Three Rs Approaches in the Quality Control of Inactivated Rabies Vaccines

The Report and Recommendations of ECVAM Workshop 48[1,2]

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Preface

This is the report of the forty-eighth of a series of workshops organised by the European Centre for the Validation of Alternative Methods (ECVAM). ECVAM's main goal, as defined in 1993 by its Scientific Advisory Committee, is to promote the scientific and regulatory acceptance of alternative methods which are of importance to the biosciences and which reduce, refine or replace the use of laboratory animals. One of the first priorities set by ECVAM was the implementation of procedures which would enable it to become well-informed about the state-of-the-art of non-animal test development and validation, and the potential for the possible incorporation of alternative tests into regulatory procedures. It was decided that this would be best achieved by the organisation of ECVAM workshops on specific topics, at which small groups of invited experts would review the current status of various types of *in vitro* tests and their potential uses, and make recommendations about the best ways forward (1).

The joint ECVAM/AGAATI (Advisory Group on Alternatives to Animal Testing in Immunobiologicals) workshop on Three Rs Approaches in the Quality Control of Inactivated Rabies Vaccines was held in Langen, Germany, on 19–21 April 2002, under the co-chairmanship of Lukas Bruckner (Institute of Virology and Immunoprophylaxis, Mittelhäusern, Switzerland) and Klaus Cussler (AGAATI, Utrecht, The Netherlands). The participants, all experts in vaccine quality control or rabies disease, came from international regulatory or government organisations, national control laboratories, vaccine manufacturers and academia.

The objectives of the workshop were: a) to review the current status of Three Rs (*replacement*, *reduction*, *refinement*) methods for the quality control of inactivated rabies vaccines; and b) to make proposals on the best way forward.

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[1]ECVAM — The European Centre for the Validation of Alternative Methods. [2]This document represents the agreed report of the participants as individual scientists.

The outcome of the discussions and the recommendations agreed by the workshop participants are summarised in this report.

Introduction

Rabies disease

Rabies is a very old viral disease which occurs all over the world, with the exception of several countries, including Australia, Japan and Hawaii, and a number of Western and Northern European countries, namely Belgium, Cyprus, Denmark, Finland, France, Great Britain, Ireland, Italy, Luxembourg, The Netherlands, Norway, Portugal, the mainland of Spain, Sweden and Switzerland. Since 1990, the number of rabies cases in animals has been reduced by 80% in European countries that have conducted campaigns to orally immunise foxes; however, the occurrence of the disease has increased in several countries in the continents of Africa, Asia and the Americas. Each year, the World Health Organization (WHO) reports at least 50,000 human rabies deaths world-wide. The actual number must be greater, since the disease is under-diagnosed and under-reported in many countries (2). Most deaths occur in countries where rabies is endemic and the delivery of healthcare is poor. It is difficult to obtain precise figures on people vaccinated either pre-exposure or post-exposure, but the WHO estimates that, each year, 10–12 million people receive one or more doses of post-exposure rabies vaccine (3).

Rabies is caused by an RNA virus, which is a member of the *Lyssavirus* genus of the *Rhabdoviridae* family. Under the electron microscope, the virus particles are seen to be bullet-shaped, with spikes on the viral envelope which are composed of a glycoprotein. The glycoprotein is responsible for the induction of virus-neutralising antibodies after vaccination. The core of the virus consists of a tightly wound helix of ribonucleoprotein, which contains structural proteins, RNA transcriptase and non-segmented negative RNA.

All mammals, including humans, are susceptible to rabies. The virus is classically inoculated into the body by a bite that results in the injection of virulent saliva. After a possible local replication, the virus enters the nervous tissue and is passively transported inside the nerve cells to the spinal cord and then to the brain. In the brain, the virus replicates, and is then distributed to other organs, including the salivary glands.

In the central nervous system, replication of the virus will induce clinical signs, depending on the areas that are infected. Some of these signs are quite specific for rabies, such as hydrophobia in humans and bi-tonal barking in dogs, whereas other signs are more typical of nervous disorders than of rabies (for example, convulsions, tetany, and absence of fear of wildlife toward humans). In the acute stage, signs of hyperactivity or paralysis predominate. Once the clinical signs are manifest, the disease leads to death. On post-mortem examination, diagnosis can be established by the detection of antigens in brain specimens (immunofluorescence) or of virulent particles with inoculation tests (on cells or mice). It is also possible to detect RNA in infected tissues, but this is not recommended by the WHO or the Office International des Epizooties (OIE) for routine diagnosis.

Vaccination

Vaccination is the only effective way of controlling rabies. Research began in 1882, when the French scientist, Louis Pasteur, discovered that rabies was transmitted by agents that were too small to be seen under a microscope. He developed techniques to cultivate and attenuate the virus in animals, and eventually developed a vaccine which was able to protect dogs against rabies. He then started to use the vaccine to successfully treat humans bitten by rabid dogs. The principle of this post-exposure treatment is that the protective immunity induced by the vaccine is in place before the virus reaches the brain.

Today, several types of product are available for the active immunisation of mammals. There are inactivated vaccines for use in humans and animals, and live attenuated or genetically modified vaccines for use in baits for wildlife such as foxes. Immunoglobulins and antisera against rabies are also used for rapid, passive immunisation in humans.

Regulatory framework for quality control

The quality control of rabies vaccines is regulated by various guidelines and monographs, for example, on a broad international level by the guidelines of the WHO (4) and the OIE (5), on a European level by the European Pharmacopoeia (*Ph. Eur.*; 6) monographs, and on an American level by the US Code of Federal Regulations (CFR; 7).

The guidelines outline tests to be performed at the different stages of production of the vaccines, which are meant to monitor quality and safety aspects of the vaccine, and, at present, some of these prescribed tests require the use of animals. For example, inactivated rabies vaccines are made from infectious material, and their production is a biological process and therefore is inherently variable. All the processes have to be strictly controlled to ensure a safe, reliably efficacious and consistent vaccine production. Consequently, each batch of vaccine is checked by a panel of quality control tests. Among them are tests for safety, inactivation,

pyrogenicity, and potency, which are legally stipulated and require the use of animals. Table 1 lists the tests performed by five companies which produce veterinary rabies vaccines for the German market.

In practice, a very large number of animals are necessary, especially for potency testing, with at least 120 mice being needed for each batch of vaccine. Table 2 gives an overview of the numbers of animals used for batch potency testing by various manufacturers and control authorities, which were provided by workshop participants.

Current policies on Three Rs alternatives in the regulatory framework

The European Pharmacopoeia

In the introduction to the *Ph. Eur.*, the European Pharmacopoeia Commission makes a clear statement on its commitment to the reduction of animal use, and encourages individuals involved in pharmacopoeial testing to seek alternative procedures. The European Pharmacopoeia Commission adopts an alternative or modified method *once it has been clearly demonstrated that it offers satisfactory control for pharmacopoeial purposes* (8).

Mechanism for reducing in vivo *testing by Official Medicines Control Laboratories (OMCLs)*

Various guidelines and notes for guidance have been written to assist the OMCLs in performing official control authority batch release testing, which is carried out independently and in addition to tests performed by the manufacturer. By agreeing to fixed common schedules of testing for specific types of products, the OMCL network has enhanced the transparency of the system and facilitated mutual confidence between laboratories. The choice of tests performed is based on scientific knowledge of the product and the technical experience of the OMCLs, and an important element of the system requires that it does not become inflexible and prescriptive.

Efforts are ongoing to replace laboratory animals in routine testing. However, the use of *in vivo* tests remains an important tool for evaluating certain products. Nevertheless, in some cases, the re-performance of *in vivo* tests by the OMCL on every production batch may not be justifiable as necessary for official batch release. OMCLs are encouraged to evaluate their testing procedures, and to identify candidates for which *in vivo* testing can be reduced without compromising product quality and safety. An OMCL performing batch release testing might identify a product where the number of *in vivo* tests performed by the OMCL could be reduced. The OMCL would then prepare a dossier giving the background and scientific rationale for this proposal, and should also indicate how it intends to maintain its expertise in the testing procedure in the light of the reduced batch testing scheme. The information provided should clearly show consistency of the product over a significant period of time.

The frequency of testing should be decided on a case-by-case basis, depending on the method of production and the characteristics of the material to be

Table 1: *In vivo* final product testing of rabies vaccines for veterinary use, as performed by different manufacturers producing for Germany

Manufacturer	Abnormal toxicity test	Identity	Safety test in dogs	Inactivation test in mice	Potency: serology in mice	Potency: challenge in mice
A	+	–	+	+[a]	–	+
B	–	+	+ (bulk)	–	–	+ (bulk)
C	–	–	+	+[a]	–	+
D	– (+)[c]	+[b]	+	+[a]	–	+
E	–	–	+	+	–	+

[a] *Final product* and *in-process*.
[b] *Performed on each sub-batch with reference to (37).*
[c] *Only when no safety test in dogs is performed.*

Table 2: Numbers of mice used for the batch potency testing of rabies vaccines

Manufacturer/control authority	Number of animals/year
Rabies vaccines for human use	
US control authority	1500–2000
European control authority	3000–4200
European manufacturer	13,000–19,500
Rabies vaccines for veterinary use	
European control authority	2000
US control authority	1500–3000
US manufacturers	30,000–40,000

tested. It should be sufficiently randomised in order to ensure effective sampling of batches, and should allow for sufficient monitoring of consistency of the product.

The final proposal and support documentation is circulated confidentially within the OMCL batch release network for evaluation. If the proposal is approved by the Member States, the reduced testing scheme can be applied by the applicant OMCL and will be recognised throughout the OMCL batch release network. The application of this procedure remains confidential within the network and may be re-evaluated at any time, given an appropriate stimulus.

The USA

Human rabies vaccine lots are submitted to the US Food and Drug Administration (FDA) for release. The release protocols are reviewed, and decisions for control testing of a submitted batch are based on data submitted in the release protocol. To reduce the number of animal-based tests conducted at the Center for Biologics Evaluation and Review (CBER), the release protocols are evaluated and decisions for testing are based on product characterisation, rather than on a prescribed number of tests. To replace the current animal-based potency test, the manufacturers would have to submit a supplement to the rabies vaccine license, detailing the new test and including a validated alternative test protocol and appropriate data to show the rationale for the new test. The CBER would review the submission and determine whether the new test procedure was an adequate alternative to the existing test.

For veterinary products, the implementation of an *in vitro* test can be accomplished if that test has been shown to correlate directly with efficacy. This usually requires a dose–response study to prove that the *in vitro* test will be able to identify an unsatisfactory product (i.e. one which does not protect vaccinees to an acceptable degree).

The WHO

By 1992, the WHO had already expressed the hope that the potency tests for inactivated rabies vaccines in animals would be replaced with an antigen quantification procedure. However, as consensus has not yet been achieved on suitable methods, the actual requirements are still based on the vaccination-challenge test (4).

The Quality Control of Inactivated Rabies Vaccines: Potency Testing and Three Rs Approaches

Potency testing

The standard method for potency testing of inactivated rabies vaccines is a multiple-dilution vaccination-challenge test in mice, i.e. groups of animals are intraperitoneally (i.p.) immunised with different dilutions of the test vaccine and, after a given period, the mice are intracerebrally (i.c.) infected with rabies virus. The test is evaluated by comparing the number of animals protected from rabies in the groups receiving the vaccine under test and the reference vaccine. In general, 50% of the animals die or show signs of rabies, which involves severe suffering.

The test is generally known as the NIH test, since it was initially developed at the National Institutes of Health (USA; 9). Table 3 shows the numerous variants of the test stipulated for regulatory purposes. All of the test methods have in common: the route of vaccination (i.p.); the route of challenge (i.c.); the challenge virus strain (CVS27); and the dose of the challenge virus used. The most important difference is in the number of vaccinations, i.e. there are two injections at an interval of 7 days in the original NIH test, whereas the *Ph. Eur.* for rabies vaccines for veterinary use stipulates only one injection. The number of animals required per dilution and the number of dilutions also differ in the various requirements, which determine the overall number of animals needed.

In practice, the NIH test or its variants are used by manufacturers and control authorities before the release of each single batch. In addition, the test is used for stability testing by manufacturers and by control authorities according to the WHO requirements (10). In conducting this testing, manufacturers and control authorities are obliged to use large numbers of mice on an ongoing basis (see Table 2).

The NIH test results are highly variable, and differences of up to 400% in the estimated potency by dif-

Table 3: Potency testing of inactivated rabies vaccines: the NIH test and its variants

	Number of tests required	Mice to be used	Number of animals/ dilution	Number of dilutions	Diluent to be used	Number of vaccinations[a]	Interval between vaccinations (days)	Interval between vaccination and challenge (days)	Challenge dose (LD50)[b]	Observation period after challenge (days)	Criteria for evaluation
Ph. Eur. for human use (38)	—	Female ± 4 weeks 11–15g	Suitable[c]	3	n.s.	2	7	7 after second	~50	5–14	Death or signs of rabies
WHO (4)	2	13–16g	≥ 16	5 (≥ 3)	PBS	2	7	14 after first	12 ≤ 25 ≤ 50	> 5–14	Death
Ph. Eur. for veterinary use (33)	—	Female ≥ 4 weeks	≥ 10	≥ 3	n.s.	1	n.a.	14	~50	5–14	Signs of rabies
SAM0308.01 (15)	1 (3, when repeated)	Female CF-1 13–15g	16	5	PBS	2	7 ± 1	14 ± 1 after first	12–50	6–14	Death or signs of rabies
OIE (5)	—	3–4 weeks	≥ 10	n.s.	n.s.	1 or 2	7 if applicable	14 days later	≥ 10	n.s.	n.s.
9CFR 113.209 (7)	2	13–16g	≥ 16	5 (≥ 3)	PBS	2	7	14 after first	12 ≤ 25 ≤ 50	> 5–14	Death
Simplified single-dilution test (12)	—	Female	10	1	—	1	n.a.	14	?	14	≥ 80% survival

[a]*Injection volume: 0.5ml; injection route: intraperitoneal.*
[b]*Challenge virus: CVS27; challenge route: intracerebral; injection volume: 0.03ml.*
[c]*A suitable number of animals should be used to meet the statistical requirements for a valid test.*

n.a. = not applicable; n.s. = not specified; PBS = phosphate-buffered saline.

9CFR = US Code of Federal Regulations, Title 9; NIH = National Institutes of Health; OIE = Office International des Epizooties; Ph. Eur. = *European Pharmacopoeia; SAM = Supplemental Assay Method; WHO = World Health Organization.*

ferent laboratories are considered to be acceptable. For example, *Ph. Eur.* monograph No. 0216 states that, for the evaluation of the potency test, the fiducial limits of error ($p = 0.95$) should not be less than 25% and not more than 400% of the estimated potency.

As an example, the *Ph. Eur.* Biological Reference Preparation (BRP) Batch Number 3 for rabies vaccine (inactivated) for veterinary use (RVIVU) was recently established in a collaborative study (11), which involved an immunogenicity test based on the NIH test, in which mice were vaccinated and the protection obtained was measured by subsequent i.c. challenge with live rabies virus. The potency of the reference preparation was calculated in international units (IU) by comparing it in parallel to the protection evident in mice vaccinated with the 5th International Standard for rabies vaccine. The data showed that potency estimates from individual assays were highly variable, and when validity criteria were strictly applied on individual assays, data from five out of the eight laboratories had to be excluded. The high variability and the difficulty of obtaining valid results in individual tests illustrate the disadvantage of using this *in vivo* test and emphasise that serious effort should be made, by using newly available technologies, to develop a more robust test that would be more reliable and would reduce the number of animals required (11).

The data provided for a 2-year period by the representative of a vaccine manufacturer at the workshop showed a standard deviation of 45% in 24 NIH tests carried out with the reference vaccine, and 115% when 38 NIH tests were carried out.

Conclusion

The current potency tests require a large number of animals and inflict great suffering on them. The tests are time-consuming and pose a risk of infection to the laboratory staff. There is an urgent need for a more reliable test, which uses fewer animals, involves less suffering and provides more-consistent results.

The disadvantages of the NIH test and its variants and, in particular, their high variability and the frequency of invalid results, make it very difficult or even impossible to demonstrate a good correlation between *in vitro* and *in vivo* data. Its use as a "gold standard" for the validation of *in vitro* methods is therefore not recommended.

Possibilities for reduction

Single-dilution test

A simplified form of the multiple-dilution vaccination-challenge test, i.e. a single-dilution test, can be used as a screening test, once experience has been gained in a given laboratory. This test (12) does not give a precise value for the vaccine, but each vaccine that passes this test at least satisfies the minimal requirements for potency. A multiple-dilution test must be applied where a vaccine fails the screening test. This testing strategy can lead to a considerable reduction in the number of animals used.

Single-dilution tests are generally performed in a control session that includes other vaccines tested with "complete" tests. This permits the same references to be used, thus reducing the numbers of animals required.

Since most laboratories have abundant data on the performance of the multiple-dilution test, a review of the historical data should enable the single-dilution test to be introduced on this basis for many rabies vaccines. However, it must be recognised that this testing scheme may not be applicable to all the rabies vaccines on the market.

This strategy has already been implemented in France for veterinary rabies vaccines. During a 3-year period (from 1999 to 2001), 203 batches of veterinary vaccines were tested in 74 control sessions. The single-dilution test was performed on 53 vaccine batches, which saved 1590 mice.

Recommendations

1. National control authorities should follow the French approach, and should investigate whether the single-dilution test could be introduced in their laboratories for the batch potency testing of veterinary rabies vaccines.

2. Whether this testing strategy could also be applied to rabies vaccines for human use should be evaluated.

Number of animals per group

At present, the regulatory requirements differ in the number of animals to be used (Table 3). Some requirements give the exact number to be used, whereas others specify a minimum number or the number is not specified, but it is indicated that the minimum number to meet statistical validity requirements should be used. The present practice is to use three to five groups of equal size, i.e. 10–16 animals per group, for the test and reference vaccines.

Recommendation

3. Depending on the type of assay used and the information already available, the use of equally sized groups is not essential in all cases. Therefore, all available information should be used in an optimised way to minimise the number of animals needed for the selection of doses and/or group sizes. Furthermore, statistical methods such as Bayesian methods or sequential

approaches, which sometimes provide for more-efficient use of the data, should be considered when planning an *in vivo* test.

Verification of the challenge dose

The current requirements stipulate that the challenge dose has to be verified for each assay. It must be high enough to kill 100% of the control animals receiving the working dilution. According to the *Ph. Eur.*, groups of ten mice are inoculated i.c. with serial dilutions of the challenge virus strain.

Recommendations

4. Whenever it is feasible, the potency testing of several vaccine batches should be performed in parallel. Thus, only one test for verification of the challenge dose and the reference vaccine would be needed, and the total number of animals used would be reduced.

5. It should be investigated whether the number of animals per group and the number of dilutions could be reduced.

Frequency of testing

The WHO and FDA require two tests for the potency testing of rabies vaccines for human use. Although the *Ph. Eur.* does not stipulate two tests, many European manufacturers perform the potency test twice, in order to meet the requirements of non-European vaccine-importing countries.

Recommendation

6. Only one potency test should be performed, since a second test does not contribute to the test precision. The WHO and the FDA should modify their requirements accordingly.

Possibilities for refinement

Immunisation and challenge dose

As an alternative to the classical NIH test, a test involving subcutaneous immunisation and intramuscular challenge was proposed by several research groups (13). However, intramuscular challenge was difficult to reproduce in different laboratories. Due to this disadvantage, further investigation of this approach was halted.

The use of anaesthetics

Intracerebral injection is considered to be a very severe and painful procedure, which could be somewhat improved with the use of anaesthetics. Currently, the anaesthetisation of mice prior to i.c. injection is only recommended by the WHO for tests for diagnostic purposes (14), and none of the requirements for the quality control of rabies vaccine stipulates the use of anaesthetics.

Recommendation

7. All regulatory authorities and other relevant organisations should stipulate the use of appropriate anaesthetics (for example, halothane, isoflurane) in their guidelines, in order to reduce the pain and distress caused by i.c. injection.

Intracerebral injection technique

Inappropriate i.c. injection may cause severe damage and death of the mice. This fact is reflected in all of the regulations, which consider death within 4 days of injection to be non-specific. There is some guidance on the injection technique in the WHO Manual (14) and in the US Department of Agriculture (USDA) Supplemental Assay Method (SAM; 15). However, it became evident during the discussion at the workshop that the procedure needs a lot of experience, and that the proper performance may even be restricted to very few persons or even a single person.

Recommendation

8. Scientists and technicians should be trained in appropriate i.c. injection techniques. A Best Practice Guide agreed by the workshop participants is presented in Appendix 1.

Criteria for evaluation of the potency test

As shown in Table 3, the various guidelines and monographs differ in their criteria for test evaluation, and they stipulate death, death or signs of rabies, or signs of rabies, as the endpoint. The *Ph. Eur.* and Title 9 CFR (9CFR) allow the use of non-lethal endpoints in order to reduce the suffering of the mice. The WHO Manual and the USDA SAM mention convulsions and paralysis as clinical signs. The clinical signs of rabies are progressive, and it may take 2–6 days for an animal to die once they have begun. Due to the severe course of rabies infection in mice and the suffering involved, death is not an appropriate endpoint.

In a recent study, it was investigated whether clinical signs, body weight and body temperature could be used as non-lethal endpoints. It was found that clinical signs, such as ruffled fur, shaky movements, trembling, and convulsions (combined with a significant reduction in body weight) form a reliable indicator for the lethal outcome of the rabies infection and could therefore be used as non-lethal

endpoints (16). Appendix 2 summarises the study and gives guidance on the application of non-lethal endpoints (for example, identification of the mice, use of score sheets, and frequency of monitoring).

Recommendations

9. Only non-lethal endpoints should be used as criteria for test evaluation. Clinical signs offer the possibility of terminating the potency test as soon as typical signs of neurological disorder are evident (for example, shaky movements, trembling, and convulsions), without any loss of scientific data, but avoiding a slow progressive death for the animals.

10. Scientific and technical staff should be trained in the application of non-lethal endpoints. The video on non-lethal endpoints for the potency testing of rabies vaccines, which has been produced by the Humane Endpoints — Lethal Parameters (HELP) Group (17), could be used for training purposes (see also, Appendix 2).

Possibilities for replacement

Current status of replacement alternatives

There is an urgent need for replacement of the NIH test and its variants for batch potency testing, stability testing and *in-process* testing. The ideal *in vitro* test should measure the functional glycoprotein which induces the production of rabies virus neutralising antibodies. It should be better than the current *in vivo* test and as good as the currently used in-house *in vitro* methods and, ideally, it should recognise all vaccine strains, whether or not in combination with an adjuvant.

Various alternative methods have been developed and reviewed by Meslin & Kaplan (13) and Weisser & Hechler (18). These methods are either based on the estimation of rabies virus neutralising antibodies in the serum of immunised mice or on the quantification of rabies virus antigen in the vaccine. Table 4 gives an overview on the current status of these methods, and summarises their advantages and disadvantages.

Alternatives based on the quantification of neutralising antibodies (serology)

Various serological tests allow the quantification of rabies virus neutralising antibodies in the serum of immunised animals. The rapid fluorescent focus inhibition test (RFFIT; 19) and the fluorescent antibody virus neutralisation test (FAVN; 20) are the reference methods recommended by the WHO and the OIE. Serially diluted test sera are pre-incubated with a given amount of rabies virus prior to inoculation on a sensitive cell culture, i.e. BHK-21 cells. After incubation, the quantity of unneutralised rabies virus is revealed by immunofluorescence.

According to the *Ph. Eur.*, the RFFIT may be used for inactivated veterinary vaccines after a suitable correlation with the mouse challenge test has been established. However, in practice, no manufacturer uses the serological method, for the following reasons.

a) The serological assay in mice, as described in the monograph, has to be performed 14 days after single immunisation with one-fifth of the recommended dose. This test has never been validated, and the data provided by participants revealed that the method in this form is not suitable. This may be due to the short time period between vaccination and blood sampling. Other approved serological assays (for example, the ToBI test for tetanus vaccine potency, and the ELISA for erysipelas vaccine potency) involve a longer time period (> 21 days).

b) As already mentioned in the introduction to the NIH test and its variants, the test has many disadvantages, which make it difficult to establish a good correlation between *in vivo* data and serological data.

Since these serological methods still require the use of animals, they are not complete replacement alternatives. It should also be considered that the antibodies estimated shortly after vaccination might not correlate directly with protection against rabies. Further disadvantages are the high degree of individual animal variability, the duration of the test (up to 3 weeks), the need to handle infectious rabies virus, and the need for a category III laboratory.

If using serological methods for potency testing, and provided that the safety testing in the target species (see below) will still be required in future, the animals involved could be used to provide blood samples for a serological potency estimation, especially if they were immunised with the recommended vaccination dose. Despite the statistically low number of animals used in this test, the data received from two or three animals of the target species may be more relevant than data from a laboratory animal test which requires far higher animal numbers.

Alternatives based on antigen quantification

The antibody binding test (ABT) was developed at the beginning of the 1970s, and became a WHO pro-

Table 4: Potency testing of inactivated rabies vaccines: summary of alternatives to the NIH test and its variants

	Status	Reference	Advantages	Disadvantages
Antibody quantification				
Rapid fluorescent focus inhibition test (RFFIT)	WHO method OIE method *Ph. Eur*. method	19 5 33		— still use animals — serology (shortly after vaccination) is not directly correlated to protection — high degree of individual animal variability
Fluorescent antibody virus neutralisation test (FAVN)	WHO method OIE method	20 5	— good interlaboratory reproducibility	— require up to 3 weeks — must be done in a category III laboratory
Antigen quantification				
Single radial diffusion	WHO method Accepted in Austria for batch release	23 24	— inexpensive — does not require special equipment	— does not differentiate — does not detect protective epitopes of the vaccines — cannot be used for adjuvanted vaccines — relatively insensitive — requires 3 days
Antibody binding test	WHO method	21		— difficult to validate — 3-day long test — precision is less than ELISA — process cannot be automated
ELISA procedures		39 40 41 42	— fast — inexpensive — highly reproducible — robust — quantitative — precise	— product-specific (not standardised across products) — reagents, by definition, are not universally available

OIE = Office International des Epizooties; Ph. Eur. = *European Pharmacopoeia; WHO = World Health Organization.*

tocol in 1973 (21). Serial dilutions of antigen are mixed with a constant dose of specific antiserum, and the amount of unbound antibody is determined by titration against live virus by using the RFFIT (22), which has several disadvantages, as listed above.

The single radial immunodiffusion (SRD) test was first described by Ferguson *et al*. in 1984 (23). The rabies virus contained in the vaccine is split by means of a detergent, and the concentration of free glycoprotein is then estimated by measurement of diffusion zones in a gel containing antibody specific for the glycoprotein. The SRD test is accepted in Austria for batch release testing of inactivated rabies vaccines for human use (24). The results of a recently conducted collaborative study in South American and Caribbean laboratories showed that the SRD test can be easily standardised and used for in-process control (25). It is rapid, inexpensive and does not require special equipment.

Several types of ELISA procedures have been developed over the past decade (Table 4), and the following variants can be distinguished: antigen capture assays involving either polyclonal or monoclonal antibodies (mAbs), antigen competition assays, and direct ELISA systems. The major advantages of ELISAs are that they are rapid, robust, precise, inexpensive, highly reproducible and quantitative. Depending on the mAb used in the system, it might be possible to differentiate between the vaccine strains. The mAbs which are currently available are described in Table 5.

There are currently several studies in progress on the potential use of these ELISA procedures for the potency testing of rabies vaccines. An ELISA competition assay test is now commercially available, which measures residual vaccine product after incubation with defined antibodies that recognise either the viral glycoprotein or the viral nucleoprotein (unpublished data). A direct ELISA has been developed by binding the test vaccine directly to the surface of the plate in the presence of detergent. The antigen content is measured

Table 5: Monoclonal antibodies used for the antigen quantification of rabies vaccines

Monoclonal antibody	Characteristics	Reference
TW-17	Murine Derived against LEP Flury laboratory strain Specific for the rabies glycoprotein Neutralising activity No cross-reactivity to other reagents in the vaccine	Available from Chiron-Behring (after agreeing to a material transfer agreement), for use in test development
TW-1	Human Derived against LEP Flury laboratory strain Specific for the rabies glycoprotein Neutralising activity The only monoclonal antibody which binds antigen	Available from Chiron-Behring Enssle *et al.* (43)
2-22-C5	Murine Derived against Pitman Moore strain Specific for the rabies glycoprotein, directed against site 2 Neutralising activity Reacts also with Pasteur, Flury and SAD strains	Used in two commercial ELISA kits from EVL and Meddens Diagnostics Bunschoten *et al.* (44)
D1	Murine IgG 1-type monoclonal antibody Specific for the rabies glycoprotein, directed against site 3 Neutralises lyssaviruses belonging to genotype 1 and genotype 6	Lafon (45)

with mAb TW-1 or mAb TW-17 (see Table 5). The direct ELISA method is currently being evaluated in several laboratories. A third test in development is a capture ELISA that utilises a polyclonal antiserum against the rabies glycoprotein. The bound vaccine is then quantified with mAb TW-17. This test is being developed in a collaborative research study. When this study is completed, this test and the necessary reagents will be made available for a defined collaborative study involving more institutes. Other ELISA tests in the course of development involve the use of mAbs as both capture and detecting reagents.

The *Ph. Eur.* monograph on veterinary rabies vaccines permits the replacement of the *in vivo* potency test for batch release testing by a suitable validated alternative. Methods based on antigen quantification are widely used for in-process control by manufacturers and also by OMCLs; for example, the French and Austrian control authorities use them for batch release testing. However, the possibility offered by the *Ph. Eur.* monograph on veterinary rabies vaccines to use the quantification of rabies virus glycoprotein for batch release testing has not yet resulted in variations. All companies (at least those which produce for the German market) only use the animal test (Table 1).

Conclusions

All of the ELISA procedures have promising properties, but none in their current form could be applied universally across vaccines. While a single uniform test that could measure the potency of both human and veterinary vaccines would be ideal, technical limitations may require the development of product-specific or strain-specific assays.

Recommendations

11. A pool of potential tests and reagents are now available. Industry, and control and other laboratories should use it for further collaborative evaluations of alternatives based on antigen quantification.

12. With regard to the validation of antigen quantification-based alternatives to the NIH test and its variants, several important issues should be considered: a) whatever test is selected should measure an antigen that correlates with protection; b) an acceptable assay should be able to distinguish potent versus sub-potent batches; and c) the development of an alternative assay should include a definition of potency and the designation of an international standard based on antigen mass units.

13. As the use of the *in vivo* potency test as a "gold standard" in developing an *in vitro* replacement method is not recommended, criteria for the acceptance of alternative methods should be defined.

The use of national and international standards in potency testing

Potency estimation of a vaccine batch with the NIH test and its variants is always performed in comparison with a reference preparation, which is calibrated in IU. The reference preparation can be an international or national standard, and in Europe, the BRP is used. A recent collaborative study to establish *Ph. Eur.* BRP No. 3 (11), emphasised the high variability of the *in vivo* assays, as described above in the introduction to NIH tests and its variants.

Some evidence indicates that reference preparations calibrated *in vivo* might not be appropriate as references for *in vitro* methods based on antigen quantification, since the manufacturers use antigenically different virus strains for the production of their rabies vaccines (4). For example, the WHO 5th International *In Vivo* Standard was also calibrated for *in vitro* tests, but the IU values obtained were somewhat confusing — the same standard was assigned 16IU and 50IU, depending on the test used for estimation (26).

Conclusion
The development of *in vitro* methods will most probably require the development of new reference preparations.

Recommendations
14. Despite the fact that national and international standards or reference preparations calibrated *in vivo* may not be suitable for potency testing with *in vitro* methods, it is recommended that future collaborative studies for their establishment could be used to evaluate, in parallel, candidate *in vitro* methods, in order to gain information and experience with these methods and to encourage the phasing out of the *in vivo* tests.

15. Standards which were calibrated *in vivo* should not be used as reference preparations in *in vitro* tests. Specific standards should be calibrated for this purpose, once an *in vitro* method has been established.

The Quality Control of Inactivated Rabies Vaccines: Safety Testing and Three Rs Approaches

Possibilities for deletion

The abnormal toxicity test/general safety test

The purpose of the abnormal toxicity test (ATT) of the *Ph. Eur.* (called the general safety test in the CFR, or the innocuity test in the WHO guidelines) is to detect any toxicity which is not related to the product. A controversial discussion about the usefulness of this test went on for many years (27, 28). As a consequence of a detailed study of the Paul Ehrlich Institute (Langen, Germany) on the relevance of this test (29), the *Ph. Eur.* has abolished the ATT as a routine batch control test (28).

The WHO still requires the test, but the WHO Expert Committee on Biological Standardisation recently recommended the initiation of an international enquiry to establish the usefulness of the ATT (30). The CFR still requires a general safety test or an ATT for all batches of human rabies vaccine submitted for release.

For veterinary vaccines, the use of the ATT has been abolished in Europe. However, elimination of the test from routine quality control is obviously a very slow process. Even 5 years after the decision to delete the test came into force, some companies still perform the test (see Table 1). The 9CFR 113.209 for veterinary rabies vaccines also does not require an ATT.

Recommendations
16. In the interests of international harmonisation, the deletion of the ATT/general safety test should be considered.

17. National control authorities, being responsible for batch release testing, should demand that companies which are still performing the ATT should cease to do so.

Possibilities for reduction and refinement

The target animal safety test (TAST) for vaccines for veterinary use

Both the *Ph. Eur.* and the 9CFR 113.209 require safety testing in target animals; however, the tests differ in detail (Table 6). The OIE does not mention a TAST for inactivated vaccines (5). According to the 9CFR requirements, three animals of the most susceptible species have to be injected with one recommended dose, if the vaccine is intended for use in more than one species. According to the *Ph. Eur.* monograph on inactivated rabies vaccines for veterinary use, the test is usually carried out in two dogs, which are injected with twice the vaccinating dose. A further difference is evident in the observation period — the *Ph. Eur.* monograph stipulates 14 days, whereas the 9CFR stipulates 28 days.

At the workshop, there was no agreement as to whether this test could lead to added value in terms of the safety of vaccine batches. The statistical relevance of the test was questioned, since experiments performed with two animals do not provide statistically sound data. Table 7 shows that, even when no

Table 6: Target animal safety test for rabies vaccines for veterinary use

	Ph. Eur.	**9CFR**
Animal Species	Target species; if carnivores included, use dogs	Most susceptible species
Animal number	2	3
Age of animals	Not specified	"Young"
Route of application	Route stated on the label	Intramuscular
Dose	Twice the dose	One recommended dose
Observation period	14 days	28 days
Evaluation criteria	No abnormal local or systemic reactions	No unfavourable reaction

9CFR = US Code of Federal Regulations, Title 9; Ph. Eur. = *European Pharmacopoeia.*

local or systemic reactions occur in a sample of two animals, it can only be concluded with 95% confidence that the true probability of no reaction is < 77.6%. For a sample size of n = 3 animals, it can only be concluded with 95% confidence that the true probability of no reaction is < 63.2%. Some of the participants felt that the test should be deleted, whereas others wanted to keep it in the monograph.

There are obviously major differences in the test conditions applied by the various manufacturers — some use commercial breeding colonies outside their facilities under well-controlled field conditions. Those animals are considered as laboratory animals only during this safety test, and are available for their original purpose after the test, which can be regarded as a part of the ordinary vaccination programme. Other manufacturers perform the test under closed laboratory Good Laboratory Practice conditions with dogs (mainly beagles), which are specially bred for experimental purposes.

Table 7: Target animal safety test: confidence intervals and sample size

n animals with reaction	**%**	**95% confidence interval**
n = 2 animals according to *Ph. Eur.*		
0	0	0.0–77.6
1	50	1.3–98.7
2	100	22.4–100.0
n = 3 animals according to 9CFR		
0	0	0.0–63.2
1	33.3	0.8–90.6
2	66.6	9.4–99.2
3	100	36.8–100.0

9CFR = US Code of Federal Regulations, Title 9; Ph. Eur. = *European Pharmacopoeia.*

The *Ph. Eur.* monograph on veterinary vaccines is currently being revised, and the draft proposal published in *Pharmeuropa* (31) stipulates that the target animal safety test should be carried out on ten consecutive batches and can then be discontinued, subject to the agreement of the Competent Authority, unless there is a change in the production conditions. Furthermore, a new chapter will be included in the *Ph. Eur.*, describing the application of the batch safety test in more detail (32).

Recommendations

18. If the TAST is to be maintained, it should be carried out as part of ordinary vaccination programmes in commercial dog breeding colonies, as is already the practice for several vaccine manufacturers.

19. Also, if the TAST is to be maintained, the test performance should be harmonised between the USA and Europe. A test using two animals and the recommended dose could provide a reduction and refinement alternative, and could also be used for potency testing, if serum antibodies were measured at the end of the observation period, with one of the serological methods given above.

Possibilities for replacement

Residual live virus testing (confirmation of inactivation)

This test is designed to detect non-inactivated rabies virus, and is required for rabies vaccines for human and veterinary use (Table 8). For human

vaccines, the WHO requires that the test on the finished product is carried out in mice, whereas the *Ph. Eur.* stipulates an *in vitro* test using cell cultures, and the CBER does not directly stipulate how the test should be conducted; for example, there is no requirement for the use of animals for this test. However, one US rabies vaccine manufacturer still uses animals for this test. For veterinary products, the USDA requirements prescribe that 20 mice and two rabbits should be injected intracerebrally with 0.25ml of the product and observed each day for 21 days. If any animals die between day 4 and day 21, material from each brain is recovered and injected into each of five mice. The OIE allows the use of a cell culture method or the test in mice. With regard to the *Ph. Eur.*, the monograph for veterinary rabies vaccines still includes the *in vivo* test in mice for the finished product, but, according to the general monograph on veterinary vaccines, the manufacturer should only perform the cell culture test on the bulk material and should omit the test in mice. However, in practice, all manufacturers still use the animal test (see Table 1), and some of the manufacturers at the workshop reported that some control authorities even ask them to carry out the test in mice on the finished product, although this is in contradiction to the general *Ph. Eur.* monograph, *Rabies Vaccine (Inactivated) for Veterinary Use* (33).

The volume injected into rabbits or mice is relatively small. The use of the cell culture method allows the testing of a much higher number of equivalent doses, and the *in vitro* test is more sensitive than the *in vivo* test. For example, the results of a study carried out by Blum *et al.* (34) demonstrate that the fluorescent antibody technique is at least as sensitive as the mouse test. The authors recommend that the test should be carried out before the addition of adjuvants and preservative.

Recommendations

20. 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.

21. The *Ph. Eur.* should clearly state that the manufacturers do not have to carry out the test in mice for residual live virus testing of the finished product.

Pyrogenicity testing

The *Ph. Eur.*, the WHO, and the FDA require that inactivated rabies vaccines for human use are tested for pyrogens with the *Limulus* amoebocyte lysate (LAL) test and the classical pyrogenicity test in rabbits, whereby the LAL test measures the endotoxin levels and the rabbit test measures non-endotoxin pyrogens.

It is questionable whether the current test in rabbits mimics the situation in humans. One concern is the route of administration: the animals are intravenously injected over a period of 3 minutes with a single vaccine dose diluted to a total volume of 10ml, which corresponds to a ten-fold dilution of the vaccine. However, the vaccine is administered intramuscularly into humans.

A number of *in vitro* methods, which are based on the human fever reaction, have been developed for the detection of pyrogens and are reviewed in ECVAM workshop report 43 (35). Six methods are currently being validated within the framework of a Shared Cost Action project funded by DG Research of the European Commission (36).

Another approach could be the direct measurement of cytokines in the vaccines, by using commercially available ELISA kits.

Recommendations

22. It should be further investigated whether the *in vitro* methods based on the human fever reaction, or commercially available kits for

Table 8: Tests stipulated for residual live virus testing of inactivated rabies vaccines

	Method	Animals
Vaccines for human use		
Ph. Eur.	Cell culture test	—
WHO	Cell culture test on bulk *and* final product test in mice	Mice
FDA	Cell culture test	—
Vaccines for veterinary use		
Ph. Eur.	Manufacturers test bulk in cell culture; control laboratories use mouse test	10 mice
USDA	Mouse/rabbit inoculation	20 mice and 2 rabbits

FDA = US Food and Drug Administration; Ph. Eur. = *European Pharmacopoeia; USDA = US Department of Agriculture; WHO = World Health Organization.*

cytokine determination, could replace the pyrogenicity test in rabbits.

23. If the pyrogen test in rabbits is maintained, the LAL test (which needs animals to produce the reagents) should be deleted.

Animal tests on virus seed lots

The current regulations in the *Ph. Eur.* for virus seed lot production for human vaccine manufacture require three different animal tests for extraneous agents. The test is conducted by neutralising live vaccine virus with antibodies, then using the mix to inoculate adult mice, suckling mice and guinea-pigs. The animals are observed for 21 days, 14 days and 42 days, respectively. The mice are observed for survival. Guinea-pigs are observed for survival, and are also analysed both microscopically and culturally for evidence of infection. This secondary analysis is performed on guinea-pigs, both for animals which die during the test period and for those which survive the observation period. In contrast, virus seed lots for veterinary vaccines are tested *in vitro* by using a cell culture system. This tissue culture system should be applicable to use for the seed lot testing of virus used to manufacture human vaccines.

Recommendation

24. According to the *Ph. Eur.* monograph, animal tests are used for the extraneous agents testing of seed lots for rabies vaccines for human use, whereas *in vitro* methods are used for veterinary vaccines. There should be an assessment of whether these *in vitro* methods could be applied to human vaccine virus strains.

In fact, the WHO has already removed the *in vivo* test from its vaccine production guidelines and allows the use of cell cultures.

List of Recommendations

Potency testing: reduction

Single-dilution test

1. National control authorities should follow the French approach, and should evaluate whether the single-dilution test could be introduced in their laboratories for the batch potency testing of veterinary rabies vaccines.

2. Whether this testing strategy could also be applied to rabies vaccines for human use should be evaluated.

Number of animals per group

3. Depending on the type of assay used and the information already available, the use of equally sized groups is not essential in all cases. Therefore, all available information should be used in an optimised way to minimise the number of animals needed for the selection of doses and/or group sizes. Furthermore, statistical methods such as Bayesian methods or sequential approaches, which sometimes provide for more-efficient use of the data, should be considered when planning an *in vivo* test.

Verification of the challenge dose

4. Whenever it is feasible, the potency testing of several vaccine batches should be performed in parallel. Thus, only one test for verification of the challenge dose and the reference vaccine would be needed, and the total number of animals used would be reduced.

5. It should be investigated whether the number of animals per group and the number of dilutions could be reduced.

Frequency of testing

6. Only one potency test should be performed, since a second test does not contribute to the test precision. The WHO and the FDA should modify their requirements accordingly.

Potency testing: refinement

The use of anaesthetics

7. All regulatory authorities and other relevant organisations should stipulate the use of appropriate anaesthetics (for example, halothane, isoflurane) in their guidelines, in order to reduce the pain and distress caused by i.c. injection.

Intracerebral injection technique

8. Scientists and technicians should be trained in appropriate i.c. injection techniques. A Best Practice Guide agreed by the workshop participants is presented in Appendix 1.

Criteria for evaluation of the potency test

9. Only non-lethal endpoints should be used as criteria for test evaluation. Clinical signs offer

the possibility of terminating the potency test as soon as typical signs of neurological disorder are evident (for example, shaky movements, trembling, and convulsions), without any loss of scientific data, but avoiding a slow progressive death for the animals.

10. Scientific and technical staff should be trained in the application of non-lethal endpoints. The video on non-lethal endpoints for the potency testing of rabies vaccines, which has been produced by the Humane Endpoints — Lethal Parameters (HELP) Group (17), could be used for training purposes (see also, Appendix 2).

Potency testing: replacement

Alternatives based on antigen quantification

11. A pool of potential tests and reagents are now available. Industry, and control and other laboratories should use it for further collaborative evaluations of alternatives based on antigen quantification.

12. With regard to the validation of antigen quantification-based alternatives to the NIH test and its variants, several important issues should be considered: a) whatever test is selected should measure an antigen that correlates with protection; b) an acceptable assay should be able to distinguish potent versus sub-potent batches; and c) the development of an alternative assay should include a definition of potency and the designation of an international standard based on antigen mass units.

13. As the use of the *in vivo* potency test as a "gold standard" in developing an *in vitro* replacement method is not recommended, criteria for the acceptance of alternative methods should be defined.

The use of national and international standards in potency testing

14. Despite the fact that national and international standards or reference preparations calibrated *in vivo* may not be suitable for potency testing with *in vitro* methods, it is recommended that future collaborative studies for their establishment could be used to evaluate, in parallel, candidate *in vitro* methods, in order to gain information and experience with these methods and to encourage the phasing out of the *in vivo* tests.

15. Standards which were calibrated *in vivo* should not be used as reference preparations in *in vitro* tests. Specific standards should be calibrated for this purpose, once an *in vitro* method has been established.

Safety testing: deletion

The abnormal toxicity test/general safety test

16. In the interests of international harmonisation, the deletion of the ATT/general safety test should be considered.

17. National control authorities, being responsible for batch release testing, should demand that companies which are still performing the ATT should cease to do so.

Safety testing: reduction and refinement

The target animal safety test (TAST) for vaccines for veterinary use

18. If the TAST is to be maintained, it should be carried out as part of ordinary vaccination programmes in commercial dog breeding colonies, as is already the practice for several vaccine manufacturers.

19. Also, if the TAST is to be maintained, the test performance should be harmonised between the USA and Europe. A test using two animals and the recommended dose could provide a reduction and refinement alternative, and could also be used for potency testing, if serum antibodies were measured at the end of the observation period, with one of the serological methods given above.

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21. The *Ph. Eur.* should clearly state that the manufacturers do not have to carry out the test in mice for residual live virus testing of the finished product.

Pyrogenicity testing

22. It should be further investigated whether the *in vitro* methods based on the human fever reaction, or commercially available kits for cytokine determination, could replace the pyrogenicity test in rabbits.

23. If the pyrogen test in rabbits is maintained, the LAL test (which needs animals to produce the reagents) should be deleted.

Animal tests on virus seed lots

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Appendix 1

Best Practice Guide for the Batch Potency Testing of Inactivated Rabies Vaccine Using Mice

Introduction

ECVAM workshop report 48 includes a number of recommendations on the application of the Three Rs in the quality control of inactivated rabies vaccines (1). With regard to the possibilities of reduction, two important aspects, which significantly reduce the overall number of animals, are repeated here: the grouping of controls, which leads to reduction when the same control groups are used for back titration of the challenge virus and for the reference vaccine; and the introduction of a single-dilution test, provided that it has been validated in the laboratory (2).

The current methods used for the potency testing of inactivated rabies vaccines for human and veterinary use are based on the intracerebral (i.c.) challenge of mice that have previously been vaccinated intraperitoneally. This guideline is not meant as a complete protocol for rabies vaccine potency testing, but focuses on techniques which should be applied in order to refine the animal procedure, thus minimising the suffering of the animals.

The following preconditions should be met:

— quality control of rabies vaccines should be a routine procedure in the laboratory, including the follow-up of the challenge virus titre, the potency of the reference vaccine and the injected dose of virus with control charts;

— the staff should be experienced in the proper handling of mice and should be regularly trained; and at least two persons experienced in the animal procedure should be working in the laboratory;

— the animal housing facilities should fulfil the legal requirements;

— animals entering the test should be of known origin and in good condition.

I.c. injection into mice is stipulated also for other pharmacopoeial tests (for example, the pertussis vaccine potency test [3] or extraneous agents testing [4, 5]), and is used for diagnostic purposes (for example, for the diagnosis of rabies [6]).

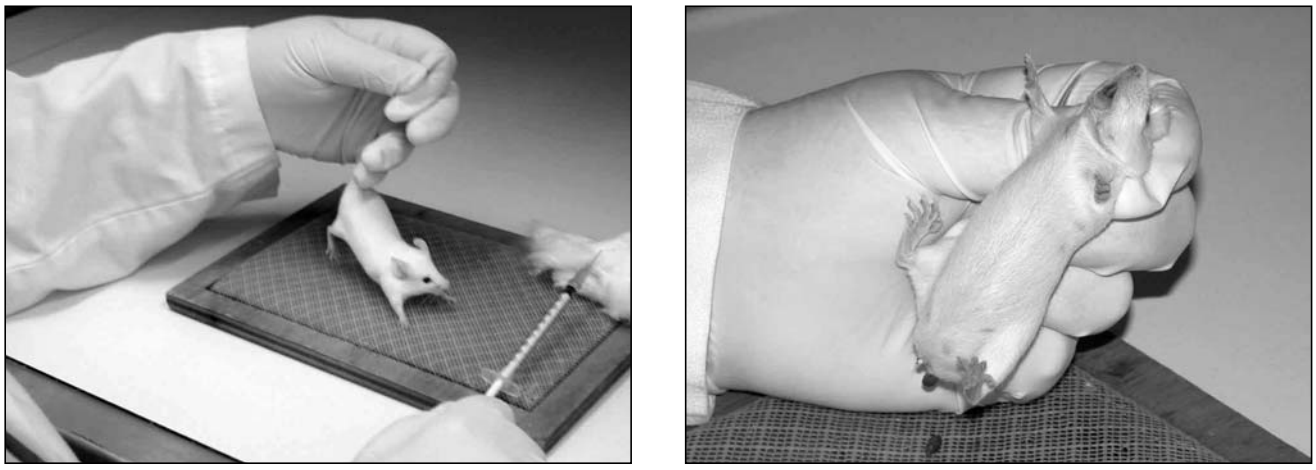
In the following paragraphs, step-by-step guidance is given for the necessary animal procedures, taking into account all possibilities for refinement of the techniques applied.

Vaccination of Mice

Groups of animals are vaccinated with different dilutions of the test vaccine. Each group is kept in a box or in several boxes, depending on the number of animals required. The animals in a group should be individually marked for identification; small spots of picric acid, applied at different sites of the body, are suitable for this purpose. Vaccination is performed by intraperitoneal injection of the vaccine, using a 25ga × 5/8 needle, as illustrated in Figures 1 and 2. This procedure is

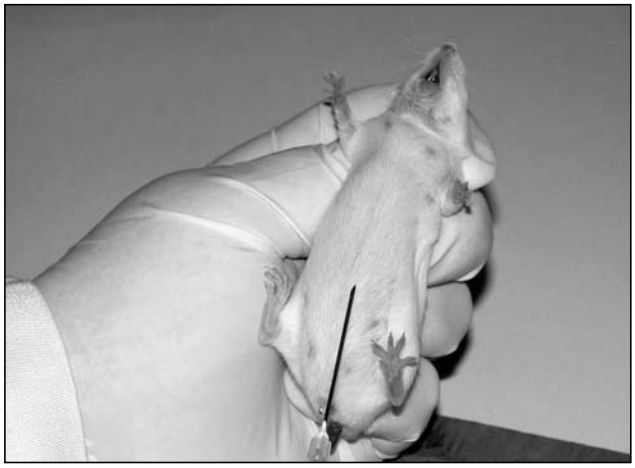
Figure 1: Catching and immobilisation of the mouse

a) b)



a) Catching; b) immobilisation.

Figure 2: Intraperitoneal vaccination



not painful, and anaesthesia of the mice is therefore not necessary.

I.c. Challenge with Virulent Rabies Virus

The i.c. challenge should be carried out only on anaesthetised animals, since it involves considerable pain and might induce shock. The technique applied should permit the anaesthetisation of the animals in their boxes and should avoid the risk of mixing animals between groups; moreover, it should be rapid and safe.

Anaesthetisation technique

Gaseous anaesthesia (for example, with isoflurane, or halothane) is routinely used, as it is necessary to have a rapid induction with a recovery time of 10–20 minutes. Premedication with an analgesic should not be performed, since the effects on the outcome of the test are not known.

Furthermore, it should be possible to anaesthetise mice in their boxes. For example, a gaseous system with a home-made box in which several cages can be installed, could be used. Among the products available, halothane has a "wake-up time" of more than 10 minutes, which allows the removal of the anaesthetisation device from the box, and the inoculation of all the mice. A trained person will need less than 2 minutes to inoculate ten mice.

I.c. injection procedure

In order to reduce the risk of contamination for the person carrying out the inoculation, the mouse should be immobilised in the abdominal position, as shown in Figures 3 and 4. The syringe (World Health Organization: 0.25–0.5ml tuberculin syringe; 7) should be held vertically, and the needle should point toward the plate and not toward the fingers of the person carrying out the inoculation. A 27ga (8) needle, which is either sheathed or very short, as shown in Figure 5, should be used to permit penetration of not more than 5mm. Modifications concerning the position for the i.c. injection were described by Koprowski (9).

Humane endpoints

The i.c. injection of even small volumes is likely to cause brain damage, even if it is performed properly. The two European Pharmacopoeia monographs on rabies vaccines for human and veterinary use (10, 11) consider death occurring before day 5 after challenge to be non-specific and not attributable to rabies infection. Therefore, it is necessary to

Figure 3: Injection site

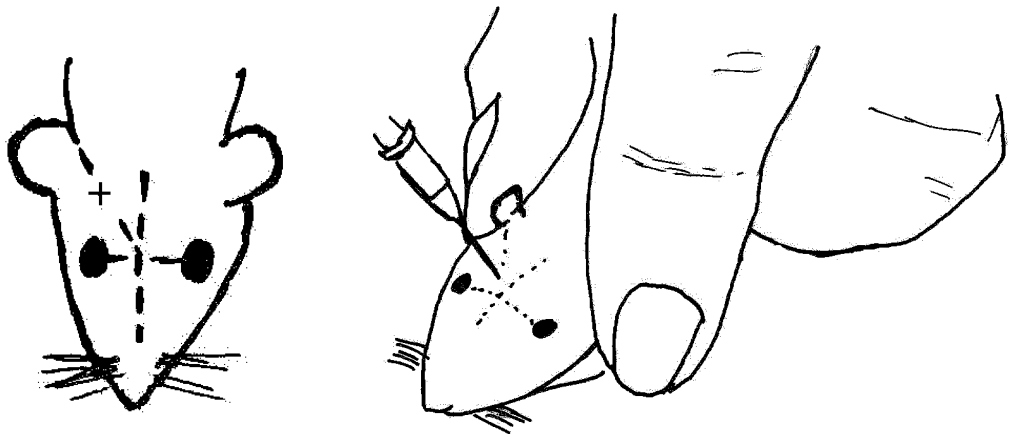


Figure 4: Immobilisation of the mouse in the abdominal position, and intracerebral inoculation

a)

b)

a) Immobilisation; b) intracerebral inoculation.

carefully observe the animals after recovery from anaesthesia. Adverse reactions can range from sudden death to mild ataxia or circling to the traumatised side. Animals showing neurological signs during the first days after infection should be excluded from the experiment and humanely killed.

Figure 5: Needles for intracerebral inoculation

The frequency of inoculation-induced adverse reactions should be carefully monitored. The use of score sheets is recommended for the documentation of clinical signs.

As has been underlined in the conclusions and recommendations of the ECVAM workshop report (1), it is possible to use humane endpoints and euthanise the mice as soon as they exhibit clinical signs of rabies, without reducing the sensitivity of the potency test (see 12). However, an exception should be made: in order to maintain the virulence of the challenge virus strain, it is passaged in mice. In this case, the animals should only be humanely killed when they are paralysed; thus, a higher virus titre can be achieved, and fewer animals will be used for the preparation of a challenge virus strain batch.

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Appendix 2

The Use of Humane Endpoints in the Quality Control of Rabies Vaccines

Introduction

Rabies vaccines for human and veterinary use are important medicinal products for preventing this fatal disease. Animal tests are the basic experiments for demonstrating the efficacy and potency of rabies vaccines. Since laboratory mice are highly susceptible to rabies infection, a virulent challenge (the so-called NIH test) is used to demonstrate the protective effect of a vaccine on a batch-to-batch basis (1).

While considerable effort has been made to replace this test, and several promising *in vitro* tests are currently being evaluated, all the regulatory requirements still include the challenge test. Rabies challenge procedures are prescribed with precise protocols for vaccine potency testing in monographs of the European Pharmacopoeia (*Ph. Eur.*), the US Code of Federal Regulations, and guidelines of the World Health Organization and the Office International des Epizooties (2–6). As it is necessary to calculate the potency in international units by means of the classical multiple-dilution assay, more than 100 animals are required to test each batch of these vaccines. The assays have to be performed in such a way that more than half of the animals succumb to infection, to ensure fulfilment of the evaluation criteria.

For animal welfare reasons, such animal tests are of great concern. Due to the length of time that is necessary for the introduction and validation of alternative techniques, progress concerning the change of testing requirements has so far been limited. Therefore, it is likely that the challenge test will continue to be used to estimate the potency of rabies vaccines for the foreseeable future, for both human and veterinary applications. The large number of mice required, together with the severity of the procedure, emphasise the relevance of the rabies vaccine challenge test to the Three Rs and to animal welfare concerns. To improve the situation on behalf of the animals, much more emphasis should be placed on the refinement of vaccine testing procedures.

In general, legislation controlling animal research requires scientists to select procedures that cause the least suffering for the animals used. One possibility for refinement is through reducing the duration of the animal tests, in order to shorten the period of suffering. For example, the humane killing of animals should be permitted when typical clinical signs of infection occur. Legal requirements (for example, the *Council of Europe Convention ETS* 123 [7] and *Directive 86/609/EEC* [8]) state that, in a choice between procedures, those methods should be selected which cause the least suffering and which are most likely to provide satisfactory results. This implies the use of humane endpoints. The monographs of the *Ph. Eur.* usually do not specify such criteria; however, a paragraph which requires the use of humane endpoints in general has recently been included in the general monographs, *Vaccines for Human Use* and *Vaccines for Veterinary Use* (9).

The possibility of using humane endpoints largely relies on the availability of typical clinical signs and other relevant parameters such as variations in body weight and body temperature. Score sheets specifically designed for the documentation of all relevant information concerning the disease under study are necessary for all these infection models (10).

A refined test that will cause less animal suffering, and which can be considered to be sufficiently well validated to replace the traditional lethal assay with mice, is described. It is proposed that clinical signs of rabies can be used as an endpoint, instead of death. Specific behaviour and loss of body weight are suitable signs, which can be used as reliable surrogate and humane endpoints to terminate an experiment at a stage much earlier than death.

Rabies Vaccine Potency Test

Routine potency tests were used to perform these studies, to avoid the use of additional animals, and were carried out in accordance with the *Ph. Eur.* monograph for *Rabies Vaccine for Human Use Prepared in Cell Cultures* (2). Animals were vaccinated intraperitoneally with several vaccine dilutions, and challenged two weeks later with an intracerebral (i.c.) injection of rabies virus under anaesthesia. The animals were then allowed to recover from the anaesthetic and were monitored carefully over the next 14 days.

Score Sheets

Score sheets were developed for the challenge procedures (11). They included data on clinical signs, body weight and body temperature (Table 1). During the experiment, the animals were observed twice daily, between 8 a.m. and 9 a.m., and between 3 p.m. and 4 p.m. In the morning, the body temperature was recorded and the animals were weighed immediately afterwards.

Table 1: Example of the use of a score sheet in the rabies vaccine potency test

	Days after infection													
	–1	**0**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12[a]**
Body weight (g)	22.2	22.3	21.6	21.9	22.1	22.3	21.8	21.2	18.5	16.9	15.4	14.2	13.9	
Temperature (°C)	38.1	38.4	37.5	37.8	37.9	37.8	37.9	37.7	37.1	36.5	35.7	34.0	33.7	
Clinical signs (score)	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**1**	**2**	**3**	**4**	**4**	**5**	
— ruffled fur								×	×	×	×	×	×	
— hunched back								×	×	×	×	×	×	
— slow movements									×					
— circular movements									×	×				
— trembling										×	×			
— shaky movements											×			
— convulsions										×	×	×		
— paresis											×	×		
— paralysis												×		
— prostration														
— agony, coma													×	

[a]*The score sheet shows the parameters for an individual mouse during the course of the potency test, beginning 1 day prior to infection, and ending on day 12 with the death of the mouse.*

Clinical Signs

The first signs of the disease do not usually appear before day 4 after challenge. Before that time, any clinical sign is most likely to have been caused by the i.c. injection procedure, so animals showing such early signs are normally excluded from the experiment. The clinical signs of rabies are progressive, and it may take between 2 and 6 days for an animal to die.

The following five stages can be recognised in mice infected with rabies virus, and which therefore have *not* been protected by the vaccine.

Stage 1: ruffled fur, hunched back (Figure 1)
Ruffled fur (sometimes referred to as a starey coat or harsh coat) indicates the first signs of clinical disease. This is a rather general sign of illness in mice and can be observed in many other diseases. The same is true for hunched back. Therefore, these clinical signs in Stage 1 are not specific indicators for rabies, but do reflect that the animal is unwell, and that its welfare is compromised.

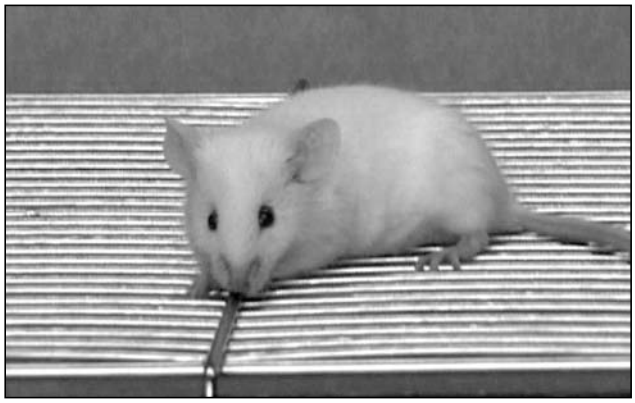
Stage 2: slow movements, circular movements (Figure 2)
During Stage 2, animals lose their alertness. They walk more slowly than usual, and if observed carefully for a while, they sometimes show circling movements, mainly in one direction. These are the first clinical indicators of neurological disorder.

Figure 1: Stage 1 — ruffled fur, hunched back

Stage 3: shaky movements, trembling, convulsions (Figure 3)
In Stage 3, the neurological signs become increasingly obvious, with trembling and shaky movements, and convulsions appear which can often be provoked. By this time, there is a marked loss of body weight. This stage, with severe and unequivocal clinical signs, clearly indicates rabies infection.

Stage 4: signs of paralysis (Figure 4)
Lameness and paresis, usually of the hind legs, are also clear indicators of progressive infection

Figure 2: Stage 2 — slow movements, circular movements



with rabies, and are soon followed by complete paralysis. The animals become clearly dehydrated.

Stage 5: moribund animals
In Stage 5, animals become moribund. They can be seen to be prostrate and recumbent, and they obviously do not feed or drink; yet they may still survive for 1 or 2 days.

Overall, rabies infection in mice is characterised by a slow onset of the disease, usually beginning between day 4 and day 6 after infection. In contrast to most other mammals with rabies, mice do not show any signs of aggression. The study showed that all mice which had developed Stage 3 signs did not recover and died a few days later. Clinical signs therefore offer the possibility of terminating the experiment as soon as typical signs of neurological disorder are evident (Stage 3 at the latest), without any loss of scientific data, but avoiding a slow progressive death for the animals.

Figure 3: Stage 3 — shaky movements, trembling, convulsions

Figure 4: Stage 4 — signs of paralysis

Body Weight

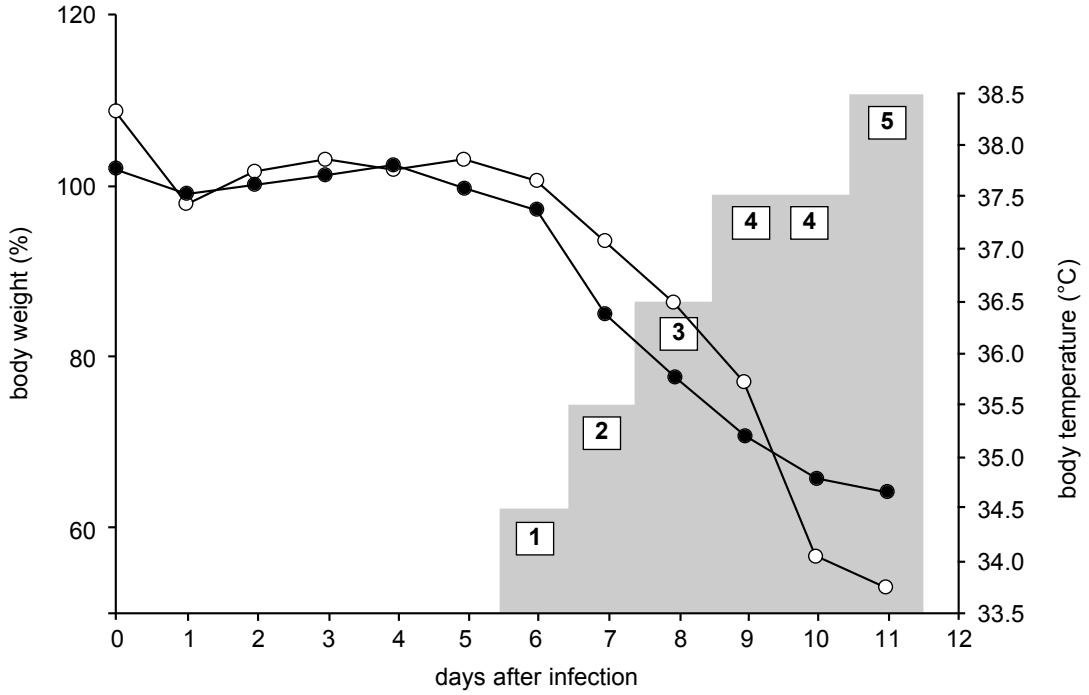
Body weight was measured with electronic scales and recorded on the score sheet. Since the anaesthetic and i.c. route of infection had a negative effect on body weight for 1 day, the weight on day 2 after the infection was used as the basis for body weight loss.

Interestingly, the measurement of body weight revealed a very early decrease, even before typical clinical signs of the disease were obvious. A significant loss of body weight (> 20%) proved to be the earliest pre-lethal endpoint for rabies in laboratory mice. Figure 5 shows that body weight loss was noted, even before other clinical signs were evident. Weight loss progressed continuously and could reach 30–50% shortly before death.

Body Temperature

To measure body temperature, temperature-sensitive transponders (incorporating a unique identifier) were implanted under the skin of the necks of the mice, and temperatures were measured twice daily. Interestingly, hyperthermia, an early sign of infection in other mammals, especially humans, never developed in the mice; hypothermia always became marked in the final stage of the disease. However, this was much too late to be considered a suitable endpoint, and other markers were seen earlier and were more reliable.

Figure 5: Body weight, temperature and development of clinical signs in a mouse during rabies infection



Body weight was normalised to day 2 after infection.

● = *body weight;* ○ = *body temperature;* ▭ = *score of clinical signs.*

Conclusions

The appearance of typical clinical signs (Stage 3), alone or Stage 2, in combination with a decrease in body weight, are suitable humane endpoints. The relatively slow progression of the disease, with an increase in severity of the clinical signs, makes the use of score sheets for rabies easy. If a decrease of body weight of more than 15% and clinical signs of neuronal dysfunction, as in Stages 2 or 3, occur in combination, a point of no return has been reached. Consideration of these two combined endpoints allows the experiment to be terminated at an even earlier phase. This reduces the duration of animal suffering by an average of 2–3 days. Figure 5 gives a typical example of the development of all three parameters.

Only a suitable balance and score sheets have to be used to identify an early humane endpoint in rabies challenge tests. The clinical signs of rabies are easy to observe, and staff can readily be trained to recognise the early stages of the disease.

A combination of clinical signs and recording of body weight are suitable humane endpoints for the rabies challenge test and would lead to a considerable reduction of animal suffering. Hopefully, many institutions and companies will rely on the use of humane endpoints instead of lethality. A video is available, which might help to change the current situation and reduce the causation of unnecessary animal suffering (12).

Acknowledgement

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