Biologicals 40 (2012) 369–381

Contents lists available at SciVerse ScienceDirect

Biologicals

journal homepage: www.elsevier.com/locate/biologicals

Meeting report

Report on the international workshop on alternative methods for human and veterinary rabies vaccine testing: State of the science and planning the way forward☆

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ARTICLE INFO

Article history:
Received 27 June 2012
Received in revised form
11 July 2012
Accepted 12 July 2012

ABSTRACT

Potency testing of most human and veterinary rabies vaccines requires vaccination of mice followed by a challenge test using an intracerebral injection of live rabies virus. NICEATM, ICCVAM, and their international partners organized a workshop to review the availability and validation status of alternative methods that might reduce, refine, or replace the use of animals for rabies vaccine potency testing, and to identify research and development efforts to further advance alternative methods. Workshop

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1045-1056/$36.00
http://dx.doi.org/10.1016/j.biologicals.2012.07.005

Keywords:
Rabies vaccines
Potency
Replacement
Refinement
Reduction
Implementation
In vitro

participants agreed that general anesthesia should be used for intracerebral virus injections and that humane endpoints should be used routinely as the basis for euthanizing animals when conducting the mouse rabies challenge test. Workshop participants recommended as a near-term priority replacement of the mouse challenge with a test validated to ensure potency, such as the mouse antibody serum neutralization test for adjuvanted veterinary rabies vaccines for which an international collaborative study was recently completed. The workshop recommended that an *in vitro* antigen quantification test should be a high priority for product-specific validation of human and non-adjuvanted veterinary rabies vaccines. Finally, workshop participants recommended greater international cooperation to expedite development, validation, regulatory acceptance, and implementation of alternative test methods for rabies vaccine potency testing.

1. Introduction

To address the availability and validation status of alternative methods that might further reduce, refine, and replace animal use for rabies potency testing, the National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM) and the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) organized an international workshop on "Alternative Methods for Human and Veterinary Rabies Vaccine Testing: State of the Science and Planning the Way Forward". The workshop was organized in collaboration with partner organizations of the International Cooperation on Alternative Test Methods, including the European Union Reference Laboratory for Alternatives to Animal Testing (EURL ECVAM), the Japanese Center for the Validation of Alternative Methods (JaCVAM), and Health Canada. The workshop was held on October 11–13, 2011 at the National Centers for Animal Health in Ames, Iowa, USA. Over 80 scientists from 14 different countries representing government, industry, and academia attended the workshop. The workshop included sixteen plenary lectures followed by three breakout sessions, and focused on the following objectives:

- Review the state of the science of currently available alternative methods, and identify any unresolved data gaps that must be addressed to allow immediate implementation of the methods in regulatory testing.
- Develop an implementation strategy and plan to address these knowledge and data gaps to achieve regulatory acceptance, implementation, and broader use of alternative methods for routine potency testing of rabies vaccines while ensuring continued protection of human and animal health.
- Define the current availability and validity of process control parameters and assays that could be used to assess lot-to-lot consistency in conjunction with *in vitro* assays for the potency testing of rabies vaccines.
- Assess and identify ways to modify procedures in the current mouse challenge test to lessen or avoid pain and distress, and reduce animal use.
- Identify best practices for current and future integrated approaches to rabies vaccine potency testing to minimize the use of animals.

Dr. William Stokes (NIEHS, NIH, USA), opened the workshop by explaining that ICCVAM is an interagency committee charged with promoting the validation and regulatory acceptance of alternative methods that can reduce, refine (less pain and distress), and replace animals for testing while maintaining the protection of human and animal health and the environment [1]. ICCVAM is composed of 15 U.S. Federal regulatory and research member agencies that require, use, generate, or distribute toxicity, safety, and other types of testing information relevant to ensuring human and animal health. ICCVAM conducts technical evaluations of new, revised, and alternative testing methods with regulatory applicability, and provides formal recommendations to agencies. In conjunction with NICEATM, ICCVAM and its member agencies have contributed to the regulatory acceptance of over 50 alternative test methods [2]. Dr. Stokes noted that this workshop represents the first in a series of specialized vaccine workshops that address priorities identified at a 2010 NICEATM-ICCVAM international workshop[1] on alternative methods for vaccine potency and safety testing [3]. Alternative methods for vaccine testing are currently one of the four highest priorities of NICEATM and ICCVAM due to the large numbers of animals used for such testing and because test animals may experience significant unrelieved pain and distress [4].

This workshop report provides highlights from the speaker presentations, followed by summaries of the discussions, conclusions, and recommendations for each of the key areas discussed. The plenary speaker presentations provided an overview of the public health and animal health significance of rabies, current U.S. and international regulatory guidelines for potency testing of human and veterinary rabies vaccines, and the current status and availability of refinement, reduction, and replacement alternatives for rabies vaccine potency testing.

1.1. Rabies – public health and animal health perspectives

Dr. Charles Rupprecht (CDC, USA) described rabies as an acute, progressive, incurable viral encephalitis with the highest case fatality rate of any conventional infectious disease. An ancient and neglected zoonosis, rabies confers significant international burden, public health, agricultural, and ecological conservation considerations. Rabies virus belongs to the Family Rhabdoviridae, Genus *Lyssavirus* and is found on every continent except Antarctica. All mammals appear susceptible to infection, thereby complicating effective disease prevention and control [5,6]. Rabies virus transmission occurs typically via a bite from an infected host [7,8]. Globally, the single most important animal reservoir is the domestic dog, with wildlife populations (e.g., foxes, raccoons, skunks, bats, etc.) providing additional reservoirs and sources of infection for sylvatic rabies in developed countries.

Each year, more than 15 million people are estimated to receive post-exposure rabies prophylaxis treatment after being exposed to rabies. Treatment includes multiple injections of rabies vaccines and immune globulin. Despite the availability of this highly effective method of prevention, rabies still accounts for over 70,000 human fatalities worldwide each year [9]. In developing African and Asian countries, human exposure occurs primarily in young children, and dogs constitute the dominant reservoirs [9,10]. In the United States, human rabies is relatively uncommon with

[1] 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. September 14–16, 2010. Information available at http://iccvam.niehs.nih.gov/meetings/BiologicsWksp-2010/BiologicsWksp.htm.

20,000–40,000 potential exposures that require the use of post-exposure prophylaxis annually, with 1–8 deaths per year [8].

In Europe, Japan, Canada, the U.S., and other developed countries, requirements for vaccination of dogs and cats have effectively eliminated enzootic rabies in these species. The coordinated, mass vaccination of dogs and wildlife is clearly the most cost-effective manner to minimize human rabies exposures and fatalities. The global elimination of canine rabies remains a realistic goal [5,6,11,12]. Ongoing elimination efforts include: 1) improved canine and wildlife vaccination programs, 2) enhanced diagnostic and surveillance techniques, 3) international communication networks promoting advocacy and awareness (e.g., World Rabies Day), and 4) availability of pure, potent, safe, and efficacious vaccines [5].

Dr. Cristina Cassetti (NIAID, USA) provided an overview of current research efforts funded by the National Institute Of Allergy and Infectious Diseases (NIAID) to develop improved and affordable rabies vaccines and treatments. These include live-attenuated vaccines that require fewer doses, new monoclonal antibody cocktails to replace rabies immune globulin for prophylaxis of exposed individuals, and thermostable wildlife vaccines. The NIAID also provides important resources, including the World Reference Center of Emerging Viruses and Arboviruses (WRCEVA) and the Biodefense and Emerging Infections Research Resource Repository (BEI).

1.2. Rabies vaccines – U.S. and international regulatory requirements

Routine lot release of nearly all inactivated human and veterinary rabies vaccines requires a mouse potency challenge test using virulent rabies virus to assure that the final product is potent and effective. A current exception is an *in vitro* ELISA potency test used in Japan to release non-adjuvanted veterinary rabies vaccines [13,14]. Potency testing requirements differ only slightly among regulatory agencies, and are historically based upon a mouse challenge test, referred to as the "NIH test" due to its origin at the National Institutes of Health [15,16]. The NIH test uses groups of mice that are injected intracerebrally with live rabies virus following immunization with several serial dilutions of a specific vaccine lot. Mice used as positive controls and those that are inadequately protected by the vaccine develop clinical signs of rabies (i.e., paralysis, paresis, and convulsions) 6–9 days following infection. In the U.S. and the EU, an estimated 50,000–70,000 mice per year are used for potency testing and release of rabies vaccines [17].

1.2.1. Human rabies vaccine potency testing requirements

Dr. Robin Levis (U.S. FDA, USA) reviewed the current U.S. requirements for potency testing of human rabies vaccines. No specific tests are defined in the Code of Federal Regulation (CFR) for determining potency of rabies vaccines for human use. 21 CFR 610.10 states: "Tests for potency shall consist of either *in vitro* or *in vivo* tests, or both, which have been specifically designed for each product so as to indicate its potency in a manner adequate to satisfy the interpretation of potency". All manufacturers of human rabies vaccines licensed in the U.S. adhere to the World Health Organization (WHO) guidelines, which define potency as the geometric mean of two valid NIH tests and state that potency must be shown to correlate with clinical efficacy. Rabies vaccines with potency $\geq$ 2.5 IU/mL correlate with clinical efficacy, being defined as protection from disease and death due to development of a serum neutralizing antibody titer equivalent to 0.5 IU/mL. The U.S. FDA has adopted the use of earlier humane endpoints (e.g., paralysis, paresis, convulsions), and their implementation is actively encouraged where applicable [Robin Levis U.S. FDA, personal communication].

The European Pharmacopoeia (Ph. Eur.) does not stipulate the number of replicate NIH tests that are required, although European manufacturers often perform two potency tests to satisfy the requirements of non-European vaccine-importing countries [18]. The use of earlier humane endpoints is clearly defined and referenced.

Dr. Sunil Gairola (Serum Institute of India, India) reported that Indian regulatory authorities may grant vaccine manufacturers permission to use a single mouse potency challenge test following the submission of consistent, valid mouse challenge test data and consistent in-process testing parameters.

As explained by Dr. Jinho Shin (WHO, Switzerland), WHO guidelines specify that all rabies vaccines licensed for human use should define potency as the geometric mean of two valid NIH potency tests [19]. To maintain clinical minimum potency throughout shelf life, the WHO recommends that the potency of each human rabies vaccine lot exceed 2.5 IU/dose, based on comparison to an international reference preparation. Briefly, the International Standard (IS) for rabies vaccines is used in the standardization of rabies vaccines in the mouse potency challenge test and for *in vitro* assays measuring glycoprotein content. The material was prepared from a bulk of Vero cell derived, Pitman Moore strain, produced by the same manufacturing process as the Fifth International Standard, RAV. The candidate standard was calibrated in international units (IU) against the Fifth International Standard in a collaborative study involving 16 participants from 10 countries. For use in the mouse potency challenge test procedure, the assigned unitage is 8 IU/ampoule (i.e., 8 IU/mL) [20].

1.2.2. Veterinary rabies vaccine testing requirements

Dr. Donna Gatewood (USDA, USA) outlined the current requirements and guidance on product-specific validation of reduction, refinement, and replacement (3Rs) alternatives for veterinary rabies vaccine potency testing in the U.S. [21,22]. The USDA currently requires one valid NIH test to demonstrate potency for lot release of all veterinary rabies vaccines. The USDA specifies minimum requirements that must be achieved for a valid relative potency assay: at least 70% of mice (11/16) receiving the most concentrated dilutions of veterinary rabies reference vaccine (VRRV) must survive; at least 70% of mice (11/16) receiving the least concentrated dilution of VRRV and the test vaccine must die; the challenge virus standard (CVS) back titration must show that between 12 and 50 LD_{50} was administered [22]. If the initial test fails to meet the minimum relative potency requirements, two additional tests are required, and the geometric mean of all 3 tests is used to evaluate relative potency. Speakers from the U.S. and international agencies noted that they recognize that while the current mouse challenge test for rabies vaccines ensures the detection of subpotent vaccine lots, they would like to see scientifically valid alternative approaches implemented. Dr. Gatewood emphasized that implementation of any alternative *in vitro* method for inactivated veterinary vaccines will require a close partnership between regulatory bodies and manufacturers, consideration of the use of assay panels, and reliance on the consistency of production methods.

Dr. Lukas Bruckner (IVI, Switzerland) explained that a serum neutralization test (SNT) assay for potency testing of inactivated veterinary rabies vaccines recently underwent a successful international interlaboratory collaboration study, and was recommended for inclusion into Ph. Eur. monograph 0451 to replace the NIH test [23,24]. At their recent 142nd session in April 2012, the European Pharmacopoeia Commission adopted the SNT for veterinary rabies vaccines.[2]

[2] European Directorate for the Quality of Medicines and HealthCare (EDQM) website at: http://www.edqm.eu/en/European-Pharmacopoeia-news-43.html.

2. The mouse potency challenge test for rabies vaccines

Dr. Peter Wunderli (NED Biosystems, USA) described the current mouse rabies challenge test, and provided a critical analysis of several aspects. The current mouse challenge test:

- Uses at least 120 mice per test, all of which receive an intracerebral injection of live rabies virus and approximately 50% experience significant unrelieved pain and distress prior to euthanasia.
- Generates highly variable results, with up to 400% differences in estimated potency [17].
- Produces a high number of invalid tests, with reports for some labs reaching 42% [25].
- Uses an unnatural route of infection (i.e., intracerebral) and vaccination route (i.e., intraperitoneal) [17,26] that, combined with the accepted challenge virus strain, can lead to artificially increased relative potency results for vaccines developed from the same (Pasteur) strain isolate.

As explained by Dr. Alexander Gaydamaka (AHI, USA), vaccine manufacturers face several considerable challenges in developing alternative methods including: resource constraints, presence of adjuvant in most veterinary rabies vaccines that can interfere with accurate antigen quantification assays, and the need to harmonize regulations to avoid duplicate testing of exported products. Dr. Holger Kost (Novartis, Germany) noted that for any proposed alternative potency method, the continued preservation (and perception) of public health and/or product safety is paramount. Therefore, many participants noted that, in the short term, the current mouse challenge test will continue to be utilized globally.

2.1. Refinement alternatives

2.1.1. State of the science

The mouse rabies potency test results in animal pain and distress from two sources. Firstly, the intracerebral injection of a live virus suspension into the cerebrum of test animals, and secondly, the development of clinical rabies in unprotected animals.

Intracerebral (IC) injection of live rabies virus is an invasive and painful procedure [17]. Guidance on appropriate IC injection techniques is available in several regulatory guidelines and publications [17,22,27], and is provided in Appendix 1. IC injection conducted in the mouse rabies potency challenge test can lead to sufficient traumatic tissue damage to cause early death of mice. Thus, regulations stipulate that deaths occurring within 5 days of injection are considered to be non-specific and not attributable to rabies virus infection. Experienced technical personnel are essential to ensure the collection of consistent and reliable potency data. Some guidance on the injection technique is provided in the WHO Manual (1996) [27], as well as the USDA SAM 308 [22], while a best practice guide for potency testing of inactivated rabies vaccine lots was presented by Bruckner et al. 2003 [17]. The use of specific needles[3] fashioned with a sleeve that provides for a uniform depth of injection are used consistently in Germany and Japan and are considered a significant improvement.

Humane endpoints are criteria for ending a test early to reduce the duration and/or severity of pain or distress that occurs during testing [3,28]. Both U.S. and European regulatory authorities have adopted specific policies that incorporate non-lethal humane endpoints as the basis for euthanizing animals when performing the current mouse rabies potency challenge test for human and veterinary rabies vaccines [17,18,21,22,29,30]. Paralysis, paresis, and/or convulsions are used as humane endpoints for the rabies potency test because they have been determined to accurately predict clinical rabies virus infection and indicate that animals exhibiting these signs would eventually die [31]. Further information and training on the recognition and use of earlier, humane endpoints for the mouse rabies potency challenge test is available in a Humane Endpoints – Lethal Parameters (HELP) Group video production titled "*Humane Endpoints Replace Lethal Parameters in Batch Potency Tests of Rabies Vaccines*" [17,32]. The video is now available on the Humane Endpoints in Laboratory Animal Experimentation website at: http://www.humane-endpoints.info/eng/, and via the NICEATM-ICCVAM homepage at iccvam.niehs.nih.gov. The use of available, scientifically valid humane endpoints wherever possible is a fundamental principle of the U.S. Public Health Service Policy on Humane Care and Use of Laboratory Animals [33], as well as current statutory animal welfare laws in the U.S. and the European Union [34,35].

The 2003 ECVAM workshop report 48 *Three Rs Approaches in the Quality Control of Inactivated Rabies Vaccines* provided a review of humane endpoints and useful score sheets for recording the progression of clinical signs during the mouse challenge test [17]. This report also recommended that a body weight decrease of 15% or more in combination with Stage 2 (loss of alertness, circling) or Stage 3 (shaky movements, trembling, convulsions) neuronal effects more precisely identified a "point of no return" and would allow an experiment to be terminated at an earlier point than using paralysis, paresis, and convulsions alone. Although body weight loss is not specified in any current guidelines for humane endpoints for rabies vaccine challenge tests, body weight loss in conjunction with early neurological signs (e.g., slow and circular movements, lack of coordination of hind legs) has been found to be a useful earlier endpoint for study termination prior to the onset of paralysis, paresis, and convulsions [36]. Body weight loss begins on day four post-injection in mice that later progress to clinical rabies and death, while the onset of significant body weight loss (>20%) occurred within 6–9 days post-injection. The average time to death was 10.8 days post-injection. These data support the use of body weight loss combined with early neuronal signs as a humane endpoint for early study termination.

2.1.2. Workshop discussion

Workshop participants recognized that the ultimate goal for rabies vaccine potency testing is the complete elimination of animal testing. However, it was universally agreed that while and where animals must still be used, they should be used in the most humane manner possible and only the minimum number of animals necessary should be used, while ensuring potency of the tested vaccine lots.

Participants discussed ways to minimize or avoid pain and distress associated with the traditional IC injection procedure. Veterinary vaccine manufacturers, a manufacturer of human vaccines, and a national control laboratory (i.e., PEI) reported a long-standing routine practice of anesthetizing animals for the IC procedure using inhalation or injectable anesthetics in their current assay protocols, with no adverse effect on test results. Participants uniformly agreed that based on this extensive experience, the use of general anesthesia for IC injections is strongly encouraged, and should be incorporated into existing protocols as well as regulatory guidelines for both human and veterinary rabies vaccines. Regulatory agency representatives indicated that additional product-specific validation studies would likely not be required by manufacturers to implement this alternative. Consistent with animal welfare laws and policies in Europe and the U.S. requiring consideration and use of 3Rs alternative

[3] Acufirm needles, Ernst Kratz GmbH, Dreieich, Germany (http://www.acufirm.de/index.php?kat=4.).

methods [33–35,37], the use of general anesthesia for any IC procedure in animals should always be considered.

Workshop participants also discussed the use of analgesics following IC injections as a potential way to minimize or avoid post-injection pain and distress, and to also further minimize pain and distress in animals experiencing early clinical signs of rabies. Sustained-release analgesic formulations of buprenorphine have been used to effectively manage pain in rodents for up to 72 h [38]. Workshop participants recommended studies to evaluate the use of analgesics during the IC procedure and before or at the onset of clinical signs, to determine if their use is compatible with generating valid study results. Some participants questioned whether analgesia might mask clinical signs used to evaluate non-lethal endpoints; however, analgesia is not expected to affect the neurological signs used as humane endpoints (i.e., paresis, paralysis, and convulsions), as these result from central nervous system tissue damage and not from the induction of pain. A concern with administering one or more injections several days after the IC procedure is the associated safety risk of handling infected animals.

Following the workshop, the USDA issued a Center for Veterinary Biologics (CVB) Notice strongly encouraging the use of general anesthesia for intracerebral inoculation of mice during rabies vaccine potency testing [39]. CVB Notice No. 12-12 also provides additional guidance on the use of humane endpoints in biological products testing, and reiterates guidance on humane endpoints for the rabies challenge test. Finally, the use of analgesics in animal studies and potency testing is also encouraged, when it can be shown this does not affect the study outcome [39].

Workshop participants also discussed two alternate inoculation routes for challenging mice with live rabies virus: the intranasal route and the rabies peripheral challenge (RPC) test. The USDA CVB commented on a study they performed to validate the use of a safer, non-invasive intranasal (i.n.) challenge method (with general anesthesia) for rabies virus infection [40]. If found to be a sufficiently valid alternative (i.e., not adversely affecting the outcome of the test), the CVB would consider publishing implementation guidance. During workshop discussions, regulatory agency representatives indicated that additional product-specific validation studies by manufacturers would not likely be required.

The RPC test confers significant advantages over the current NIH test, including: eliminating the IC challenge; a more natural route of infection; increased sensitivity through measurement of a vaccines primary immunogenicity; and reduced variability by using older mice and higher viral challenge dose [41]. Briefly, the RPC test uses a single dose of vaccine administered intramuscularly (i.m.) followed by an i.m. rabies virus challenge 28 days later [41]. A single dose i.m. vaccination more closely reflects routine vaccine practices and has been shown to increase protection of mice compared to single dose i.p. vaccinations with either human or veterinary rabies vaccines [26,41]. The RPC test therefore confers additional advantages for rabies vaccine testing, in addition to those stated above. However, implementation of the RPC test would likely require product-specific validation. Although workshop participants considered that future efforts focus on replacing the vaccination-challenge test, they recommended that the validity and use of the RPC test be considered by the WHO Expert Committee on Rabies.

2.1.3. Recommendations

The following guidelines are suggested when it is necessary to conduct the mouse rabies potency challenge test:

- Where not already implemented, regulatory agencies and global standards-setting organizations should encourage:
  - Immediate incorporation of humane endpoints in all national and international testing regulations and guidelines for mouse rabies challenge testing for both human and veterinary rabies vaccines.
  - The routine use of anesthetics and appropriate techniques to reduce the pain and distress associated with IC administration of live rabies virus during the mouse challenge procedure should be stipulated in all regulatory guidelines.
  - Provision of analgesics to avoid or minimize post-procedural pain and distress associated with the rabies virus IC injection, contingent upon studies to determine that there is no interference with the outcome of the study. Similarly, investigations should be conducted to determine if analgesics can be provided to avoid or minimize pain and distress associated with the development of clinical rabies in unprotected animals without interfering with study objectives.

2.2. Reduction alternatives

2.2.1. State of the science

A comparison of various international regulatory requirements for the rabies challenge test is provided in Table 1. There are variations in several aspects of the mouse challenge test protocol for potency testing of inactivated rabies vaccines as currently utilized by various international regulatory authorities, including: the minimum number of vaccine dilutions, the minimum number of mice tested per dilution dose group, whether mice receive single or repeated doses of vaccine, and test validity criteria. For example, the current requirements for the number of mice used per dilution dose group ranges from 10 to 18. Considering that at least 3 dilutions are used per test, use of the highest group size can result in 80% more animals used as compared to the lowest required group size.

2.2.2. Workshop discussion

Workshop participants considered several approaches to reduce the number of mice used for the current mouse potency test, including: 1) reducing the number of mice tested per dilution, 2) reducing the number of dilutions tested, 3) eliminating duplicate testing, and 4) testing multiple lots simultaneously.

Due to the inherent variability associated with the current mouse potency test, many participants expressed reluctance to reduce the numbers of mice in all dilutions tested. However, some vaccine manufacturers suggested that fewer mice may be used in the vehicle control group, the positive control group, and in vaccine dilutions where 100% or 0% mortality, respectively, is expected. This approach would provide an immediate opportunity to reduce the number of animals tested for different dilutions. Indeed, one vaccine manufacturer reported the incorporation of a reduced number of animals for different dilutions into their test protocols without adversely affecting test outcomes. However, as noted above, the mouse challenge test can require significant re-testing with up to 42% of tests failing to meet the validity criteria [25]. Efforts to reduce the number of dilutions or the number of animals per dilution could potentially result in more invalid tests. Therefore, the 3Rs benefits of such reductions should be evaluated for the potential to increase animal use due to increased test failures and subsequent retesting. Laboratories testing numerous vaccine lots per year, however, have sufficient expertise and testing history that may allow them to aggressively pursue reducing the number of mice and/or dilutions used.

In the 2003 ECVAM workshop report 48 *Three Rs Approaches in the Quality Control of Inactivated Rabies Vaccines,* participants recommended that national control authorities investigate whether a single dilution version of the mouse potency test could be

Table 1
International regulatory requirements for the mouse potency challenge test.[a]

	Number of valid tests required	Number of animals/dilution	Number of dilutions	Number of vaccinations	Mice used	Interval between vaccinations (days)	Interval between vaccination and challenge (days)	Challenge dose (LD50)	Observation period after challenge (days)	Criteria for evaluation	Available alternative methods
USDA SAM 308[b,c] (veterinary use)	1 (3, if original test fails)	16	5	2 (i.p.)	Female 13–15 g	7 ± 1	14 ± 1	12–50	5–14 (daily)	Death, clinical signs of rabies (paralysis, paresis, convulsions)	Humane endpoints; Anesthesia during i.c. injection
U.S. FDA[d] (human use)	2	16	5	2 (i.p.)	13–16 g	7	14, from first	12–50	5–14	Death, clinical signs of rabies (paralysis, paresis, convulsions)	Humane endpoints
Ph. Eur. (human use)[e]	–	Suitable to meet the requirements for validity of the test	3	2 (i.p.)	Female 11–15 g	7	7, from second	~50	5–14 (twice daily)	Death, clinical signs of rabies (paralysis, paresis, convulsions)	Humane endpoints; Single dilution
Ph. Eur. (veterinary use)[f]	–	≥10	≥3	1 (i.p.)	Female ~ 4 weeks	N/A	14	~50	5–14 (twice daily)	Death, clinical signs of rabies (paralysis, paresis, convulsions)	Serum neutralization test[h]; humane endpoints; single dilution
WHO (1996)[d] (human use)	2	≥16	≥3 (usually 5)	2 (i.p.)	13–16 g	7	14, from first	12–50	5–14	Death; clinical signs of rabies (paralysis, paresis, convulsions)	Humane endpoints (if validated) single dilution
OIE[g] (veterinary use)	–	≥10	A sufficient number	1 or 2	3–4 weeks	7, if required	14	12–50	N/A	N/A	–

[a] Update of a table originally provided in the ECVAM workshop report 48 (Bruckner et al. 2003) [17].
[b] USDA. 2007. Standard Requirements. SAM 308: Supplemental Assay Method for Potency Testing of Inactivated Rabies Vaccines in Mice Using the National Institutes of Health Test. 9 CFR 113 [22].
[c] U.S. Code of Federal Regulations, Title 9, Chapter I: Animal and Plant Health Inspection Service, Department of Agriculture, Subchapter E – Viruses, Serums, Toxins, and Analogous Products; Organisms and Vectors, Part 113.209, Rabies Vaccine, Killed Virus. Washington D.C., USA: U.S. Government Printing Office. (Updated 2010) [21].
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[f] European Pharmacopoeia. 2011. Rabies Vaccine (Inactivated) for Veterinary Use, Draft Monograph for Comment. Pharmeuropa 2011; 23(1):128–131 [24].
[g] OIE. 2011. Rabies. In: Manual of Diagnostic Tests and Vaccines for Terrestrial Animals (World Organization for Animal Health, eds.) [42].
[h] Adopted by European Pharmacopoeia Commission in April 2012.

introduced for potency testing of veterinary and human rabies vaccines, since it had been successfully established by a European control authority [17,43]. However, participants at this workshop did not encourage future validation efforts aimed towards implementing the single-dilution assay, as it produces no quantitative data, and since it generates a pass/fail result, cannot assign a potency value. In addition, vaccine lot stability testing is difficult to interpret without a potency value.

As noted previously, the current guidelines for human rabies vaccines in many countries require duplicate testing using the geometric mean of two valid mouse potency tests [19]. This requirement is driven by the historical nature of the mouse challenge test, with acceptable $P = 0.95$ confidence limits between 25% and 400% of the estimated potency. Dr. Sunil Gairola (Serum Institute of India, India) reported a 50% reduction in the number of mice used following regulatory approval of a single test based upon submitted data of manufacturing and testing consistency. Manufacturing consistency was demonstrated with over 75 lots, with test data (i.e., two tests per lot) submitted to the relevant National Control Laboratory from 2007 to 2010. Regulatory approval was granted on the condition that every sixth lot continues to be tested for potency using two tests.

Vaccine manufacturers were encouraged to investigate and/or provide historical data to support the elimination of duplicate testing where it is currently required. Acceptance of a single test would substantially reduce the number of mice used in the lot-release testing of human rabies vaccines up to 50%. Criteria for when a single test would be considered acceptable should be established, such as criteria provided by the USDA that allow for a single potency test [22]. Total confirmatory testing of human and veterinary rabies vaccines by U.S. and European control authorities is estimated to account for 6000 to 9200 mice per year [17]. Workshop participants suggested that duplicate testing be eliminated through strict manufacturing controls that could obviate the need for confirmatory testing by regulatory authorities.

Currently, regulatory authorities allow testing of multiple vaccine lots at one time. This permits a single reference vaccine and a single back-titration of challenge virus (confirmation of virulence) to suffice for testing several vaccine lots, providing significant reductions in the use of mice. For example, the same positive control and vehicle control groups can serve as the controls for several lots of vaccine, eliminating the need for each lot to have separate control groups. Vaccine manufacturers confirmed that they currently test multiple vaccine lots at one time, in conjunction with stability samples when conducting the mouse potency challenge test.

2.2.3. Recommendations

- Additional validation efforts for the alternative single dilution assay for rabies vaccines are not encouraged. However, manufacturers should consider reducing the number of dilutions tested, provided that this does not increase the number of test failures and the number of retests performed.
- Manufacturers and regulatory authorities are encouraged to investigate ways to reduce the number of mice used per vaccine dilution, especially at higher and lower vaccine dilutions, and for vehicle and positive control groups. Bio-statistical analysis of lot release potency data should be evaluated to determine whether the number of dilutions and/or the number of mice tested per dilution can be reduced.
- Human rabies vaccine manufacturers should review historical test data to determine if this supports eliminating the need for duplicate mouse potency testing on each vaccine lot.
- To further reduce animal use, manufacturers should, where feasible, test multiple lots at the same time, using a single reference test vaccine and a single back-titration of challenge virus.
- Regulatory authorities should discuss establishment of criteria that could avoid additional duplicate potency testing for vaccines imported to the respective country.

3. *Refinement and reduction:* serological potency assays – antibody quantification methods

3.1. State of the science

Serological potency methods measure the amount of antibody produced in vaccinated animals, which is compared to a reference antibody value known to provide protection in the original challenge model. Serology methods provide a significant refinement to the mouse rabies challenge test by avoiding IC injection of live rabies virus and its associated unrelieved pain and distress, as well as avoiding the development of clinical disease in unprotected vaccinates and control animals [3].

Dr. Lukas Bruckner (IVI, Switzerland) provided an overview and current status of an international collaborative study investigating a modified Rapid Fluorescent Focus Inhibition Test (RFFIT) assay, now termed the Serum Neutralization Test (SNT), for potency testing inactivated veterinary rabies vaccines [23]. The European Directorate for the Quality of Medicines and HealthCare (EDQM) sponsored an international collaborative study of 13 laboratories from 10 countries, including laboratories in the EU, Canada, and the U.S., that confirmed the accuracy and interlaboratory transferability of the SNT. The SNT was able to distinguish lots identified as subpotent in the current mouse challenge test [25]. Serological testing, while not completely eliminating animal use, addresses 3Rs goals by: reducing animal use per test vaccine by up to 10-fold, (8–10 vs ~80) and avoiding the pain and distress of the IC challenge method. Compared to the challenge test, the SNT is also more cost and time effective (3 weeks vs more than 4 weeks). In 2011, the European Pharmacopoeia (Ph. Eur.) Group of Experts 15V recommended the SNT assay for inclusion into a revised Ph. Eur. Monograph 0451 [24]. Workshop participants agreed that this monograph provides a critical framework that can be used by industry to conduct product-specific validation necessary to implement the SNT. Subsequent to the workshop, the European Pharmacopoeia Commission approved the revised monograph in April 2012.[2]

Dr. Elisabeth Kamphuis (PEI, Germany) explained that the SNT proposed for lot release of veterinary vaccines is a qualitative, and at best semi-quantitative test, with a pass-fail endpoint (Appendix 2). An additional validation approach was presented with a multi-dilution SNT that can establish parallelism with the reference and can be used to calculate IU. The multi-dilution SNT will likely be preferred to evaluate stability of test and reference vaccine lots, to calibrate new standards, and to evaluate changes in the manufacturing process. In addition, Dr. Kamphuis also described a development study for an alternative multi-dilution SNT specific for potency testing of human rabies vaccines, with regulatory authorities and manufacturers providing both material and assay design support. Current development efforts are focused on investigating and optimizing the protocol, including: identifying the optimal age of the mice used, the number of immunizations, and potentially using i.m. rather than i.p. inoculation. Regulatory agencies are likely to require quantitative data before accepting a pass-fail test to release human rabies vaccine lots.

3.2. Workshop discussion

There was considerable discussion among workshop participants regarding the specific parameters and associated variables

within the SNT. Specifically, older mice possess a more mature immune system that could potentially yield more uniform serological responses to vaccination than younger animals used in the validation study. Bruckner et al., 1988 [44] reported higher uniform antibody responses in 5–7 week old mice following vaccination. In addition, the variance of serum neutralization titers was reduced 2-fold in 9–11 week old vaccinated mice when compared to standard 3–4 week old mice [45].

The ability to discriminate between potent and subpotent vaccines was considered optimal when the serologic response is measured 14 days after a single, non-boosted vaccination. However, Dr. Wunderli stated that currently available data indicate that extending the time of immunization to 21–28-days, as opposed to the current recommendation of 14-days, can provide a more accurate measurement of potency and/or confer a reduction in associated variability. Workshop participants discussed the SNT variables and suggested international standards that should be considered (Table 2).

Workshop participants generally considered the SNT assay to have an acceptable, standardized protocol that has undergone sufficient validation to be used for assessing the potency of inactivated veterinary rabies vaccines. However, some concerns were raised regarding the assay's ability to adequately detect subpotent lots, and it was suggested that product-specific validation studies include additional lots identified as subpotent by the current mouse challenge test. This may necessitate the artificial creation of subpotent vaccine lots.

Individual manufacturers are required to demonstrate product-specific validation of an alternative assay prior to regulatory approval. Historically, the replacement of an existing assay requires comparative studies that demonstrate statistical equivalence between the two methods. Due to the inherent variability associated with the mouse challenge test, quantitative correlation studies demonstrating such equivalence may not be possible. Although the ability of the SNT to detect subpotent vaccine lots must be demonstrated, regulatory authorities conveyed that they recognize the complexities associated with quantitative correlation and invited an open dialogue with manufacturers as they proceed with their product-specific validation. Regulatory authorities also encouraged manufacturers to consider supplementing their assay validation with evidence of manufacturing consistency through detailed SOPs and in-process testing controls. Guidelines were suggested for the product-specific validation of the SNT (Table 3).

3.3. Recommendations

- The use of serological methods (i.e., vaccination and measurement of neutralizing antibodies) instead of the challenge test for potency testing will avoid significant pain and distress and avoid worker safety issues associated with using live rabies virus in animals. It could also use significantly fewer animals compared to the challenge test.
- Based on results of the SNT interlaboratory validation study [23] and acceptance of the method described in the Ph. Eur. Monograph 0451 [29] for inactivated veterinary rabies vaccines, the SNT is considered sufficiently standardized to provide the framework to substitute for the mouse challenge test. Therefore, the following are suggested:
  - Veterinary rabies vaccine manufacturers in collaboration and consultation with appropriate regulatory authorities are encouraged to initiate product-specific validation using the SNT serological method. Validation should include determining whether the SNT can identify subpotent lots and the extent that the SNT results correlate to the current challenge test.
  - Establishment of regulations and guidelines based on Ph. Eur. Monograph 0451 can facilitate the global implementation of this method.
  - Validation of the multi-dilution SNT for both veterinary and human vaccines should continue for quantitative assessment of test and reference vaccine stability, to calibrate new standards, and to evaluate changes in the manufacturing process.
  - Where feasible, specifically for non-adjuvanted vaccines, manufacturers are encouraged to consider moving directly to an antigen quantification test for lot release potency testing.

4. Replacement alternatives: *in vitro* antigen quantification assays

4.1. State of the science

In recent years, several *in vitro* assays have been developed that quantify rabies virus antigens in vaccines against a suitable reference standard. Workshop participants reviewed these assays and discussed the opportunities and challenges for the validation and global implementation of each method.

The rabies virus spike glycoprotein (G Protein) is the primary rabies virus antigen shown to induce rabies virus neutralizing antibodies in animals [46] (Fig. 1). The natively folded form of rabies virus G protein is virion-associated, trimeric, and highly immunogenic [47]. Therefore, any *in vitro* assay and the associated detecting monoclonal antibody that attempts to equate glycoprotein content with vaccine

Table 2
Suggested parameters for the international standardization of the serum neutralization test (SNT) for rabies vaccine potency testing.

Parameter	Recommendation	Comments
Age of mice	4–7 weeks	5–7 weeks is ideal
Sex of mice	Female	
Strain of mice	Outbred	e.g. CF-1
Cell line	BHK-21 (C13) (ATCC #CCL-10)	Mouse neuroblastoma cells also acceptable
Cell Line Passages	Limited and should be specified	Should adhere to Master Cell principles
Cell substrate and format	96-well microtiter plates	
Reference vaccine	EDQM, CVB, or internal reference	Calibrated against WHO standard
Reference serum	WHO 2nd international standard	Available from NIBSC
Cell culture challenge virus	CVS-11 strain adapted to cell culture	Recommend that the same challenge strain be used; Should adhere to Master Cell principles
Endpoint determination	50% fluorescence-positive wells	Fluorescent-cell counting not recommended
Allowable number of non-responder mice	1/6 or 2/8	
Number of mice per test	8–10	
Number of injections	One	Booster dose not recommended
Volume of injection	0.2 ml (1/5 dose)	
Immunization time	14-day bleeding	21–28-day bleeding may reduce variability
Immunization route	i.p.	i.m. Vaccination may yield higher responses

Table 3
Recommendations for product specific validation of the alternative serum neutralization test (SNT) as a rabies vaccine potency test.

	Recommendation	Comments
Required parameter		
Reference vaccine	Internal reference or standard reference such as from EDQM or, CVB, etc.	Internal reference is the vaccine shown to be potent by immunogenicity study
SNT calibration	Based upon standard reference serum	Calibrated to the WHO 2nd standard
Discrimination	Must be able to identify subpotent lots	Lots that have failed the mouse challenge test must fail the SNT potency test
Comparison with the mouse challenge test	Evaluate on a pass/fail basis rather than in units or relative potency	
Parallelism	For validation (not routine testing), suggest testing multiple dilutions of test and reference vaccines in SNT. The endpoint titer is the dilution with 50% of mice seroconverting	Testing dilutions of reference and test vaccine in the SNT can establish parallelism and can allow calculation of relative potency or international units by comparison with a reference of known potency.
Suggested parameter		
Ability to detect manufacturing process errors	Construct subpotent lots based upon common manufacturing errors	
Required parameter		
Manufacturing consistency	Use in-process antigen quantification, assessment of antigen quality, and manufacturing consistency to augment validation	Calibrated against WHO standard
Additional data	Consider evaluating banked sera from host immunogenicity study	

potency must be able to distinguish between the highly immunogenic (virion-associated, trimeric) and poorly immunogenic (non-associated or soluble, monomeric) forms of the G protein. Establishing a direct correlation between the quantity and quality of the antigen and its immunogenicity and protective response represents a significant and enduring problem. Furthermore, this barrier signifies a greater challenge when the potency testing of rabies vaccines containing adjuvant is considered. To date, an ELISA using a monoclonal antibody (National Veterinary Assay Laboratory (NVAL) mAb in Table 4) to quantify the trimeric form of the G protein antigen is only used for potency release testing of one non-adjuvanted veterinary rabies vaccine produced and tested in Japan (Dr. Koichiro Gamoh, NVAL, Japan).

Dr. Claudia Lopez-Yomayuza (Justus Liebig University, Germany) summarized the available approaches to developing alternative *in vitro* assays for rabies vaccine potency testing, which included: genomic and proteomic characterization of the vaccine, measurement of antigen/immunogen content, and assessment of antigen structure and the integrity of the virus particles. Note that the G protein mass does not correlate with immunogenicity or efficacy. Therefore, a G-protein ELISA requires neutralizing monoclonal antibodies that correctly quantify the amount of relevant antigen (virion associated, trimeric) present in the vaccine lot. Dr. Lopez-Yomayuza recommended that proposed *in vitro* assays be correlated against serological tests in target species.

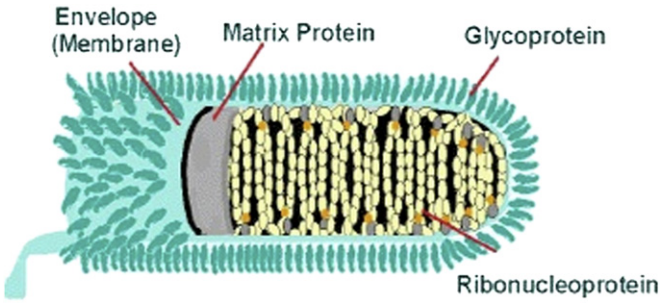

Fig. 1. Schematic diagram of a rabies virus particle. Rabies virions are bullet-shaped with 10-nm spike-like glycoprotein peplomers covering the surface (image available at: http://www.cdc.gov/rabies/transmission/virus.html).

Three types of alternative *in vitro* assays for determining the potency of inactivated rabies vaccines were considered during the workshop: Enzyme-Linked Immunosorbent Assays (ELISA), single radial immunodiffusion (SRID) tests, and Antibody Binding Tests (ABT).

4.1.1. Enzyme-linked immunosorbent assays (ELISA)

The Immunocapture ELISA (IC-ELISA) uses anti-glycoprotein antibody-coated (monoclonal or polyclonal) microtiter plates to capture antigen in vaccine or in-process samples. Bound antigen is detected using a specific monoclonal antibody for the trimeric form of the G protein, thereby quantifying only conformationally correct antigen [48–50].

Dr. Koichiro Gamoh (NVAL, Japan) provided an overview and detailed protocol of an IC-ELISA approved for lot release potency testing of non-adjuvanted veterinary rabies vaccines in Japan since 1996 [13,14]. This alternative method involves removal of soluble monomeric G protein via gel filtration prior to performing the ELISA. The monoclonal antibody utilized (mAb 13-10 in Table 4) binds to native trimeric glycoprotein but not soluble monomeric G. As noted by Dr. Gamoh, no efforts have yet been made to apply this ELISA to potency release testing of human rabies vaccines in Japan.

According to Dr. Jean-Michel Chapsal (Sanofi Pasteur, France) a D1 mAb (Table 4) is currently utilized for both antigen capture and antigen detection in a sandwich ELISA assay at the French National Security Agency of Medicines (Agence Nationale de Sécurité du Médicament et des produits de santé [ANSM]), formerly the French Agency for the Medical Safety of Health Products (AFSSAPS). The mAb-D1 reacts with native G protein and not soluble, monomeric Gs [51]. The ELISA is currently used in France to quantify G protein in non-adjuvanted human rabies vaccines. As a major correlate of the efficacy of rabies vaccines is the induction of neutralizing antibodies against the transmembrane G protein, immunogenicity is dependent upon its preserved three-dimensional structure. As such, Dr. Chapsal indicated that chemico-physical tests, including microcalorimetry, Surface Plasmon Resonance, and Dynamic Light Scattering may be used to characterize a vaccine during its development, particularly to ensure that the G protein has retained the correctly folded structure. Dr. Chapsal also reported on the development of a sandwich ELISA using one antibody for coating and mAB-D1 for detection. The titration was performed against an internal reference calibrated in IU

Table 4
Rabies virus monoclonal antibodies.

Monoclonal antibody	Isotype	Affinity	Properties	Reference	Availability for licensing (mAb/ Hybridomas)	Source (mAb/Hybridomas)
mAb-13-10	IgG2b	Antigenic site II-8	Neutralizing antibody to glycoprotein. Reacts with native glycoprotein and not Gs. Reacts to RC-HL, ERA, Ni, HEP-Flury, and CVS strains.	Gamoh et al. 1996 [13]; Luo et al. 1998 [54]	May be available for license	Japan Veterinary Products Association – National Veterinary Assay Laboratory
mAb-D1 (also known as mAb D1-25)	IgG1	Antigenic site III on Glycoprotein (aa 330–338)	Reacts with native glycoprotein and not Gs. Neutralizes PV, PM and CVS strains.	Jallet et al. 1999 [51]	Available for license	Office of Technology Transfer Institut Pasteur, Paris
mAb-M5B4	IgG1	Antigenic site III	Reacts with native glycoprotein and not Gs. Reacts with PV, PM, Flury LEP. Does not react with CVS in RFFIT.	Nagarajan et al. 2006 [55]	Available for license	Indian Immunologicals Ltd. Andhra Pradesh, India
mAb SO4	IgG1	G Protein Antigenic Site III	Neutralizes all vaccine strains and most street rabies strains	B Dietzschold, Personal Communication 2012	Available for license (also available as expression vector)	Office of Technology Transfer Thomas Jefferson University Philadelphia, PA, USA
Human mAb SO 57	Unknown	G Protein	Neutralizes all vaccine strains and most street rabies strains	B Dietzschold, Personal Communication 2012	Available for license (also available as expression vector)	Office of Technology Transfer Thomas Jefferson University Philadelphia, PA, USA
Various	–	G Protein	React with various sites	H Ertl, Personal Communication 2012	May be available for license	Business Development Office Wistar Institute Philadelphia, PA USA
Various	–	G Protein	React with most vaccine strains	C Rupprecht, Personal Communication 2012	Available for license	Technical Transfer Office Centers for Disease Control and Prevention (CDC) Atlanta, GA USA
mAb-1112 mAb-E559.14 mAb-62-7-13 mAb-M727-5-1 mAb-M777-16-3	IgG1 IgG1 IgG2b IgG2a IgG1	Antigenic site II Antigenic site II Antigenic site III Antigenic site II Antigenic site II	All neutralizing	Müller et al., 2009 [56]	These 5 mAbs may be reserved for use in PEP	Thomas Jefferson University, USA FLI, Germany CDC, US CFIA, Canada CFIA, Canada
5B12 (1,2,3) 4G4 (1,2,3) 6041 (1,2) Rab-50 (1,2,3) RV1C5 (1,2,3) O.N.541 (1,2) MA1-21549 (1) Pierce (1) Santa Cruz (2) Novus (3)	IgG2a IgG2a IgG2a IgG2b IgG2a IgG2b IgG2a	Unknown		Máčiková et al., 1996 (Rab-50) [57]	These seven mAbs are available from commercial sources.	Commercial Sources: 1) Pierce mouse mAbs against purified Rabies virus including at least one mAb (Rab-50) with neutralizing properties. These are sold as Protein A purified globulin from ascites fluid. http://www.pierce-antibodies.com/search/searchResults.cfm 2) Santa Cruz http://www.scbt.com/table-rabies_virus.html 3) Novus Biologicals http://www.novusbio.com/Rabies-Virus-Antibody-Rab-50_NB100-63045.html
Commercial ELISA Kit Commercial ELISA Kit			Quantification of nucleoprotein content Quantification of glycoprotein content		Commercially available including positive and negative controls	DRG International Mountainside, NJ, USA. http://www.drg-international.com/index.php?id=15&broad=Rabies&button=&formid=ProductRequest

Abbreviations: CDC – Centers for Disease Control and Prevention, FLI – *Friedrich Loeffler Institute*, CFIA – Canadian Food Inspection Agency, HEP-Flury – high egg passage strain of rabies virus, Ni – Nishigahara strain of rabies virus, Flury LEP – low egg passage strain of rabies virus, CVS – challenge virus standard strain of rabies virus, ERA – Evelyn Rokitniki Abeseth strain of rabies virus, PE – Post-exposure prophylaxis, PM – Pitman-Moore strain of rabies virus, RC-HL – avirulent varient of the Nishigahara rabies virus strain used in veterinary rabies vaccines in Japan.

against the 6th WHO Internal Standard for Rabies vaccine. Finally, Dr. Chapsal stated that antigen quantification may represent an adequate replacement for the NIH test, provided vaccines are sufficiently characterized and undergo stringent consistency of manufacturing protocols.

Dr. Fabrizio de Mattia (MSD Animal Health, Netherlands) described validation studies associated with sodium citrate treatment of aluminium phosphate adjuvanted veterinary vaccines to desorb the rabies virus antigen for quantification in a sandwich ELISA. Test results indicate that sodium citrate treatment did not alter the G protein epitope, that assay results were linear regardless of vaccine antigen content, and that the assay could distinguish between potent and subpotent lots.

4.1.2. Single radial immunodiffusion (SRID) test

Dr. Lorraine McElhinney (AHVLA, UK) discussed the poor correlation of the SRID test to the NIH test and its inability to be used with adjuvanted vaccines. Consistently higher vaccine potency values were found with the SRID test, as it does not distinguish between highly immunogenic virion associated G protein and the poorly immunogenic monomeric form. Furthermore, an investigation of expired human vaccine lots that failed the mouse challenge test (with <2.5 IU/mL), successfully passed when the SRID assay was utilized [52]. Finally, Lyng et al. 1992 [53] demonstrated poor correlation of SRID results with mouse challenge tests during stability studies. Accordingly, the SRID is not proposed as a replacement for potency testing of final vaccine lots, although it may be considered for in-process testing to estimate overall virus protein. Dr. Gairola reported their use of an adapted 7-day SRID as an in-process test for bulk vaccine lots.

4.1.3. Antibody binding test (ABT)

The ABT is performed by incubating serial dilutions of reference or test vaccine lots with a standardized neutralizing polyclonal antiserum and uses a fluorescent focus reaction to detect unabsorbed (un-neutralized) antibody. As explained by Dr. McElhinney, even though the ABT may be preferable to the mouse challenge test, its correlation to the *in vivo* test is quite poor. Furthermore, there are no data regarding the ability of the ABT to distinguish native, trimeric G protein from the poorly immunogenic form, and the presence of adjuvants is known to interfere with the assay through inhibition of antibody binding. Due to these issues and the development of IC-Capture ELISA assays, further development of the ABT was not recommended.

Dr. Gairola summarized a manufacturer's perspective of the application and use of consistency parameters and integrated approaches to replace animal use for rabies vaccine potency testing. The consistency approach model for vaccine lot release aims to: 1) identify critical indicators of safety and efficacy that can be accurately measured with non-animal tests and thereby replace animal use for final lot release testing; 2) make use of quality control procedures throughout the manufacturing process to identify when there are unacceptable changes in critical parameters of product consistency; and 3) encourage the application of newer concepts (e.g., quality by design (QBD), process analytical technology (PAT) approaches) for the vaccine quality assurance. A successful consistency approach for determining the potency and safety of rabies vaccines will require several in-process assays to accurately determine the quantity and integrity of relevant rabies virus G proteins. These may include 2-D gel electrophoresis and a nucleoprotein ELISA assay that can determine the ratio of structurally intact (nucleoprotein not detectable) to degraded virions (detects released nucleoprotein). Regulatory authorities support the development and validation of manufacturing consistency parameters and/or integrated quality control strategies for the routine lot release of rabies vaccines.

4.2. Workshop discussion

Workshop participants agreed that the use of mAbs in an ELISA appears to be the best quantitative method for measuring the virus-associated conformationally intact glycoprotein. Soluble G protein should either be removed or blocked (or both) if its presence will interfere with the detection of the immunologically relevant protein. The mAb specific for the conformationally correct G protein will need to be correlated with protection for each specific rabies vaccine.

Workshop participants recommended that a table indicating the affinity, properties, source, and availability of mAbs specific to native rabies virus G protein would be a valuable resource for the development and facilitation of alternative *in vitro* potency testing methods for rabies vaccines. This information is detailed in Table 4; however not all potentially useful mAbs may be available for either assay development or for commercial therapeutic purposes due to intellectual property and licensing issues. Workshop participants noted that since rabies vaccines are produced from different virus strains, one mAb may not be suitable for all products.

Consistent availability of reagents, including the standard reference, is critical for validation of the ELISA. Currently, the EDQM and WHO rabies standards are non-adjuvanted but have been compared to adjuvanted products. Workshop participants stated that the successful development of an ELISA will require an in-house standard (i.e., an internal vaccine lot with its potency calibrated to one of the international rabies standards).

Workshop participants suggested that a pass/fail correlation to the mouse challenge test through the use of subpotent lots is necessary for successful implementation and regulatory approval of any alternative method. Data to support correlation with the mouse test results may require that subpotent lots be generated through detergent, pH, or heat treatment of potent lots, or other acceptable method. Comparisons may also need to be made to serological titers considered protective in humans or animals. Additionally, some workshop participants recommended that active post-market surveillance to determine that vaccines are generating an adequate neutralizing response should be considered for, minimally, a defined period after implementation of *in vitro* antigen quantification potency tests.

Workshop participants did not encourage further development of either the ABT or SRID assay as replacement potency tests due to poor correlation with the mouse challenge test and inability to distinguish between highly immunogenic virion-associated G protein and poorly immunogenic monomeric G protein, respectively. However, workshop participants did agree that the SRID assay may be useful in measuring total G protein during in-process testing.

4.3. Recommendations

- Monoclonal antibodies in an ELISA appear to be the best quantitative method for measuring the virus-associated conformationally intact G protein necessary to determine the potency of rabies vaccines. Soluble G protein should either be removed or blocked (or both) if its presence will interfere with the detection of the immunologically relevant protein. The mAb specific for the conformationally correct G protein may need to be correlated with protection for each specific rabies vaccine
- Manufacturers are encouraged to develop, validate, and implement *in vitro* antigen quantification methods to replace the mouse challenge test. Human rabies vaccines that are non-adjuvanted and monovalent (e.g., U.S. and EU) should be high priorities for the development of *in vitro* potency assays

- Final product *in vitro* methods will require identification and use of appropriate reagents (e.g., mAbs) with specificity for the neutralizing epitope of the virus-associated trimeric form of G protein
- Validation of *in vitro* replacement tests needs to include identification of subpotent lots. Comparison of *in vitro* results to serological titers may also be necessary
- *In vitro* antigen quantification methods currently used by rabies vaccine manufacturers as in-process tests include ELISA and SRID. However, the SRID is not considered adequate to identify the specific trimeric G protein considered necessary for protective immunity
- The following guidelines should be considered to implement *in vitro* methods for *non-adjuvanted* vaccines:
  - Communicate early and often with regulatory authorities
  - Define reagents including reference and secondary standards relevant to in-house virus strains
  - Generate high quality data that:
    - Demonstrate the specificity of mAb for the protective, conformational epitope
    - Demonstrate the ability to discriminate between potent and subpotent lots
    - Compare the ELISA to the mouse challenge test
      - Qualitative rather than statistically-significant correlation is expected
  - Compare data to the SNT in mice or host animal
  - Generate in-process data to demonstrate manufacturing consistency
  - Review data with regulatory authorities to ensure relevance of reagents and appropriate validation
  - Harmonize with international organizations early in the process
- The following guidelines should be considered to implement the use of *in vitro* methods for *adjuvanted* vaccines:

  Manufacturers should consider the steps listed above, as well as:
  - Develop methods to remove adjuvant:
    - Develop data confirming that the removal of adjuvant does not interfere with mAb detection of immunogenic *G protein*
  - Develop methods to test adjuvanted vaccines:
    - Understand the quality and quantity of the G protein antigen that is assembled into the final product
  - Consider the use of adjuvanted reference standards to overcome the problem of non-parallelism between non-adjuvanted reference standard and adjuvanted vaccine.

5. Conclusions

This international workshop reviewed the current state of the science for alternative methods that can reduce, refine, and replace animal use for human and veterinary rabies vaccine potency testing, and developed recommendations to further advance alternative methods and approaches. Participation by academic, industry, and regulatory representatives in both the human and veterinary rabies vaccine fields provided the opportunity to share important insights on the current similarities and differences in human and veterinary rabies vaccine potency testing. The workshop also afforded the opportunity for information exchange and detailed discussion between international experts. Continuing the productive interactions, communication, and cooperation established at the workshop is expected to accelerate implementation of the workshop recommendations and advance the development, validation, and implementation of alternative test methods for rabies vaccine testing.

With regard to animal welfare, workshop participants encouraged immediate actions to further minimize or avoid pain and distress where and while it is still necessary to conduct the rabies virus challenge test. The routine use of anesthetics, post-procedural analgesics, and earlier human endpoints can have an immediate impact on alleviating the significant unrelieved pain and distress experienced by mice in this procedure.

Workshop participants also recognized that the SNT potency test has undergone sufficient preliminary validation and should therefore be considered for product-specific validation by vaccine manufacturers for both adjuvanted and non-adjuvanted vaccines. Implementation of the serologic methods could provide for complete refinement by avoiding the pain and distress associated with the challenge test. The serologic assays could also significantly reduce animal use.

Workshop participants also recognized the successful validation, implementation, and use of a completely *in vitro* rabies potency assay in Japan for over ten years. Accordingly, the workshop recommended that the *in vitro* IC-ELISA should be a high priority for product-specific validation for non-adjuvanted vaccines. Another priority activity to facilitate the implementation of *in vitro* assays for adjuvanted vaccines is the development of methods that remove adjuvant or procedures that ensure that the adjuvant does not interfere with quantification of the immunogenic G protein.

Vaccine manufacturers were encouraged to seek a collaborative, synergistic alliance with regulatory authorities throughout the development and product specific validation of new alternative methods. Finally, regulatory agencies noted the importance for vaccine manufacturers to develop and implement in-process production quality control testing to characterize and assure the consistency of final products.

The workshop highlighted how the application of new science and innovative technologies provides enhanced opportunities for the development, validation, and implementation of alternative methods for potency testing of veterinary and human vaccines. Implementation of the workshop recommendations is expected to advance alternative methods for rabies vaccine potency testing that will benefit animal welfare by refining and reducing animal use, while ensuring safe and effective vaccines for people and animals.

Acknowledgements

The authors extend their sincere appreciation to all participants in the international workshop for their contributions leading to the workshop conclusions and recommendations. The members of the Rabies Workshop Organizing Committee, ICCVAM Interagency Biologics Working Group, and NICEATM staff are acknowledged for their contributions to the planning of the workshop, and all the invited experts are acknowledged for their contributions to breakout group discussions and workshop proceedings. Participating national/international validation organizations from the International Cooperation on Alternative Test Method (ICATM) are also gratefully acknowledged for their scientific contributions and financial support, including NICEATM-ICCVAM, the European Union Reference Laboratory for Alternatives to Animal Testing (EURL ECVAM), Health Canada, and the Japanese Center for the Validation of Alternative Methods (JaCVAM). Finally, the authors gratefully acknowledge Dr. Rajendra Chhabra and Dr. Michael Humble from the National Institute of Environmental Health Sciences, National Institutes of Health, USA for their review of the manuscript.

Appendix A. Supplementary material

Supplementary data related to this article can be found online at http://dx.doi.org/10.1016/j.biologicals.2012.07.005.

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