry vaccines for which *in vivo* extraneous agent testing is still performed, adjuvanted vaccines that cause a site reaction, and vaccines that are well characterized. Vaccines identified as the highest priorities were those for avian diseases, rabies, *Clostridium spp.*, and subunit protein and DNA vaccines, in addition to modified live viral products that do not contain

[#] The National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods and the Interagency Coordinating Committee on the Validation of Alternative Methods

* Corresponding author *e-mail address*: stokes@niehs.nih.gov

This article may be the work product of an employee or group of employees of the National Institute of Environmental Health Sciences (NIEHS), National Institutes of Health (NIH), or European Commission, however, the statements, opinions or conclusions contained therein do not necessarily represent the statements, opinions or conclusions of NIEHS, NIH, the United States government, European Commission, or other organizations.

excipients. Workshop participants recommended priority research, development, and validation activities to address critical knowledge and data gaps, including opportunities to apply new science and technology. Recommendations included further assessment of the need for a general safety test; expanded application of primary cell culture and polymerase chain reaction (PCR) techniques to replace *in vivo* chicken tests for extraneous agents; development of in-process safety testing to verify detoxification of selected vaccines; and further investigation of cell-based assays to measure residual toxicity. Implementation of the workshop recommendations is expected to advance alternative methods for veterinary vaccine post-licensing safety testing that will benefit animal welfare and reduce or replace animal use while ensuring continued protection of human and animal health.

© 2011 Published by Elsevier Ltd. Selection and/or peer-review under responsibility of the National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM).

Keywords: veterinary vaccines; vaccine safety testing; alternative methods; refinement, reduction; replacement; ICCVAM

1. Introduction

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

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

To promote and advance the development and use of scientifically valid alternative methods for human and veterinary vaccine testing, NICEATM and ICCVAM organized the International Workshop on Alternative Methods to Reduce, Refine, and Replace the Use of Animals in Vaccine Potency and Safety Testing: State of the Science and Future Directions. The workshop was held at the National Institutes of Health in Bethesda, Maryland, on September 14–16, 2010. It was organized in conjunction with the European Centre for the Validation of Alternative Methods (ECVAM), 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]. Each report incorporates the contributions of invited experts and the general public during the various plenary presentations and dedicated breakout group sessions [3, 4, 5, 6, 7, 8]. This report addresses methods and strategies for the reduction, refinement, and replacement of animal use for post-licensing safety 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, 15]. Authorities emphasized that, following the approval of a vaccine, testing is required to ensure that each subsequent production lot is pure, safe, and potent enough to generate a protective immune response in people or animals [11, 12].

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

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

3. Requirements for veterinary vaccine post-licensing safety testing

For both human and veterinary vaccines, strict regulatory guidelines ensure that vaccines released for sale are pure, safe, potent, and effective [31]. In the United States alone, approximately 18,000 serials (batches) are released annually for approximately 2000 different products that protect animals from 213 different animal diseases [12].

Veterinary vaccines are necessary to (1) ensure a safe and efficient global food supply, (2) control diseases of companion animals and domestic animals, and (3) reduce the transmission of zoonotic and food borne infections to people [9]. In addition, safe and effective vaccines reduce the need for low-level antibiotics to treat sick animals and control some diseases in food animals [9].

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 potency and safety product release testing [12, 14, 22].

Post-licensing safety testing of each vaccine production batch is intended to address a number of potential safety issues, confirming that (1) there has been complete inactivation of virulent organisms in products containing killed viruses and bacteria; (2) the product does not contain toxic substances, such as incompletely neutralized inactivating agents; (3) unknown toxic compounds were not inadvertently introduced during the vaccine manufacturing process; and (4) unknown virulent extraneous agents or other contaminants were not inadvertently introduced during the manufacturing process. Safety implies the absence of residual virulence (for live attenuated vaccines), freedom from extraneous infectious agents, and the absence of toxicity.

4. Veterinary vaccine post-licensing safety testing: priority vaccines for future research, development, and validation activities

Workshop participants identified criteria that should be considered in prioritizing veterinary vaccine post-licensing safety tests for further development and validation of 3Rs methods. Criteria included safety tests for vaccines that:

- Use large number of animals per test
- Cause animal pain and distress
- Have a large number of serials produced annually
- Use additional animals for safety testing (i.e., a safety assessment cannot be performed using animals from the *in vivo* potency test)
- Are used for poultry and for which *in vivo* extraneous agent testing is still performed
- Contain adjuvants that cause an injection site-related reaction
- Have the protective antigen identified and adequately characterized

Based on these criteria, the highest priority vaccines for further development of alternative methods for safety testing include:

- Avian vaccines
- Rabies vaccines
- *Clostridium spp.* vaccines
- Subunit protein and DNA vaccines
- Modified live viral products that do not contain excipients

Avian, rabies, and *Clostridium spp.* vaccines were identified as the highest priorities because their required safety tests use large numbers of animals, and the vaccines are produced in large numbers each year. Another reason for prioritizing avian vaccines is a need to replace *in vivo* extraneous agent testing with *in vitro* (i.e., polymerase chain reaction [PCR]) methods. Subunit protein and DNA vaccines, as well as modified live products without excipients, were also prioritized because they have a lower likelihood of adverse reactions. Workshop participants emphasized that cell-based alternatives may not be appropriate for many inactivated vaccines due to the presence of adjuvants or other excipients that are nontoxic *in vivo* but are toxic to cells in culture.

5. Alternative methods for veterinary vaccine post-licensing safety testing: state of the science; knowledge gaps; and priority research, development, and validation activities

5.1. State of the science

5.1.1. General safety test

5.1.1.1. United States

In the United States, all veterinary vaccine batches must meet the safety criteria defined in Title 9 of the U.S. Code of Federal Regulations [32] prior to release. If potency testing procedures require the use of target (host)

animals, those animals are also observed for adverse reactions. Separate safety testing is generally not required. However, if potency testing is conducted in laboratory animal models or *in vitro*, separate target (host) or laboratory animal batch release safety tests are typically required. Safety testing serves multiple purposes; and strategies for reducing, refining, and replacing the use of animals must address all of them on a case-by-case basis. As specified in U.S. codified safety tests, a minimum number of target animals (e.g., dog, cat, calf, horse, pig, chicken, salmon) are tested at 1x, 2x, or 10x doses. In the mouse safety test [33], 0.5 mL of each dose is administered (intraperitoneal or subcutaneous) to groups of eight animals that each are then observed for seven days. By comparison, in the guinea pig safety test [34], 2.0 mL of each dose is administered (intramuscular or subcutaneous) to groups of two guinea pigs that each are then observed for seven days.

5.1.1.2. Europe

In Europe, the general safety test in laboratory animals, typically known as the abnormal toxicity test (ATT), has been eliminated for veterinary vaccines because of the test's inability to detect unsafe batches and thus its lack of relevance as a criterion for vaccine release testing. Therefore, only target animals are used for safety testing now. The European target animal safety test (TAST) typically uses two animals for mammalian vaccines, 10 for chicken vaccines, and at least 10 for fish vaccines [14]. Target animals are injected with either a 2x dose of an inactivated vaccine or a 10x dose of a live vaccine. Test animals are then observed for any abnormal or systemic reactions to the vaccine over a given period of time (typically 14–28 days).

European Pharmacopoeia Monograph 062 – Vaccines for Veterinary Use (Ph. Eur. 062) [35] currently allows for elimination of the target animal batch release safety test on a case-by-case basis. Manufacturers request a “waiver by the competent authority in the interests of animal welfare when a sufficient number of consecutive batches have been produced and found to comply with the test, thus demonstrating consistency of the manufacturing process. Significant changes to the manufacturing process may require resumption of routine testing to re-establish consistency”. A report from an independent third-party expert addresses inherent variability, intrinsic safety margin, and validation data in support of quality and safety. If the manufacturing process is significantly changed, it may be necessary to reinstate the target animal batch release safety test to reestablish consistency. To date, target release safety tests have been waived for some products based on consistency and alternative quality controls [28].

The “consistency approach” implies that the routine release of vaccines is based upon the principle that the quality of vaccine is a consequence of a quality system of consistent production of batches with similar characteristics to those batches that have been shown to be safe and effective in the target species [36, 37, 38]. This concept includes standardized manufacturing procedures, the use of master seed and master cell banks, in-process controls, minimum and maximum limits set for antigen content in vaccines, and a system for pharmacovigilance.

In a preliminary analysis of target animals used for testing of batches released in the United Kingdom from 2007 to 2009, roughly 50% were chickens, 25% fish, and the remaining 25% divided between cattle, pigs, horses, and sheep [14]. Based on these data, the greatest decrease in animal use (i.e., removing or waiving of safety tests) may be achieved with fish and chickens, which accounted for 75% of the animals used for target safety testing procedures during this period. Because these tests can currently be waived in the Europe with the demonstration of consistency after safe production of 10 consecutive batches, no further research or validation is necessary, except the initiative of the vaccine manufacturer to apply for a waiver with their respective data.

5.1.1.3. Japan

In Japan, batch safety testing is performed in target species for all vaccines except inactivated vaccines used for large animals. Inactivated vaccines for other species are tested with a 1x dose injection, and live vaccines for all species are tested with an overdose [39].

5.1.2. Extraneous agent testing

More appropriate and specific vaccine safety testing procedures are now available to address the presence of extraneous agents. For extraneous agent testing in poultry vaccines, the European Pharmacopoeia (Ph. Eur.) requires that products be monitored for many potential contaminants. The specific tests are described in individual monographs and Ph. Eur. 2.6.25 [40] (Avian live virus vaccines: tests for extraneous agents in batches of finished product). The safety methods currently in use include both animal and non-animal tests performed on each batch

(serial) of vaccine. The tests utilize eggs, cell culture, and SPF chicks to cover the range of potential contaminants. Briefly, the *in vivo* test in chicks involves the inoculation of 10 2-week old SPF chicks with multiple injections of the vaccine product by both intramuscular and eye drop injection. Multiple injections significantly improve the sensitivity of the test. Serum is collected from each chick prior to inoculation and after the testing period. Animals are observed for mild signs of infectious disease, and the serum samples are tested for antibodies against a list of infectious agents. The vaccination must not induce the production of antibody against specific viruses. The *in vivo* test in chicks encompasses 16 infectious diseases [41].

Recent development of more technologically advanced molecular methods has increased their use in the quality control of veterinary vaccine products. For example, the utilization of a PCR technique for extraneous agent testing of both live and inactivated poultry vaccines has been identified as a valuable alternative testing method to replace the use of animals in quality control testing [41, 42, 43]. Results so far indicate that the sensitivity of the PCR procedure is at least equal to or, in most cases, higher than that of conventional *in vivo* testing. In addition to the higher sensitivity, PCR tests also eliminate the use of animals and have faster turnaround times [44].

PCR tests are now available for 15 different extraneous agents tested in avian vaccines, including avian adenoviruses, avian infectious bronchitis virus, chicken anemia virus, infectious bursal disease virus, and Newcastle disease virus [44]. Following the acceptance and implementation of these methods, the number of chickens used in the United Kingdom between 2007 and 2009 was reduced by approximately 50% (Woodland personal communication). A complete list of available alternative assays including PCR, primary cell culture methods, and egg methods is provided in the ECVAM workshop report “Three Rs Approaches in the Production and Quality Control of Avian Vaccines” [41].

For nonavian vaccines, the extraneous agent test can be combined with the target animal safety test. Examples include selected bovine vaccines (corona, rotavirus) and porcine vaccines (parvo) that utilize serological testing of test animals used in the general safety tests.

5.1.3. *Inactivation and residual toxicity tests*

A residual live virus test is required to confirm that inactivated human and veterinary rabies vaccines are in fact inactivated [45]. For veterinary rabies vaccines, the USDA requires that 20 mice be injected intracerebrally with 0.25 mL of vaccine product and observed daily for 21 days. If any mice die between Day 4 and Day 21, brain material is recovered and injected into five additional mice [46]. The World Organisation for Animal Health (OIE) guidelines includes the use of an *in vitro* cell culture test, as well as the *in vivo* mouse test [47]. The cell-based method can accommodate a much higher number of equivalent doses than can be injected into mice or rabbits. Results to date indicate that the fluorescent antibody technique in cell culture is at least as sensitive as the mouse test; however, the test should be performed prior to the addition of adjuvants and preservatives [48]. The report and recommendation of ECVAM Workshop 48 stated that “the test for residual live virus should be conducted on the bulk material by using cell cultures, and the test in mice and rabbits should be deleted as a finished product test” [45]. However, to date, Ph. Eur. Monograph 0451 [49] still includes the *in vivo* test in mice for the finished product.

Workshop participants emphasized that any proposed reduction, refinement, and replacement alternative method must adequately address the safety objective for the original test. Extensive studies may be necessary to replace currently implemented *in vivo* safety methods. However, implementation and use of stringent in-process controls during the vaccine manufacturing process may help reduce animal use. Vaccine manufacturers who can demonstrate highly refined, consistent manufacturing processes and appropriate biocontainment and disinfection processes may also be able to justify exemptions on a product-by-product basis.

Some workshop participants expressed significant concerns regarding this strategy. There may be insufficient data to support a relationship between stringent manufacturing processes and safety risks at this time. In addition, some regulatory agencies may not have adequate resources to develop and apply consistent approaches for conducting risk assessment on a product-by-product basis. Furthermore, post-marketing adverse event reporting by veterinary vaccine manufacturers is not currently a global requirement. Therefore, safety problems with individual batches of vaccine products may go undetected. For this reason, many veterinary vaccine manufacturers still hesitate to release batches of vaccine product that have not been tested for safety in animals. Even with the implementation of revised safety testing regulations, manufacturers may continue to conduct animal testing for liability and ethical concerns. In addition, U.S. manufacturers are typically allowed a broader range of antigenic material with a clear minimum, but not a maximum, as defined in the outline of production. Consequently, it is anticipated that the upper

limit would be set both by cost considerations and by vaccine safety testing data, further outlining the perceived value of the general safety test in the United States.

A simple alternative to reduce animal use in vaccine safety testing would be to combine it with potency testing. For example, if potency testing of a veterinary vaccine product requires the use of target animals, these animals may also be observed for adverse reactions/clinical signs. Additional safety testing would therefore not be required. Specific examples of vaccines for which this approach has been implemented include mink enteritis vaccine [50] Newcastle disease vaccine [51], bovine virus diarrhea vaccine [52], canine distemper virus [53], and canine parainfluenza vaccine [54] (**Table 1**). In addition, potency and safety testing procedures for avian influenza vaccine are performed simultaneously in chickens. Relative potency of this vaccine product is determined by hemagglutination inhibition titers in blood samples collected three weeks after vaccination [28]. The same test animals are also observed for safety effects during the 3-week observation period. However, if relative potency testing is conducted in laboratory animals or *in vitro*, separate host animal safety tests are still required.

Table 1: Examples of veterinary vaccine post-licensing safety tests that incorporate reduction, refinement, and replacement alternative methods

Vaccine Product/ Test	Available 3Rs Method	Traditional Safety Test	Regulatory References	Scientific References
General				
Vaccines for veterinary use	Submission of data to support waiver of target animal safety test as stated in the current Ph. Eur.: "For an established vaccine the routine application of the safety test may be waived by the competent authority in the interests of animal welfare when a sufficient number of consecutive batches have been produced and found to comply with the test, thus demonstrating consistency of the manufacturing process. Significant changes to the manufacturing process may require resumption of routine testing to re-establish consistency" ^a	Target animal safety	Ph. Eur. Monograph 62 [52]; Ph. Eur. 5.2.6 [55]; Ph. Eur. 5.2.9 [56]	Weisser and Hechler 1997 [57]; Cussler and Poessnecker 2000 [58]; AGAATI 2002 [59]
Viral Vaccines				
Mouse safety	Intraperitoneal or subcutaneous inoculation of mice for live virus vaccines	Intracerebral and intraperitoneal inoculation of mice for live virus vaccines	9 CFR 113.33 [33]; CVB Notice No. 05-01 [60]	-
Avian live virus vaccines	Embryonated hen's eggs ^b , cell culture ^b , PCR ^b	Chicken extraneous agent test ^c	Ph. Eur. 2.6.25 [40]	Bruckner et al. 2000 [41]; Ottiger 2006 [43]; Ottiger 2010 [44]
Canine distemper vaccine (CDV)	Combining potency and safety tests eliminates need for separate target animal testing for general safety test	Observation of vaccinated dogs used in potency test	9 CFR 113.201 [53]	-
Mink enteritis vaccine; Mink enteritis virus	Combining potency and safety tests eliminates need for separate target animal testing for general safety test	Observation of vaccinated mink used in potency test	9 CFR 113.204 [50]	-
Newcastle disease vaccine: chickens	Combining potency and safety tests eliminates need for separate target animal testing for general safety test	Observation of vaccinated chickens used in potency test	9 CFR 113.205 [51]	-

Vaccine Product/ Test	Available 3Rs Method	Traditional Safety Test	Regulatory References	Scientific References
Bovine virus diarrhea vaccine; BVD virus	Combining potency and safety tests eliminates need for separate target animal testing for general safety test	Observation of vaccinated calves used in potency test	9 CFR 113.215 [52]	-
Canine parainfluenza vaccine	Combining potency and safety tests eliminates need for separate target animal testing for general safety test	Observation of vaccinated dogs used in potency test (For live virus vaccines, a mouse safety test is also conducted unless the virus or agent in the vaccine is inherently lethal for mice.)	9 CFR 113.316 [54]	-

^aThe target animal safety test can be waived after demonstration of consistency, i.e., the product passes the target animal safety test in at least 10 consecutive batches.

^bPublished in the European Pharmacopoeia

^cExtraneous agent test for live avian vaccines no longer uses animals in Europe

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

A critical knowledge gap is a definitive understanding of the range of possible causes that result in a failed general vaccine safety test for each vaccine. Workshop participants recommended that the historical basis for instituting the general safety test be carefully reviewed in order to define the need for its continuation. Specifically, the basis for failed safety tests and/or the relative frequency of their occurrence must be determined. These data are not readily available because veterinary vaccine manufacturers generally do not provide regulatory agencies with detailed safety testing failure information. Workshop participants recommended that a collaborative study between vaccine manufacturers and global regulatory agencies be initiated to evaluate the value of and need for general safety testing.

The global differences in standardization of the maximum allowable antigen content should be further discussed. For example, antigen maximum limits are clearly defined in the Europe but not in the United States. As stated earlier, veterinary vaccine manufacturers in the United States are typically allowed a broader range of antigenic material with a clear minimum, but not a maximum, as defined in the outline of production. If a maximum allowable antigen content limit was to be implemented in the United States, it might eliminate the need for 10x and 2x vaccine safety testing. However, this resolution may be at odds with a vaccine manufacturer's desire to increase antigen content in selected veterinary vaccines in order to maintain the vaccines' stability and increase shelf-life.

Another knowledge gap identified at the workshop involved an understanding of the relationship between endotoxin content and/or mouse toxicity test results and field adverse reporting. Workshop participants recommended defining the correlation between endotoxin testing and the prelicensing serial data to set an upper limit on endotoxin content. Users would conduct additional toxicity testing only if that upper endotoxin limit is exceeded in specific batches of veterinary vaccines.

A final concern discussed at the workshop was the ability, or inability, to correlate proposed alternative assays with currently implemented vaccine safety tests. Participants noted that extensive research, development, and validation studies would be required to ensure that substitute assays accomplish the same objectives as current *in vivo* vaccine safety testing procedures.

The highest priority research, development, and validation activity identified by workshop participants was critical analysis of target species and laboratory safety data to more accurately determine the basis of why and how often vaccine safety tests fail. As stated earlier, the safety testing of each batch (serial) of vaccine product in target and/or laboratory animals remains a global requirement for vaccine release.

A second priority is expanding the application of primary cell culture and PCR techniques to poultry vaccines. These techniques represent promising approaches to replace the *in vivo* chicken extraneous agent test [44].

Another priority is determining if the *in vivo* veterinary rabies inactivation test could be replaced with primary or other cell culture techniques. Such an approach is now included in EDQM guidelines for testing of virus inactivation for human rabies virus vaccines [61]. This approach has yet to be applied to veterinary rabies vaccines and was therefore identified as a key priority.

Finally, workshop participants recommended the use of in-process safety testing to verify detoxification of selected vaccines (e.g., *Clostridium spp.*). They recommended that cell-based assays be considered to measure residual toxicity [62].

6. Application of human vaccine post-licensing safety testing to veterinary vaccines: reduction, refinement, and replacement alternative methods

Progress has recently been achieved in the development and validation of alternatives that reduce, refine, and replace the use of animals in the safety testing of human vaccine products. These alternatives should be reviewed and applied as appropriate to veterinary vaccines (and vice versa). In general, human vaccines are better characterized than veterinary vaccines and contain fewer excipients and/or less complex adjuvants that could interfere with *in vitro* testing procedures. Therefore, modified live veterinary vaccines (instead of inactivated vaccines that contain complex adjuvants and excipients) are the favored candidates to consider for the application of human vaccine safety testing 3Rs methods. However, one possible approach for inactivated bacterial veterinary vaccines is to assess *in vitro* tests currently in development to detect residual toxicity in human tetanus vaccines. Several *in vitro* methods, including binding and enzymatic assays, have been described [62].

7. Achieving broader acceptance and use of currently available reduction, refinement, and replacement methods for veterinary vaccine post-licensing safety testing

Workshop participants agreed that broader acceptance and use of 3Rs alternative methods for veterinary vaccine safety testing requires the successful harmonization of quality control and assurance procedures by both national and international regulatory agencies. An existing program that can assist in this effort is the International Cooperation on Harmonisation of Technical Requirements for Registration of Veterinary Medicinal Products (VICH). The VICH is a collaborative program primarily between regulatory authorities and animal health industries of the EU, 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 following extensive input and review from global regulatory agencies.

The VICH was originally established under the auspices of the OIE. The OIE participates as an associate member by supporting and disseminating VICH recommendations and guidelines on a global stage (<http://www.vichsec.org>). As stated earlier, VICH guidelines are developed through collaborations with the international scientific community, thereby ensuring broader review and acceptance of the regulatory guidance principles and facilitating a more rapid and uniform implementation. Specific examples of VICH guidelines that have been adapted by the USDA's Animal and Plant Health Inspection Service (APHIS) include VICH Guideline number GL 41: Target Animal Safety – Examination of Live Veterinary Vaccines in Target Animals for Absence of Reversion to Virulence [63] (2008) (adopted in the United States in 2008) and GL 44: Target Animal Safety for Veterinary Live and Inactivated Vaccines [64] (2009) (adopted in the United States in 2010).

Workshop participants recommended that all VICH harmonization initiatives on product safety be comprehensively reviewed and that relevant data be provided to fill existing knowledge gaps. Specifically, a review and endorsement of the draft guidelines/criteria for requirements to request a waiver for target animal safety testing was considered a high priority (Harmonisation of Criteria to Waive Batch Safety Testing for Inactivated Vaccines for Veterinary Use). In addition, workshop participants encouraged the broad adoption of the VICH guidelines for pharmacovigilance (Guidelines 24, 29, 30, 35, and 42) [65].

Finally, there was extensive dialogue regarding application of the consistency approach to the removal of the target animal batch release safety test. During the workshop, participants raised concerns about how consistency can be adequately demonstrated and what specific data and performance are required for a safety test or any other test to be waived. In general, the participants agreed that, to facilitate its successful global implementation, a consistency

approach for waiving vaccine testing would require the application of stringent process controls throughout the manufacturing process with suitability assessed on a product-by-product basis.

8. Other issues to be addressed to facilitate the reduction, refinement, and replacement of animals in veterinary vaccine post-licensing safety testing

A key issue identified at the workshop was replacing the general batch release safety test performed in target and/or laboratory animals. There was consensus that further discussion will require (1) a review of product release historical data and (2) broader access to in-process data such as endotoxin testing results. In addition, these discussions will require the active participation of every major regulatory agency in order to achieve the successful elimination of the vaccine safety test at a global level. Workshop participants also proposed that regulatory agencies should remain flexible as better-defined veterinary products are developed. They suggested that it may not be necessary to proceed automatically to traditional animal-based safety tests for future vaccine products.

Additionally, the participants requested that information describing successfully implemented testing methods be more openly accessible. Accordingly, a specific proposal was introduced requesting public internet access and free availability of the Ph. Eur. monographs.

The key issues of insufficient research funding and adequate incentives for vaccine manufacturers need to be addressed in order to reduce animal use for veterinary vaccine safety testing. Funding support from government granting agencies, industry associations, and animal welfare advocacy groups could significantly accelerate this process. Also, increased academic research into the development of alternative safety testing procedures needs to be promoted and encouraged.

Further incentives for industry stakeholders to develop, validate, and implement alternative vaccine safety testing methods need to be clearly conveyed and implemented by regulatory agencies. Workshop participants identified incentives that may be considered attractive to relevant vaccine manufacturers, including (1) an expedited regulatory review time, (2) waiver of the variation fee (if applicable), and (3) the opportunity to utilize intermittent *in vivo/in vitro* parallel data to expedite validation of new *in vitro* methods.

9. Discussion

This was the first international workshop in the United States to focus on the reduction, refinement, and replacement of animal use for potency and safety 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 veterinary vaccines. Participants, particularly/especially vaccine manufacturers and regulatory authorities, agreed that the workshop facilitated information exchange not only between global regions but also between regulatory authorities in the same country. This interaction leads to potential acceleration in the development of alternative methods once priorities are firmly established.

Workshop participants addressed the state of the science of 3Rs alternative methods for veterinary vaccine post-licensing safety testing and provided recommendations for priority research and other activities necessary to advance the development and/or implementation of these alternative methods.

At this time, all veterinary vaccine products still require a batch release safety test in target or laboratory animals prior to release. In the Europe the target animal test may be waived with the demonstration of manufacturing consistency and the successful safety testing and release of 10 consecutive batches. For regulatory authorities outside Europe to consider waiving either the laboratory or target animal safety test a broad international assessment of historical safety data (both in-process and release testing) would be necessary to determine the extent to which the alternative approaches can adequately identify unsafe batches (serials) of vaccine on a product-specific basis. This process will require extensive collaboration between interested stakeholders (e.g., vaccine manufacturers, regulatory agencies) and may lead to the standardization of safety test methods. Participants recognized that any implementation initiative should commence with nonadjuvanted, better-characterized vaccines. Furthermore, as reduction, refinement, and replacement alternative methods are validated and accepted, the methods should be published and made available in the public domain.

Continued efforts should be made to assess general safety as part of the *in vivo* potency release test, which could significantly reduce the number of animals used for safety testing. A recommended first priority was to assess the

general safety test for fish vaccines. In addition, it may be necessary to supplement the *in vivo* safety test with *in vitro* methods of safety assessment. Methods such as serology, cell-based assays, and ELISAs, may help to develop sufficient information on residual virulence (for live attenuated vaccine), freedom from extraneous agents, and the absence of toxicity to support elimination of the general safety test. A key initiative of the workshop was to encourage the broader adaptation of PCR testing for extraneous agents in regions where *in vivo* techniques are still in use. To achieve this goal, opportunities for technical training must be provided. In some cases, the necessary equipment to conduct the testing must be made available.

Workshop participants gave high priority to further investigation to determine if the *in vivo* veterinary rabies inactivation test may be replaced with cell culture techniques already in use for the testing of virus inactivation for human rabies virus. With an *in vitro* test already validated and in use, its application to veterinary vaccines could be rapidly explored to reduce animal use as well as to improve test sensitivity and improve review times for product release.

A key recommendation of the workshop was that all VICH harmonization initiatives on product safety should be comprehensively reviewed. In particular, a review and endorsement of the draft guidelines/criteria for requirements to request a waiver for target animal safety testing was considered a high priority. There was broad support for adoption of the VICH guidelines for pharmacovigilance to ensure that there are universal criteria for the reporting of adverse events to ensure a safe and effective veterinary vaccine supply. Adoption of the VICH guidelines would in turn support changes in the safety assessment process by enhancing the ability to detect any failures in an alternative process (<http://www.vichsec.org>). Such actions would support the larger goal of international harmonization of safety testing procedures. As part of these efforts, workshop participants recommended broader access to information describing vaccine safety testing methods that have already been successfully implemented. A specific workshop recommendation was that there should be public internet access and free availability of the Ph. Eur. monographs and other regulatory guidelines.

The key issues of insufficient research funding and adequate incentives for vaccine manufacturers need to be addressed in order to reduce animal use for veterinary vaccine safety testing. Funding support by government granting agencies, industry associations, and animal welfare advocacy groups could significantly accelerate this process. Furthermore, increased academic research into the development of alternative safety testing procedures needs to be actively encouraged.

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 incentives that may be considered attractive to relevant vaccine manufacturers, including (1) an expedited regulatory review time, (2) waiver of the variation fee (if applicable), and (3) the opportunity to utilize intermittent *in vivo/in vitro* parallel data to expedite validation of new *in vitro* methods.

The process of reduction, refinement, and replacement of animal use for veterinary vaccine safety testing will require collaboration among manufacturers, regulators, and international organizations such as the VICH and the OIE. Incentives to streamline the regulatory process will assist in more rapid implementation.

10. Conclusion

This workshop session reviewed the current status of veterinary vaccine post-licensing safety testing and identified research, development, and validation activities necessary to further advance testing methods and strategies that would use fewer or no animals. The session identified opportunities for expanded use of *in vitro* safety tests such as PCR for extraneous agent testing, cell culture techniques to confirm virus inactivation, and cell-based assays to assess residual toxicity. Expanded application and implementation of these available alternative methods are expected to further reduce animal use for safety testing. Workshop participants also acknowledged the opportunity for developing batteries of validated parameters to assess final product consistency as a potential substitute for *in vivo* safety testing. Finally, workshop participants recommended stronger collaboration among the global human and veterinary vaccine communities to expedite further reduction and replacement of animals for vaccine testing while ensuring the safety of people and animals.

Acknowledgements

The authors extend their sincere appreciation to all participants in the international workshop for their enthusiastic contributions leading to the workshop recommendations and conclusions. The members of the ICCVAM Interagency Biologics Working Group and NICEATM staff are acknowledged for their contributions to the planning of the workshop, and the many invited experts are acknowledged for their contributions to breakout group discussions and workshop proceedings. Finally, the authors thank David Allen and Nelson W. Johnson for their assistance in the preparation of this manuscript.

References

- [1] Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM). The NICEATM-ICCVAM five-year plan (2008-2012): a plan to advance alternative test methods of high scientific quality to protect and advance the health of people, animals, and the environment. National Institute of Environmental Health Sciences, 2008. NIH Publication No. 08-6410. <http://iccvam.niehs.nih.gov/docs/5yearplan.htm>
- [2] Stokes WS, Kulpa-Eddy J, McFarland RM. Introduction and summary of the international workshop on alternative methods to reduce, refine, and replace the use of animals in vaccine potency and safety testing: state of the science and future directions. *Proc Vaccinol* 2011;5:1-15.
- [3] McFarland R, Verthelyi D, Casey W, Arciniega J, Isbrucker R, Schmitt M, Finn T, Descamps J, Horiuchi Y, Sesardic D, Stickings P, Johnson NW, Lipscomb E, Allen D. Non-animal replacement methods for human vaccine potency testing: state-of-the-science and future directions. *Proc Vaccinol* 2011;5:16-32.
- [4] Kulpa-Eddy J, Srinivas G, Halder M, Hill R, Brown K, Roth J, Draayer H, Galvin J, Claassen I, Gifford G, Woodland R, Doelling V, Jones B, Stokes WS. Non-animal replacement methods for veterinary vaccine potency testing: state-of-the-science and future directions. *Proc Vaccinol* 2011;5:60-83.
- [5] Casey W, Schmitt M, McFarland R, Isbrucker R, Levis R, Arciniega J, Descamps J, Finn T, Hendriksen C, Horiuchi Y, Keller J, Kojima H, Sesardic D, Stickings P, Johnson NW, Lipscomb E, Allen D. Improving animal welfare and reducing and refining animal use for human vaccine potency testing: state-of-the-science and future directions. *Proc Vaccinol* 2011;5:33-46.
- [6] Stokes WS, Brown K, Kulpa-Eddy J, Srinivas G, Halder M, Draayer H, Galvin J, Claassen I, Gifford G, Woodland R, Doelling V, Jones B. Improving animal welfare and reducing animal use for veterinary vaccine potency testing: state-of-the-science and future directions. *Proc Vaccinol* 2011;5:84-105.
- [7] Isbrucker R, Levis R, Casey W, McFarland R, Schmitt M, Arciniega J, Descamps J, Finn T, Hendriksen C, Horiuchi Y, Keller J, Kojima H, Sesardic D, Stickings P, Johnson NW, Allen D. Alternative methods and strategies to reduce, refine, and replace animal use for human vaccine post-licensing safety testing: state of the science and future directions. *Proc Vaccinol* 2011;5:47-59.
- [8] Kulpa-Eddy J, Srinivas G, Halder M, Hill R, Brown K, Draayer H, Galvin J, Claassen I, Gifford G, Woodland R, Doelling V, Jones B, Stokes WS. Alternative methods and strategies to reduce, refine, and replace animal use for veterinary vaccine post-licensing safety testing: state of the science and future directions. *Proc Vaccinol* 2011;5:106-119.
- [9] Roth J. Veterinary vaccines and their importance to animal health and public health. *Proc Vaccinol* 2011;5:127-136.
- [10] Schuchat A. Human vaccines and their importance to public health. *Proc Vaccinol* 2011;5:120-126.
- [11] Finn T. U.S. FDA Requirements for human vaccine safety and potency testing. *Proc Vaccinol* 2011;5:137-140.
- [12] Hill RE. USDA requirements for veterinary vaccine safety and potency testing. *Proc Vaccinol* 2011;5:141-145.
- [13] Isbrucker R, Sontakke S, Smith D. Health Canada's human vaccine lot release program: impact on the 3Rs. *Proc Vaccinol* 2011;5:147-150.
- [14] Woodland R. European regulatory requirements for veterinary vaccine safety and potency testing and recent progress toward reducing animal use. *Proc Vaccinol* 2011;5:151-155.
- [15] Horiuchi Y, Ochiai M, Kataoka M, Yamamoto A, Yuen C-T, Asokanathan C, Corbel M, Kurata T, Xing D. Strategic approaches for developing alternative tests for safety and potency of vaccines. *Proc Vaccinol* 2011;5:156-163.
- [16] Shin J, Lei D, Conrad C, Knezevic I, Wood D. International Regulatory Requirements for Vaccine Safety and Potency Testing: A WHO perspective. *Proc Vaccinol* 2011;5:164-170.
- [17] Draayer H. Overview of currently approved veterinary vaccine potency testing methods and methods in development that do not require animal use. *Proc Vaccinol* 2011;5:171-174.
- [18] Claassen I. Case study of development, validation, and acceptance of a non-animal method for assessing veterinary vaccine potency. *Proc Vaccinol* 2011;5:175-183.
- [19] Levis R. Overview of the current status of human vaccine potency testing methods that replace animals. Presented at: International Workshop on Alternative Methods to Reduce, Refine, and Replace the Use of Animals in Vaccine Potency and Safety Testing: State of the Science and Future Directions. NICEAM. Bethesda, MD. September 2010. Available at: <http://iccvam.niehs.nih.gov/meetings/BiologicsWksp-2010/BiologicsWksp-present.htm>
- [20] Descamps J, et al. A Case study of development, validation, and acceptance of a non-animal method for assessing human vaccine potency. *Proc Vaccinol* 2011;5:184-191.

- [21] Keller JE. Overview of currently approved serological methods with a focus on diphtheria and tetanus toxoid potency testing. *Proc Vaccinol* 2011;5:192-199.
- [22] Srinivas G. Refinement alternatives for veterinary vaccine potency testing: overview of currently approved serological methods. Presented at: International Workshop on Alternative Methods to Reduce, Refine, and Replace the Use of Animals in Vaccine Potency and Safety Testing: State of the Science and Future Directions. NICEAM. Bethesda, MD. September 2010. Available at: <http://iccvam.niehs.nih.gov/meetings/BiologicsWksp-2010/BiologicsWksp-present.htm>
- [23] Stickings P, Rigby P, Coombs L, Hockley J, Tierney R, Sesardic D. Animal refinement and reduction: alternative approaches for potency testing of diphtheria and tetanus vaccines. *Proc Vaccinol* 2011;5:200-212.
- [24] Arciniega JL, Dominguez-Castillo RI. Development and validation of serological methods for human vaccine potency testing: case study of an anthrax vaccine. *Proc Vaccinol* 2011;5:213-220.
- [25] Hendriksen CFM. Humane endpoints in vaccine potency testing. *Proc Vaccinol* 2011;5:221-226.
- [26] Kulpa-Eddy J, Srinivas G. Approaches to reducing animal numbers in vaccine potency testing. *Proc Vaccinol* 2011;5:227-231.
- [27] Kulpa-Eddy J, Dusek D. Application of the consistency approach in the United States to reduce animal use in vaccine potency testing. *Proc Vaccinol* 2011;5:232-235.
- [28] Gifford G, Agrawal P, Hutchings D, Yarosh O. Veterinary vaccine post-licensing safety testing: overview of current regulatory requirements and accepted alternatives. *Proc Vaccinol* 2011;5:236-247.
- [29] Arciniega JL, Corvette L, Hsu H, Lynn F, Romani T, Dobbelaer R. Target alternative vaccine safety testing strategies for Pertussis toxin. *Proc Vaccinol* 2011;5:248-260.
- [30] Rubin SA. Toward replacement of the monkey neurovirulence test in vaccine safety testing. *Proc Vaccinol* 2011;5:261-265.
- [31] United States Public Law 430 of 1913 and Public Law 99-198 (1985 amendments to the Virus-Serum-Toxin Act); Title 21 U.S. Code, Chapter 5 §§ 151 et. seq.
- [32] United States Department of Agriculture. Code of Federal Regulations, Title 9, Animal and Animal Products. Part 113-Standard Requirements. <http://law.justia.com/us/cfr/title09/9-1.0.1.5.50.html>
- [33] United States Department of Agriculture. Code of Federal Regulations, Title 9, Animal and Animal Products. Part 113-Standard Requirements. Section 133.33. Mouse Safety Tests. <http://law.justia.com/us/cfr/title09/9-1.0.1.5.50.0.69.19.html>
- [34] United States Department of Agriculture. Code of Federal Regulations, Title 9, Animal and Animal Products. Part 113-Standard Requirements. Section 113.38. Guinea Pig Safety Test. <http://law.justia.com/us/cfr/title09/9-1.0.1.5.50.0.69.24.html>
- [35] European Pharmacopoeia Monograph 01/2008:0062. Vaccines for Veterinary Use. 6th ed. Strasbourg, France; European Department for the Quality of Medicine within the Council of Europe. 2008.
- [36] Metz B, Hendriksen C, Jiskoot W, Kersten G. Reduction of animal use in human vaccine quality control: opportunities and problems. *Vaccine* 2002;20:2411-2430.
- [37] Hendriksen C. Refinement, reduction, and replacement of animal use for regulatory testing: Current best scientific practices for the evaluation of safety and potency of biologicals. *ILAR Journal* 2002; 43 Suppl:S43-48.
- [38] Hendriksen C, Arciniega JL, Bruckner L, Chevalier M, Coppens E, Descamps J, Duchene M, Dusek DM, Halder M, Kreeftenberg H, Maes A, Redhead K, Ravetkar SD, Spieser J-M, Swam H. The consistency approach for the quality control of vaccines. *Biologicals* 2008;36:73-77.
- [39] Office International des Epizooties. The role of official bodies in the international regulation of veterinary biologicals. In: *Manual of Standards for Diagnostic Tests and Vaccines*, Fourth Ed. Paris, France, Office International des Epizooties, 2000: pp.63-73.
- [40] European Pharmacopoeia Monograph 01/2008:20625. Avian Live Virus Vaccines: Tests for Extraneous Agents in Batches of Finished Product. 6th ed. Strasbourg, France; European Department for the Quality of Medicine within the Council of Europe. 2008.
- [41] Bruckner L, Bongers J, Castle P, Flore PH, Guittet M, Halder M, Jungback C, Le Gros FX, Tollis M, Nair VK, Wilhelm M, Zeegers J, Zigterman G. Three Rs approaches in the production and quality control of avian vaccines. The report and recommendations of ECVAM workshop 41. *ATLA* 2000; 28:241-258.
- [42] Ottiger HP. Use of PCR for extraneous agent testing in avian vaccines. In: *Advancing Science and Eliminating the Use of Laboratory Animals for Development and Control of Vaccines and Hormones*. Basel, Karger; *Dev Biol* 2002;111:23-26.
- [43] Ottiger HP. Monitoring veterinary vaccines for contaminating viruses. In: Vannier P, Espeseth D, editors. *New Diagnostic Technology: Applications in Animal Health and Biologics Controls*. Basel, Karger, *Dev Biol* 2006;126:309-319.
- [44] Ottiger HP. Development, standardization and assessment of PCR systems for purity testing of avian vaccines. *Biologicals* 2010;38:381-388.
- [45] Bruckner L, Cussler K, Halder M, Barrat J, Castle P, Duchow K, Gatewood DM, Gibert R, Groen J, Knapp B, Levis R, Milne C, Parker S, Stükel K, Visser N, Volkers P. Three Rs approaches in the quality control of inactivated rabies vaccines. The report and recommendations of ECVAM Workshop 48. *ALTA* 2003;31:429-454.
- [46] United States Department of Agriculture. Code of Federal Regulations, Title 9, Animal and Animal Products. Part 113-Standard Requirements. Section 113.209. Rabies Vaccine, Killed Virus. <http://law.justia.com/us/cfr/title09/9-1.0.1.5.50.0.73.81.html>
- [47] Office International des Epizooties. Rabies. In *Manual of Standards for Diagnostic Tests and Vaccines*, 4th edn, Paris, France. 2000. pp. 276-291.
- [48] Blum S, Braunschweiger M, Kramer B, Duchow K, Cussler K. Inactivation test for rabies vaccines: A comparison of test results from *in vivo* and *in vitro* methods. *Pharmeuropa Special Issue, Proceedings Biologicals Beyond 2000*, 2001;390.

- [49] European Pharmacopoeia. Monograph 01/2010:0451. Rabies Vaccine (Inactivated) for Veterinary Use. 7th ed. Strasbourg, France: European Department for the Quality of Medicines within the Council of Europe; 2010.
- [50] United States Department of Agriculture. Code of Federal Regulations, Title 9, Animal and Animal Products. Part 113-Standard Requirements. Section 113.204. Mink Enteritis Vaccine, Killed Virus. <http://law.justia.com/us/cfr/title09/9-1.0.1.5.50.0.73.76.html>
- [51] United States Department of Agriculture. Code of Federal Regulations, Title 9, Animal and Animal Products. Part 113-Standard Requirements. Section 113.205. Newcastle Disease Vaccine, Killed Virus. <http://law.justia.com/us/cfr/title09/9-1.0.1.5.50.0.73.77.html>
- [52] United States Department of Agriculture. Code of Federal Regulations, Title 9, Animal and Animal Products. Part 113-Standard Requirements. Section 113.215. Bovine Virus Diarrhea Vaccine, Killed Virus. <http://law.justia.com/us/cfr/title09/9-1.0.1.5.50.0.73.87.html>
- [53] United States Department of Agriculture. Code of Federal Regulations, Title 9, Animal and Animal Products. Part 113-Standard Requirements. Section 113.201. Canine Distemper Vaccine, Killed Virus. <http://law.justia.com/us/cfr/title09/9-1.0.1.5.50.0.73.73.html>
- [54] United States Department of Agriculture. Code of Federal Regulations, Title 9, Animal and Animal Products. Part 113-Standard Requirements. Section 113.316. Canine Parainfluenza Vaccine. <http://cfr.vlex.com/vid/316-canine-parainfluenza-vaccine-19609135>
- [55] European Pharmacopoeia 5.2.6. Evaluation of Safety of Veterinary Vaccines and Immunosera. 6th ed. Strasbourg, France; European Department for the Quality of Medicine within the Council of Europe.
- [56] European Pharmacopoeia 5.2.9. Evaluation of Safety of Each Batch of Veterinary Vaccines and Immunosera. 6th ed. Strasbourg, France; European Department for the Quality of Medicine within the Council of Europe.
- [57] Weisser K, Hechler U. Animal welfare aspects in the quality control of immunobiologicals. A critical evaluation of animal tests in pharmacopoeial monographs. Nottingham, UK, FRAME for ECVAM and the Paul-Ehrlich-Institute, 1997.
- [58] Cussler K, Possnecker A. Is there still a need for the target animal safety test beyond 2000? *Pharmeuropa Special Issue, Proceedings Biologicals Beyond 2000*. 2000;00:343-347.
- [59] Advisory Group on Alternatives to Animal Testing in Immunobiologicals. The target animals safety test – is it still relevant? *Biologicals* 2002;30:277-287.
- [60] U.S. Department of Agriculture, Center for Veterinary Biologics (CVB) Notice 05.01. Mouse Safety Testing. 2005. Available at: www.aphis.usda.gov/animal_health/vet.../notice_05_01.pdf
- [61] European Pharmacopoeia Monograph 04/2008:0216. Rabies Vaccine for Human Use Prepared in Cell Culture. 6th ed. Strasbourg, France; European Department for the Quality of Medicine within the Council of Europe. 2008.
- [62] Leung T, Corran PH, Gee C, Ekong TAN, Sesardic D. Application of an *in vitro* endopeptidase assay for detection of residual toxin activity in tetanus toxoids. Brown F, Hendriksen C, Sesardic D, Cussler K, editors. In: *Advancing Science and Elimination of the Use of Laboratory Animals for Development and Control of Vaccines and Hormones*. Basel, Karger, *Dev Biol* 2002;111:327-332.
- [63] International Cooperation on Harmonization of Technical Requirements for Registration of Veterinary Products (VICH): GL 41; TARGET ANIMAL SAFETY: EXAMINATION OF LIVE VETERINARY VACCINES IN TARGET ANIMALS FOR ABSENCE OF REVERSION TO VIRULENCE. 2007. Available at: <http://www.vichsec.org/en/guidelines2.htm>
- [64] International Cooperation on Harmonization of Technical Requirements for Registration of Veterinary Products (VICH): GL 44; Target Animal Safety for Veterinary Live and Inactivated vaccines. 2008. Available at: <http://www.vichsec.org/en/guidelines2.htm>
- [65] International Cooperation on Harmonization of Technical Requirements for Registration of Veterinary Products Guidelines; Available at: <http://www.vichsec.org/en/guidelines2.htm>