CHAPTER 2.1.13.

RABIES

SUMMARY

Rabies is a major zoonosis for which diagnostic techniques have been standardised inter-nationally. As there are neither gross pathognomonic lesions nor specific and constant clinical signs for rabies, accurate diagnosis can only be made in the laboratory. Laboratory techniques are preferably undertaken on central nervous system (CNS) tissue removed from the cranium (specifically, brain stem, Ammon's horn, thalamus, cerebral cortex and medulla oblongata). A composite of CNS samples should be tested and the brain stem is the most important component of the sample.

Identification of the agent: *Agent identification is preferably undertaken using the fluorescent antibody test (FAT). A drop of purified immunoglobulin previously conjugated with fluorescein isothiocyanate (FITC) is added onto an acetone-fixed brain tissue smear, preferably made from several parts of the central nervous system. FAT provides a reliable diagnosis in 98–100% of cases for all rabies virus strains if a potent conjugate is used. For a large number of samples, as in an epidemiological survey, the polymerase chain reaction (PCR) can provide rapid results in specially equipped laboratories.*

Infected neuronal cells have been demonstrated by histological tests and these procedures will reveal aggregates of viral material ('Negri bodies') in the cytoplasm of neurones. However, histological techniques are much less sensitive than immunological methods, especially in the case of autolysed specimens. Consequently, histological techniques can no longer be recommended for primary diagnosis.

In cases of inconclusive results from FAT, or in all cases of human exposure, further tests (cell culture or mouse inoculation tests) on the same sample or repeat FAT on other samples are recommended. A monolayer culture of susceptible cells is inoculated with a pool of several CNS tissues, including the brain stem. FAT undertaken after appropriate incubation will demonstrate the presence or absence of viral antigen. Alternatively, newborn or 3–4-week-old mice may be inoculated intracerebrally with a similar pool of tissues and then kept under observation for 28-days. For any mouse that dies between 5 and 28-days post-inoculation, the cause of death should be confirmed by FAT. Wherever possible, virus isolation in cell culture should replace mouse inoculation tests.

The identification of the agent can be supplemented in specialised laboratories by identifying any variant virus strains through the use of monoclonal antibodies, specific nucleic acid probes, or the polymerase chain reaction followed by DNA sequencing of genomic areas. Such techniques can distinguish between field and vaccine strains, and identify the geographical origin of the field strains. These very sensitive tests should be used by well trained personnel in specialised laboratories.

Serological tests: *Virus neutralisation (VN) assays in cell cultures are prescribed tests for checking vaccination responses prior to international animal movement or trade. Results are expressed in International Units relative to an international standard antiserum. Alternatively, use may be made of validated tests that are known to correlate with these, notably enzyme-linked immunosorbent assays using antibody to the G protein or the whole virus.*

Requirements for vaccines: *Rabies vaccines for use in animals contain either live virus attenuated for the target species (such as Flury low egg passage, Flury high egg passage, Street-Alabama-Dufferin or Kelev), or virus inactivated by chemical or physical means, or recombinant vaccines. The virus is cultivated in embryonated egg, or in cell cultures.*

Rabies vaccines are usually lyophilised, but inactivated virus vaccines, preferably with an adjuvant, may be stored in liquid form.

Before newly developed vaccines can be licensed, the duration of immunity resulting from their use should be determined in vaccinated animals of the target species. Vaccines should confer protective immunity for at least 1 year.

For live virus vaccines, the minimum virus content that will elicit a protective immune response must be established.

The potency of inactivated virus vaccines is established and controlled using tests formulated by the United States Department of Agriculture in the United States of America or the European Pharmacopoeia elsewhere. The final products of both types of vaccine are subjected to tests for innocuity and absence of toxicity.

For live vaccines that are prepared for oral vaccination of wild (or domestic) animals, safety and efficacy in target animals and safety in non-target species must be demonstrated.

A. INTRODUCTION

Rabies is caused by neurotropic viruses of the genus *Lyssavirus* in the family Rhabdoviridae, and is transmissible to all mammals. As the viruses are transmissible to humans, all suspected infected material must be handled under the appropriate safety conditions specified by the World Health Organization (WHO, 1996).

Eleven distinct species can be distinguished within the genus, namely classical rabies virus (RABV), Lagos bat virus (LBV), Mokola virus (MOKV), Duvenhage virus (DUVV), European bat lyssaviruses type-1 (EBLV1) and type-2 (EBLV2), Australian bat lyssavirus (ABLV) and four lyssaviruses (Aravan virus [ARAV], Khujand virus [KHUV], Irkut virus [IRKV], and West Caucasian bat virus [WCBV]), which have been isolated from Eurasian bats, and have recently been ratified as new lyssavirus species (ICTV). In addition, a newly identified lyssavirus (Shimoni bat virus) has been isolated from a bat in Kenya (Kuzmin *et al.* 2010) and is awaiting official classification. RABV is found worldwide, and is responsible for the overwhelming majority of reported animal and human rabies cases. Other lyssaviruses appear to have more restricted geographical and host range, with the majority having been isolated from bats. However, all lyssaviruses tested to date cause clinical disease indistinguishable from classical rabies. Conserved antigenic sites on the nucleocapsid proteins permit recognition of all lyssaviruses with modern commercial preparations of anti-rabies antibody conjugates used for diagnostic tests on brain tissue.

The Lyssaviruses have been divided into two phylogroups with distinct pathogenicity and immunogenicity (Badrane *et al.*, 2001). For RABV, DUVV, EBLV and ABLV, conserved antigenic sites on the surface glycoproteins allow cross-neutralisation and cross-protective immunity to be elicited by rabies vaccination. A reduced protection with pre-exposure vaccination and with conventional rabies post-exposure prophylaxis was observed against IRKV, ARAV, and KHUV (Hanlon *et al.,* 2005) and all of the above-mentioned lyssavirus species were assigned to phylogroup 1. Little or no cross-protection against infection with the members of phylogroup 2 (MOKV and LBV) is elicited by rabies vaccination and most anti-rabies virus antisera do not neutralise these lyssaviruses (Badrane *et al.*, 2001). WCBV does not cross-react serologically with any of the two phylogroups. Laboratories working with lyssaviruses or suspect material must comply with national biocontainment and biosafety regulations and they should also comply with the guidelines for Risk Group 3 pathogens in Chapter 1.1.3 *Biosafety and biosecurity in the veterinary microbiological laboratory and animal facilities.*

The WHO recommends the preventive immunisation of all staff handling infected or suspect material. The immunisation protocol includes three injections, e.g. at days 0, 7, and 28. The serological evaluation of immunisation is made 1–3 weeks after the last injection, and checked every 6 months in the case of laboratory workers or every 2 years for other diagnosticians. Booster vaccination must be given when the titre falls below 0.5 International Units (IU) per ml. In the absence of serological monitoring, the vaccination regimen should consist of a booster vaccination at 1 year and thereafter every 1–3 years.

As no clinical sign or gross post-mortem lesion can be considered pathognomonic in domestic or wild animals, the diagnosis of rabies has to rely on laboratory testing. Serological testing is rarely useful for ante-mortem diagnosis because of late seroconversion and the high mortality rate of host species, but is very useful for assessing sero-conversion following vaccination and for epidemiological studies.

B. DIAGNOSTIC TECHNIQUES

1. Identification of the agent

Clinical observation may only lead to a suspicion of rabies because signs of the disease are not characteristic and may vary greatly from one animal to another. The only way to undertake a reliable diagnosis of rabies is to identify the virus or some of its specific components using laboratory tests.

As rabies virus is rapidly inactivated, refrigerated diagnostic specimens should be sent to the laboratory by the fastest means available. Shipment conditions must be considered to be part of the 'rabies diagnostic chain' and should follow international guidelines.

Several laboratory techniques may be used, and have been detailed and standardised in the fourth edition of the WHO's *Laboratory Techniques in Rabies* (WHO, 1996). The methods vary in their efficiency, specificity and reliability. They are classically applied to brain tissue, but they can also be applied with variable sensitivity and specificity to other organs (e.g. salivary glands). In the brain, rabies virus antigen is particularly abundant in the thalamus, pons and medulla. It is recommended that a pool of brain tissues that includes the brain stem should be collected and tested (Bingham & van der Merwe, 2002). The most widely used test for rabies diagnosis is the fluorescent antibody test (FAT), which is recommended by both WHO and OIE, and is sensitive, specific and cheap.

Precautions should be taken when handling central nervous system tissues from suspected rabies cases. Gloves should always be worn and precautions must be taken to prevent aerosols. Cutting tools, scissors and scalpels, should be used with care to prevent injury and contamination.

a) Collection of brain samples

Usually the brain is collected following the opening of the skull in a necropsy room, and the appropriate samples are collected preferably Ammon's horn, thalamus, cerebral cortex and medulla oblongata. Under some conditions (e.g. in the field or when sampling for large epidemiological studies, this step may be impractical. In such cases, there are two possible methods of collecting some brain samples without opening the skull:

- **Occipital foramen route for brain sampling**

A 5 mm drinking straw (Barrat & Blancou, 1988) or a 2 ml disposable plastic pipette is introduced into the occipital foramen in the direction of an eye. Samples can be collected from the rachidian bulb, the base of the cerebellum, hippocampus, cortex, and medulla oblongata. When using a straw it should be pinched between the fingers to prevent material escaping when withdrawing. Brain specimens from cattle can also be sampled using the 'brain scoop or tool' developed for bovine spongiform encephalopathy (BSE) tissue sampling , yielding a sample suitable for diagnosis of both BSE and rabies.

- **Retro-orbital route for brain sampling**

In this technique (Montano Hirose *et al.*, 1991), a trocar is used to make a hole in the posterior wall of the eye socket, and a plastic pipette or straw is then introduced through this hole. The sampled parts of the brain are the same as in the former technique, but they are taken in the opposite direction.

b) Shipment of samples

Suspect material should be shipped according to the International Air Transport Association (IATA) Dangerous Goods Regulations. These regulations are summarised in Chapter 1.1.2 *Transport of specimens of animal origin*.

When it is not possible to send refrigerated samples, other preservation techniques may be used. The choice of the preservative is dependent on the tests to be used for diagnosis:

- Formalin inactivates the virus, thus virus isolation tests cannot be used and diagnosis depends on using a modified direct fluorescent antibody test (FAT), polymerase chain reaction (PCR), (less sensitive than these tests on fresh tissue), immunohistochemistry or histology (Warner *et al.*, 1997);

- Infectivity at room temperature may be extended for several days if brain material is kept in a mixture of 50% glycerol in phosphate buffered saline (PBS). Glycerol/PBS slows bacterial action and therefore protects against the chemical and biological effects of putrefaction. It does not protect against titre decline due to thermal conditions and therefore, because rabies is thermo-labile, the virus titre will

decline during glycerol/PBS storage. Under normal transport conditions in the tropics, this protection may only be effective for a matter of several days. Therefore, whenever possible samples in glycerol/PBS should be kept refrigerated. As the virus is not inactivated by glycerol/PBS, all laboratory tests can be used on these samples.

- An alternative for the transport of samples for molecular techniques is the use of FTA Gene Guard system (Picard-Meyer *et al.*, 2007). The FTA paper preserves rabies virus RNA within the fibre matrix allowing the transport of samples at ambient temperature without specific biohazard precautions for further characterisation of rabies strains.

c)   Laboratory tests

i)   Immunochemical identification of rabies virus antigen

- *Fluorescent antibody test*

The most widely used test for rabies diagnosis is the FAT, which is recommended by both WHO and OIE. This 'gold-standard' test may be used directly on a smear, and can also be used to confirm the presence of rabies antigen in cell culture or in brain tissue of mice that have been inoculated for diagnosis. The FAT gives reliable results on fresh specimens within a few hours in more than 95–99% of cases. The FAT is sensitive, specific and cheap. The sensitivity of the FAT depends on the specimen (the degree of autolysis and how comprehensively the brain is sampled, see Section B.1) (Barrat & Aubert, 1995), on the type of lyssavirus and on the proficiency of the diagnostic staff. For direct rabies diagnosis, smears prepared from a composite sample of brain tissue, that includes the brain stem, are fixed in 100% high-grade cold acetone for at least 20 minutes, air dried and then stained with a drop of specific conjugate for 30 minutes at 37°C. Anti-rabies fluorescent conjugates available commercially are either polyclonal or monoclonal antibodies (MAbs), specific to the entire virus or to the rabies nucleocapsid protein, conjugated to a fluorophore such as fluorescein isothiocyanate (FITC). FAT slides should then be examined for specific fluorescence using a fluorescent microscope and filter appropriate for the wavelength of the fluorescent conjugate used, for instance FITC, the most commonly used, is excited at 490 nm and re-emits at 510 nm. Aggregates of nucleocapsid protein are identified by specific fluorescence of bound conjugate. It is recommended that two independent trained operators read each FAT slide. Fluorescent antibody conjugates may be made locally, but should be fully validated for specificity and sensitivity before use, including its ability to detect lyssaviruses other than rabies.

The FAT may be applied to glycerol-preserved specimens after a washing step. If the specimen has been preserved in a formalin solution, the FAT may be used only after the specimen has been treated with a proteolytic enzyme (Warner *et al.*, 1997). However, the FAT on formalin-fixed and digested samples is always less reliable and more cumbersome than when performed on fresh tissue (Barrat, 1992).

In cases of inconclusive results from FAT, or in all cases of human exposure, further tests on the same sample or repeat FAT on other samples are recommended. This is particularly important where sample autolysis is confirmed or suspected.

- *Immunochemical tests*

Immunoperoxidase methods can be used as an alternative to FAT with the same sensitivity (Lembo *et al.,* 2006), but attention should be paid to the risk of nonspecific false-positive results. This risk is considerably reduced by the thorough training of the technicians. It must also be emphasised that this technique needs one incubation step more than the FAT.

Peroxidase conjugate may also be used on fresh brain tissue or sections of formalin-fixed tissue for immunohistochemical tests.

- *Enzyme-linked immunosorbent assay* (*ELISA*)

An ELISA that detects rabies antigen is a variation of the immunochemical test. It is useful for large epidemiological surveys (Xu *et al.,* 2007). The specificity and sensitivity of such tests for locally predominant virus variants should be checked before use. In case of human contact these tests should be used in combination with confirmatory tests such as FAT or virus isolation

- *Rapid immunodiagnostic test* (*RIDT*)

A rapid immunodiagnostic test (RIDT) was developed recently (Kang *et al.,* 2007). This simple test can be used under field conditions and in developing countries with limited diagnostic resources.

Generally, tests other than the gold standard FAT should only be used after validation in multiple laboratories.

ii) Detection of the replication of rabies virus after inoculation

These tests detect the infectivity of a tissue suspension in cell cultures or in laboratory animals. They should be used if the FAT gives an uncertain result or when the FAT is negative in the case of known human exposure. Wherever possible, virus isolation on cell culture should be considered in preference to the mouse inoculation test (MIT). Cell culture tests are as sensitive as MIT (Rudd & Trimarchi, 1989) but are less expensive, give more rapid results and avoid the use of animals.

- *Cell culture test*

Neuroblastoma cells e.g. N2a, CCL-131 in the American Type Culture Collection (ATCC)[1] are highly susceptible to infection with lyssaviruses. The cells are grown in Dulbecco's modified Eagle's medium (DMEM) with 5% fetal calf serum (FCS), incubated at 36°C with 5% CO_2. Baby hamster kidney (BHK-21) cells are also sensitive to most street isolates without any adaptation step, but should be checked for susceptibility to locally predominant virus variants before use. Cell culture tests may be undertaken in multi-well plastic plates, multi-chambered glass slides or on glass cover-slips. The use of one 4-day passage in four wells of a 96-well microtitre plate has been shown to have comparable sensitivity to MIT for rabies strains (Rudd & Trimarchi, 1989). However additional passages could be considered to increase sensitivity. Cytotoxicity is a commonly reported factor limiting test robustness. Techniques proposed to reduce cytotoxicity include adding antibiotics, reducing the time before changing media (to as short as 35 minutes) and dilution of samples. Cell culture tests and their variations should be fully validated before use.

Suggested protocol for a 96-well plate: 100 µl of clarified brain homogenate (20% in phosphate buffered saline, 0.1 M, pH 7.4) is added to 200 µl of a 2×10^5 cells/ml suspension, freshly prepared from a sub-confluent flask in four wells of a 96-well plate. After 24 hours incubation at 5% CO_2 and 37°C, the supernatant from each well is removed and 200 µl of fresh medium is added to each well. After a further 72 hours incubation the supernatant is removed by pipette and kept. The cells are fixed with 80% acetone and stained with fluorescent antibody according to manufacturers' recommendations. Variations include reduced incubation time before changing media to reduce cell toxicity, the use of cell permeability agents (e.g. DEAE-dextran), and further passages. Up to three passages may be considered to increase sensitivity.

Suggested protocol for use in 8-chamber Lab-Tek ® slides: 50 µl of clarified brain homogenate (20% in a grinding substrate made of PBS, 0.1 M, pH 7.4 with heat-inactivated new-born calf serum) is added to 400 µl of a 10^5 cells/ml suspension, freshly prepared from a subconfluent flask. After 24 hours incubation at 5% CO_2 and 35.5°C, the supernatant from each chamber is removed and 400 µl of fresh medium is added to each chamber. After a further 24 hours incubation (or more) the supernatant is removed, chamber structure removed, cells layer dried and fixed with pure high grade cold acetone. The fixed cell layer is then stained with fluorescent antibody according to laboratory procedures. Variations include incubation time, use of cell permeability agents and further passages. Removed supernatants should be kept for possible further passage.

- *Mouse inoculation test*

Three -to-ten mice, 3–4 weeks old (12–14 g), or a litter of 2-day-old newborn mice, are inoculated intracerebrally. The inoculum is the clarified supernatant of a 10–20% (w/v) homogenate of brain material including brainstem (e.g. cortex, Ammon's horn, thalamus, medulla oblongata) in an isotonic buffered solution containing antibiotics. Mice should be anaesthetised when inoculated. The mice are observed daily for 28 days, and every dead mouse is examined for rabies using the FAT. For faster results in newborn mice, it is possible to check one baby mouse by FAT on days 5, 7, 9 and 11 post-inoculation. Any deaths occurring during the first 4 days are regarded as nonspecific (due to stress/bacterial infection etc.).

Once a validated and reliable cell culture unit exists in the laboratory, consideration should be given to replacing the mouse inoculation test with cell culture whenever possible as it avoids the use of live animals, is less expensive and gives more rapid results. However, advantages of MIT are that when the test is positive, a large amount of virus can be isolated from a single mouse brain for strain identification purposes and that it can be easily and practicably applied in situations where skills and facilities for other tests (e.g. cell culture) are not available. MIT may also detect viruses other than rabies virus.

---

1   American Type Culture Collection (ATCC), P.O. Box 1549, Manassas, Virginia 20108, United States of America (USA)

iii) Molecular techniques

Various molecular diagnostic tests, e.g. detection of viral RNA by reverse transcription PCR (RT-PCR), PCR-ELISA, hybridisation *in situ* and real-time PCR are used as rapid and sensitive additional techniques for rabies diagnosis (Fooks *et al.*, 2009). The principle of lyssavirus-specific PCRs is a reverse transcription of the target RNA (usually parts of the N gene) into complementary DNA followed by the amplification of the cDNA by PCR. Although those molecular tests have the highest level of sensitivity, their use is currently not recommended for routine post-mortem diagnosis of rabies (WHO, 2005) due to high levels of false positive or false negative results without standardisation and very stringent quality control. Nevertheless, they are useful for confirmatory diagnosis, as a first step in virus typing (see below).

iii) Histological identification of characteristic cell lesions

Negri bodies correspond to the aggregation of viral proteins, but the classical staining techniques detect only an affinity of these structures for acidophilic stains. Techniques that stain sections of paraffin embedded brain tissues (e.g. Mann's technique) are time consuming, less sensitive and more expensive than FAT. Seller's method on unfixed tissue smears has a very low sensitivity is only suitable for perfectly fresh specimens. These methods are no longer recommended for routine diagnosis. Immunohistochemical tests are the only histological methods specific to rabies.

d) Other identification tests

The tests above describe methods to accurately diagnose rabies and to isolate and identify the virus. Typing of the virus can provide useful epidemiological information and should be undertaken in specialised laboratories (such as OIE or WHO Reference Laboratories). These techniques would include the use of MAbs, nucleic acid probes, or the PCR, followed by DNA sequencing of genomic areas for typing the virus (Bourhy *et al.,* 1993). These characterisations enable, for instance, a distinction to be made between vaccine virus and a field strain of virus, and possibly identify the geographical origin of the latter.

Participation in inter-laboratory proficiency testing is highly encouraged as part of quality assurance schemes; such tests should be organised for Regional Laboratories by the National Reference Laboratories, while the latter in turn should participate in international proficiency tests organised by OIE reference laboratories.

2. Serological tests

The main application of serology for classical rabies is to determine responses to vaccination, either in domestic animals prior to international travel, or in wildlife populations following oral immunisation. In accordance with the WHO recommendations (WHO, 1985), 0.5 IU per ml of rabies antibodies is the minimum measurable antibody titre considered to represent a level of immunity in humans that correlates with the ability to protect against rabies infection. The same measure is used in dogs and cats to confirm a satisfactory response to vaccination. As neutralising antibodies are considered a key component of the adaptive immune response against rabies virus (Hooper *et al.*, 1998) the gold standard tests are virus neutralisation (VN) tests. However, indirect ELISAs have been developed that do not require high-containment facilities and produce rapid results. Care should be taken when correlating results between virus neutralisation tests and ELISAs owing to the inherent differences between them. Multiple publications demonstrate a variable sensitivity and specificity for ELISAs in both humans and animals. Although VN tests are recommended where specific assessment of protection is required, both tests are useful for detecting responses to vaccination if appropriate cut-offs are used. Poor quality sera can cause cytotoxicity in VN tests, which could lead to false-positive results. For such samples, the use of an indirect ELISA has been shown to be as sensitive and specific as the VN test (Servat *et al.*, 2007).

Serological surveys have also been used to provide information on dynamics of lyssaviruses in bats although standardisation of serological tests for bats is still needed.

a) Virus neutralisation test in cell culture: fluorescent antibody virus neutralisation test (a prescribed test for international trade)

The principle of the fluorescent antibody virus neutralisation (FAVN) test (Cliquet *et al.,* 1998) is the *in vitro* neutralisation of a constant amount of rabies virus ('challenge virus standard' [CVS-11] strain adapted to cell culture) before inoculating cells susceptible to rabies virus: BHK-21 C13 cells (ATCC number: CCL-10).

The serum titre is the dilution at which 100% of the virus is neutralised in 50% of the wells. This titre is expressed in IU/ml by comparing it with the neutralising dilution of the OIE serum of dog origin under the

same experimental conditions. The WHO standard for rabies immunoglobulin [human][2] No. 2, or an internal control calibrated against the international control may be used. The WHO standard or internal control should only be used as a control in the test and should not be used to calculate the IU/ml titre of the sera.

This microplate method uses 96-well plates, and is an adaptation of the technique of Smith *et al.* (1973). The FAVN test and the rapid fluorescent focus inhibition test (RFFIT) give equivalent results (Cliquet *et al.*, 1998).

- **Essential equipment**

Humidified incubator at 35°C/37°C with 5% CO_2; dry incubator at 37°C; biocontainment cabinet; fluorescence microscope suitable for FITC fluorescence equipped with ×10 eye-piece and ×10 objective. The global magnification of the microscope ranges between ×100 and ×125 due to the extra magnification of some epi-fluorescence systems.

- **Reagents and biologicals**

PBS buffer, pH 7.2, without Ca^{2+} and Mg^{2+}, stored at 4°C;

Trypsin ethylene diamine tetra-acetic acid (EDTA);

High-grade acetone 80% (diluted with deionised water), stored at 4°C;

Dulbecco's modified Eagle's medium (DMEM) + 10% heat-inactivated FCS;

FITC anti-rabies conjugate;

Cells: BHK-21 C13 (ATCC CCL-10) maintained in GMEM with 10% FCS and antibiotics;

Virus: CVS-11 (previously ATCC reference VR 959) strain, which is available from the ATCC or the OIE Reference Laboratory for Rabies, Nancy, France (see Table given in Part 4 of this *Terrestrial Manual*). Vials are stored at –80°C;

OIE Standard Serum of dog origin (OIE Reference Laboratory for Rabies, Nancy, France [see Table given in Part 4 of this *Terrestrial Manual*] stored at +4°C and diluted to 0.5 IU/ml with sterile deionised or distilled water according to the titre of the batch). This control serum may be used to calibrate an internal control that is used for regular FAVN testing;

Naive serum: The pool of negative dog sera is stored at –20°C.

- **CVS production**

i)   *Cell growth:* the BHK-21 C13 cells (ATCC CCL-10) used to produce the CVS virus (ATCC VR 959 CVS-11) are trypsinised during the rapid growth phase, i.e. cells are in the exponential phase of their kinetic growth. If the confluence of the layer is complete, a new passage should be made. The cells in the cell suspension should not be aggregated; 2×10^7 cells are needed for a 75 cm^2 cell culture flask. Cells are collected within a volume of 20–30 ml in cell culture medium with 10% heat-inactivated FCS.

ii) *Infection of cells:* the multiplicity of infection (number of infective particles per cell) is adjusted to between 0.1 and 0.5. The glass bottle containing the virus/cell suspension is incubated for 60 minutes at 35.5–37°C. The contents of the bottle are gently stirred every 10–15 minutes.

iii) *Virus growth:* the virus/cell suspension is then centrifuged at 800–1000 *g* for 15 minutes and the cell pellet is resuspended in cell culture medium mixed with 10% heat-inactivated FCS. Virus is harvested 2 days later.

iv) *Harvest and storage:* the supernatant is centrifuged at 800–1000 *g* for 15 minutes at 4°C. If several flasks have been used, the different centrifuged supernatants are mixed and then aliquoted and frozen at –80°C. The infective titre of the harvest is established at least 3 days after freezing.

- **Titration of virus in TCID$_{50}$ (50% tissue culture infective dose)**

This titration method uses BHK-21 C13 cells (ATCC CCL-10) in microtitre plates.

Different steps in this procedure may be adapted according to the safety requirements and to the working practices of the laboratory, but the following should not be changed:

- inoculation of a 24-hour cell layer,

- tenfold dilutions prepared using 0.9 ml of diluent and 0.1 ml of virus suspension,

- four to six 50 µl replicates per dilution,

- incubation for 72 hours,

- qualitative reading (i.e. the well is positive or negative),

- in every titration session, a vial of a control batch of virus is titrated and this titre is integrated in a control card to validate the titration process,

- calculation according to neoprobit graphic or Spearman–Kärber methods.

i) *Cell suspension:* the day before titration, a cell suspension containing 10^5 cells/ml is prepared in cell culture medium containing 10% heat-inactivated FCS, and is distributed, 200 µl per well, into 96-well microtitre plates. The plates are then incubated for 24 hours at 35.5°C–37°C with 5% CO_2.

ii) *Dilution of the virus:* the serial dilutions are performed in 5 ml tubes using a cell culture medium without FCS as diluent. Ten-fold dilutions from 10^{-1} to 10^{-12} are prepared (0.9 ml of diluent with 0.1 ml of the previous dilution).

iii) *Infection of the cells:* the medium in the microtitre plates is discarded using an aspiration system. Fifty µl of each virus dilution is distributed per well. Six replicates are used per dilution. The microtitre plate is then incubated for 1 hour at 35.5–37°C with 5% CO_2. Then 200 µl of cell culture medium, containing 5% FCS, is added.

iv) *Incubation:* incubate for 3 days at 35.5–37°C in 5% CO_2.

v) *Staining and calculation of titre:* The cells are stained using the FAT, as detailed below. Reading is qualitative, every well that shows specific fluorescence is considered to be positive. The titre calculation is made using either the neoprobit graphic method or the Spearman–Kärber formula (WHO, 1996).

vi) The CVS titration must be performed by FAVN test to establish the infective dose in $TCID_{50}$.

- **Test procedure**

i) The microplates are used according to the pattern shown in Figure 1. Plate No. 1 is used for the titration of CVS (rows 1 to 4), and for the controls, standard sera and naive dog serum are used. All other plates are used for the sera to be tested.

ii) Medium is added to the wells as follows: plate 1, rows 1 to 4 and cells A9 to A12: add 150 µl per well; in the other plates, rows 6 and 12: add 200 µl per well; all other wells: add 100 µl.

iii) Sera to be tested are heat inactivated for 30 minutes at 56°C. As indicated in Figure 1, 50 µl of each undiluted serum to be tested is added to four adjacent wells.

iv) Dilutions of sera are conducted in the microplates as follows:

OIE serum, the WHO serum, the internal control and the naive dog serum: with a 50–200 µl multichannel pipette, mix the first dilution wells by sucking in and out at least eight times, transfer 50 µl from one row to the next one, until the last one is reached. Discard 50 µl from the last row.

If there is a serum to be tested on the control plate, see below for the dilution step.

A minimum of four three-fold dilutions is required.

Sera being tested (all plates): as above, transfer successively 50 µl from one row to the following one until rows 5 and 11 (dil. $10^{-2.39}$). With a 5–50 µl multichannel pipette, transfer 10 µl from rows 5 and 11 to rows 6 and 12, respectively (from dil. $10^{-2.39}$ to dil. $10^{-4.23}$). Using a multichannel pipette adjusted to 90 µl, mix rows 6 and 12 and discard 180 µl. Then add 70 µl of medium to these rows. This final step does not lend itself to high throughput testing. To attain or exceed the recommended final dilution alternative procedures may be used. These may require modifications to the plate layout.

Fig. 1. *Proposed use of microplates for the fluorescent antibody virus neutralisation test. Wells to which undiluted sera must be added are filled with the indicated '50 µl'. Wells to which 50 µl of diluted challenge virus standard must be added are shaded. Dilutions are given in* log_{10}.

Plate 1: Controls

	H	G	F	E	D	C	B	A		
Challenge virus									1	
standard									2	
titration									3	
									4	
OIE	50 µl					50 µl			5	**Naive dog**
standard serum	50 µl					50 µl			6	**serum**
(0.5 IU/ml)	50 µl					50 µl			7	**(negative)**
	50 µl					50 µl			8	
Serum or internal	50 µl								9	
positive control	50 µl								10	
or WHO Standard	50 µl								11	
serum	50 µl								12	
log (dilution)	0.48	0.95	1.43	1.91	2.39	2.87	CVS virus control	Cells control		

Plate 2: Sera to be tested

log dilution		0.48	0.95	1.43	1.91	2.39	*4.23*	0.48	0.95	1.43	1.91	2.39	*4.23*	
		1	2	3	4	5	6	7	8	9	10	11	12	
	A	50 µl						50 µl						
Serum 1	B	50 µl						50 µl						**Serum 3**
	C	50 µl						50 µl						
	D	50 µl						50 µl						
	E	50 µl						50 µl						
Serum 2	F	50 µl						50 µl						**Serum 4**
	G	50 µl						50 µl						
	H	50 µl						50 µl						

- **Addition of challenge virus standard**

i) Stock CVS is stored in 1 ml microtubes at –80°C. One tube is thawed rapidly under cold running water, and placed in melting ice.

ii) One dilution from this tube is prepared in order to obtain 100 $TCID_{50}$ in 50 µl. Of this dilution, 50 µl is added to each serum-filled well (see Figure 1). For virus titration, 50 µl is added to wells H1 to H4 (plate 1). Next, transfer 50 µl from row to row (plate 1, lines 1–4). Discard 50 µl from the last row (plate 1, wells A1 to A4). No virus is added to wells A9 to A12 of plate 1 (controls). The range allowed for the virus dose titre must be between 30 and 300 $TCID_{50}$/50 µl.

iii) Incubate the microplates at 35–37°C in a humid incubator with 5% CO_2 for 1 hour.

iv) *Addition of cells:* trypsinise a subconfluent culture of BHK-21 cells. Resuspend the cells to obtain a 4×10^5 cells/ml suspension in DMEM supplemented with 10% heat-inactivated FCS. Add 50 µl of the cell suspension to each well.

v) Incubate the microplates for 48 hours at 35–37°C in a humid incubator with 5% CO_2.

- **Fixation and staining**

i) After the 48-hour incubation period, the medium is discarded, and the microplates are rinsed once in PBS, pH 7.2, and once in 80% acetone. The microplates are then fixed in 80% acetone at room temperature for 30 minutes, and are dried at room temperature for at least 30 minutes.

ii) Add 50 µl of the FITC anti-rabies conjugate, at the working dilution, to each well, gently rock the microplates and incubate at 35–37°C for 30 minutes. Discard the fluorescent conjugate and rinse the microplates twice with PBS. Excess PBS is removed by briefly inverting the microplates on absorbent paper.

- **Reading and interpreting the results**

i) The total surface of each well is observed. The reading evaluation is qualitative (plus or minus): no fluorescent cell – a minus score is recorded for the well; fluorescent cells (one cell or more) – a plus score is recorded for the well.

ii) Cell and virus controls are read first. For titration of CVS, naïve serum, and OIE standard serum, titres are calculated according to the Spearman–Kärber method or the neoprobit graphic method (WHO, 1996).

iii) Results of titration of CVS ($TCID_{50}$), naive serum (D_{50} [median dose]) and positive standard (D_{50}) are reported on a control card for each of these three controls. The control results of the current test are compared with the accumulated control test results from previous tests using the same batch of control. The test is validated if the values obtained for the three controls in the current test are not statistically different from the mean (± 2 SD) of all the values obtained in the tests conducted previously according to this technique.

iv) The result of the test corresponds to the non-neutralised virus after incubation with the reference serum or with the serum to be tested. These titres are calculated with the neo-probit graphic method or with the Spearman–Kärber formula (WHO, 1996).The comparison of the measured titre of the tested sera with that of the OIE positive standard serum of a known neutralising titre allows determination of the neutralising titre of the tested sera in IU/ml. The conversion to IU/ml can be made by using either the log D_{50} value of the day or the mean value of the OIE standard serum.

- Formula to convert the log D_{50} value in IU/ml titre:

$$\text{Serum titre (IU/ml)} = \frac{[(10^{(\text{serum log } D_{50} \text{ value})}) \times \text{theoretical titre of OIE serum 0.5 IU/ml}]}{(10^{(\log D_{50} \text{ of OIE serum 0.5 IU/ml})})}$$

Example of conversion:

- log D_{50} of the serum = 2.27

- theoretical titre of OIE serum 0.5 IU/ml = 0.5 IU/ml

- log D_{50} of OIE serum = 1.43

(for the log D_{50} of OIE, the value of the day or the mean value can be considered)

$$\text{Serum titre (IU/ml)} = \frac{10^{2.27} \times 0.5}{(10^{1.43})} = 3.46 \text{ IU/ml}$$

The following parameters have to be strictly respected:

- Rabies virus: only the CVS-11 strain should be used.

- Cells culture: only BHK-21 cells (ATCC number – CCL 10) should be used.

- The FAVN test must be performed only in 96 wells microplate.

- Control charts should be used for rabies virus, naïve serum and positive standard serum of dog origin.

- The back titration of the CVS virus, as well as naïve serum and positive standard serum of dog origin, must be present on control plate.

- A minimum of four three-fold dilutions of sera are required. The reading method is 'all or nothing' only.

- Four replicates of each serum should be diluted.

- For the conversion of log D_{50} in IU/ml, the laboratories should use only the log D_{50} value of the positive standard serum of dog origin.

b) **The rapid fluorescent focus inhibition test (RFFIT) for determining rabies virus-neutralising antibody (a prescribed test for international trade)**

- **Standard procedure (from WHO Laboratory Techniques in Rabies, 1996)**

   - **Preparation of seed virus suspension**

   i)   Trypsinise one 3-day-old 150 ml flask culture of mouse neuroblastoma (MNA) cells. A similar cell line (CCL-131) may be obtained on request from the ATCC (see footnote 1).

   ii)   Resuspend 3×10^7 cells in a 50 ml conical centrifuge tube in 2.7 ml of Eagle's minimal essential medium supplemented with 10% fetal bovine serum (EMEM-10).

   iii)   Using standard rabies safety procedures, add 1×10^7 infectious units of CVS-11 rabies virus (previously ATCC reference VR959) and vortex/mix once. Incubate the cells and virus for 15 minutes at 37°C; vortex/mix the cells once during this time.

   iv)   Add 10 ml EMEM-10, vortex/mix, and centrifuge the cells at 500 *g* for 10 minutes.

   v)   Discard the supernatant. Resuspend the cells in 30 ml of growth medium and transfer to a 150 ml flask.

   vi)   Gently rock the flask to mix the cell suspension, and then prepare three eight-well tissue-culture chamber slides by pipetting 0.2 ml of the cell suspension into one well of each slide.

   vii)   Incubate the flask and slides at 37°C in a humidified incubator with 0.5% carbon dioxide (CO_2). The flask should be incubated as a closed culture (tighten the cap).

   viii) At 20, 40 and 64 hours after infection, acetone fix and stain one slide using an immunofluorescence technique (Cliquet *et al.,* 1998) to determine the virus infectivity. The supernatant should be harvested 24 hours after the cells reach 100% infectivity (typically 40 hours after infection).

   ix)   Transfer the supernatant to a 50 ml centrifuge tube and centrifuge at 4000 *g* for 10 minutes.

   x)   Distribute the supernatant into 0.5 ml aliquots and store at –70°C.

   - **Titration of seed virus suspension**

   i)   Thaw one aliquot of the seed virus and prepare serial tenfold dilutions (from 10^{-1} to 10^{-8}) in EMEM-10.

   ii)   Distribute 0.1 ml of each virus dilution into one well of an eight-well tissue-culture chamber slide. Add 0.2 ml of MNA cells suspended in EMEM-10 (concentration 5×10^4 cells per 0.2 ml) to each well.

   iii)   Mix the cells and virus by gently rocking the slide, then incubate at 37°C in a humidified incubator with 0.5% CO_2 for 40 hours.

   iv)   Acetone fix and stain the slide using an immunofluorescence technique. Evidence of virus infection should be observed at the 10^{-6} dilution of virus, indicating a virus stock suspension containing at least 1×10^6 infectious units per 0.1 ml. Prepare sufficient seed virus so that frequent serial passage of the virus is unnecessary.

   - **Preparation of stock virus suspension**

   i)   Infect 3×10^7 MNA cells with 1×10^7 infectious units of the seed virus preparation (see above).

   ii)   Harvest the supernatant 24 hours after the cells reach 100% infectivity (typically 40 hours after infection).

   iii)   Distribute the supernatant into 0.5 ml aliquots and store at –70°C.

   - **Titration of stock virus suspension**

   i)   Thaw one aliquot of the stock virus and use this to prepare serial tenfold dilutions (from 10^{-1} to 10^{-6}) in EMEM-10.

   ii)   Distribute 0.1 ml of each virus dilution into one well of an eight-well tissue-culture chamber slide. Add 0.2 ml of MNA cells suspended in EMEM-10 (concentration 1×10^5 cells per 0.2 ml) to each well.

   iii)   Mix the cells and virus suspension by gently rocking the slide, then incubate at 37°C in a humidified incubator with 0.5% CO_2 for 20 hours.

   iv)   Acetone fix and stain the slide using an immunofluorescence technique.

Each well of an eight-well tissue-culture chamber slide contains 25–50 distinct microscopic fields when observed at ×160–200 magnification. One unit of virus for the RFFIT is determined as the dilution at which 50% of the observed microscopic fields contain one or more foci of infected cells (the focus-forming dose, FFD_{50}). The stock virus suspension should contain at least $1 \times 10^4\ FFD_{50}$ per 0.1 ml (i.e. the well with cells infected with the 10^{-4} dilution of the virus should contain at least one focus of infected cells in 50% of the observed microscopic fields). A stock virus suspension of this titre can then be diluted to $10^{-2.3}$ to obtain a challenge virus containing 50 FFD_{50}.

- **Reference sera**

A national or international reference serum standard diluted to a potency of 2.0 IU/ml should be included in each test. The reference serum used at the Centres for Disease Control and Prevention is the second international standard for rabies immunoglobulin (Lyng, 1994), which may be obtained from the NIBSC (see footnote 2). The reference serum should be maintained as frozen aliquots in amounts sufficient for 1 week of tests. A positive serum control standard diluted to a potency of 0.5 IU/ml and a negative serum control standard with a potency of <0.1 IU/ml should also be prepared by the laboratory and included in each test.

- **Test sera**

Serum samples should be heated at 56°C for 30 minutes before testing in order to inactivate complement. If sera are frozen, they should be reheated after thawing. Serial dilutions of test sera may be prepared in an eight-well tissue-culture chamber slide. Screening dilutions of 1/5 and 1/50 are sufficient for routine evaluation of vaccination efficacy and may be made as follows:

i)   Prepare a 1/2.5 dilution by adding 0.1 ml of inactivated serum and 0.15 ml of EMEM-10 to one of the slides. Mix by gently rocking the slide.

ii)   Transfer 0.05 ml of the 1/2.5 dilution to a second well containing 0.45 ml of EMEM-10. Discard all but 0.1 ml from the well containing the 1/2.5 dilution.

iii) Mix the second well and discard all but 0.1 ml.

iv)   Add 0.1 ml of the challenge virus preparation (containing 32–100 FFD_{50}) to all serum dilutions.

v)   Mix and incubate at 35°C in a humidified incubator with 0.5% CO_2 for 90 minutes.

- **Addition of cells**

i)   During the incubation period, trypsinise a stock culture of 3–5-day-old MNA cells.

ii)   Resuspend the cells in EMEM-10 to give a final concentration of 1×10^5 cells per 0.2 ml.

iii) Distribute 0.2 ml of the cell suspension into each well of the slide and incubate at 35°C in a humidified incubator with 0.5% CO_2 for a further 20 hours.

- **Acetone fixation and staining by immunofluorescence**

i)   After 20 hours, remove the slides from the incubator and pour off the medium into a virucidal solution.

ii)   Rinse the slides once in PBS and then fix for 10 minutes at room temperature in cold acetone (–20°C).

iii) Leave the slides to dry for 10 minutes before adding FITC-conjugated anti-rabies serum. The conjugate may be prepared in EMEM-10 or PBS; there is no need to adsorb the conjugate with tissue or cells. The working dilution of the conjugate should be determined by titration. The slides should be stained for 20–30 minutes at 37°C and then rinsed in PBS and distilled water, respectively.

iv)   Observe the slides under a fluorescence microscope.

- **Calculation of virus-neutralising antibody titres**

Residual virus is detected using a standard fluorescence microscope. The serum neutralisation end-point titre is defined as the dilution factor of the highest serum dilution at which 50% of the observed microscopic fields contain one or more infected cells (i.e. a 97% reduction in the virus inoculum). This value may be obtained by mathematical interpolation. Alternatively, a 100% neutralisation titre may be determined by recording the highest serum dilution at which 100% of the challenge inoculum is neutralised and there are no infected cells in any of the observed fields. For both titration methods, the titre of antibody in the test serum (in IU/ml) can be obtained by comparison with the titre of the national reference standard included in each test. It should be noted that it is also valid to perform the RFFIT using BHK-21 cells instead of neuroblastoma cells. A modified protocol for this has been published (WHO, 1996).

The following parameters have to be strictly adhered to:

- Rabies virus; only the CVS-11 strain should be used.

- Cells cultures: only BHK-21 cells (ATCC number CCL10) or MNA cells (ATCC number CCL131) should be used.

- The test should be performed only on Lab-tek chamber slides.

- Control charts should be used for rabies virus, naïve serum and positive standard dog serum.

- The back titration of the CVS virus, as well as the naïve serum and positive standard dog, must be present on control plate.

- Reading method for the test: each chamber slide should contain 25–50 fields and be observed at ×160–200 magnification.

- A minimum of three-to-five-fold dilutions of sera is required.

- For the conversion of log D_{50} to IU/ml, only the log D_{50} value of the positive standard serum of dog origin should be used.

c) Virus neutralisation in mice

This method is no longer recommended by either OIE or WHO.

d) Enzyme-linked immunosorbent assay (a prescribed test for international trade)

The ELISA provides a rapid (approximately 4 hours) test that avoids the requirement to handle live rabies virus. Commercial indirect ELISA kits are available that allow detection of rabies antibodies in individual dog and cat serum samples following vaccination. In 2007, the International Committee adopted such methods as Prescribed Tests for evaluating vaccine responses in dogs and cats prior to international movement, provided a kit is used that has been validated and adopted on the OIE Register as fit for such specific purposes[3]. The validation for this purpose should include a comparison with, and calibration against prescribed methods for virus neutralisation.

Other ELISA methods or kits should not be regarded as prescribed but may be useful for monitoring of vaccination campaigns in wildlife populations, provided the kit used has been validated for the wildlife species under study.

C. REQUIREMENTS FOR VACCINES

1. General background

The prevention and control of rabies is usually a national responsibility and, in many countries, the vaccine may be used only under the control of the Competent Authority. Guidelines for the production of veterinary vaccines are given in Chapter 1.1.6 *Principles of veterinary vaccine production*. The guidelines given here and in chapter 1.1.6 are intended to be general in nature and may be supplemented by national and regional requirements. Varying requirements relating to quality, safety and efficacy apply in particular countries or regions for manufacturers to obtain an authorisation or licence for a veterinary vaccine. Where possible, manufacturers should seek to obtain such a licence or authorisation for their rabies vaccines as independent verification of the quality of their product.

Virulent rabies virus may be used to produce inactivated rabies vaccine; consequently, the rabies vaccine production facility should operate under the appropriate biosafety procedures and practices. The facility should meet the requirements for containment outlined in chapter 1.1.3 and WHO (2005).

Rabies vaccines are defined as a standardised formulation containing defined amounts of immunogens. These immunogens are either inactivated (killed), live-attenuated or biotechnology-derived as described in chapter 1.1.6.

Licensed vaccines for the parenteral vaccination of domestic animals and oral vaccines for the immunisation of wild animals are available. These vaccines are frequently used off-label in captive and free-ranging wild animals. Oral vaccines for dogs are in the experimental stage (WHO, 2007).

---

3   see http://www.oie.int/vcda/eng/en_VCDA_registre.htm?e1d9

Lyssaviruses are classified into different viral species as described in Section A of this chapter. All rabies vaccines are produced from rabies virus (Type species RABV).

Most rabies vaccines are prepared from Pasteur's original 1882 strain and its derivative strains (Pasteur Virus, Challenge Virus Standard [CVS], Pitman-Moore), and strains isolated in the 20th century (Flury, low egg passage [LEP], high egg passage [HEP], Street-Alabama-Dufferin [SAD], Vnukovo) (Geue *et al.,* 2008; Tao *et al.,* 2010; Wu *et al.,* 2011). Rabies vaccines produced in compliance with OIE requirements protect against all variants of rabies virus including other phylogroup 1 lyssaviruses (Brookes *et al.,* 2005).

Table 1. *Current rabies viruses used for challenge or for vaccine manufacturing*
(Müller *et al,* 2009; Pastoret *et al.,* 1997)

Pasteur strain	Street Alabama Dufferin	Flury strain	Other strains

1882 France from a rabid cow infected by a dog
 	
Passages in rabbits and mice then passages in cells at different levels:
  Pasteur virus (PV-12)
  Kissling (CVS-11)
  CVS challenge virus strain (CVS-27
  Pitman-Moore (PM)
  RV-97
	1935 USA from a dog
	
	Primary cells of hamsters & pigs (10 passages) = ERA virus
	BHK 21 cell line passages:
	  SAD Vnukovo (USSR Russia)
	  SAD Vnukovo-32
	  SAD Bern (Switzerland)
	  SAD-B19
	  SAG2
	  ERA 333
		1939 USA from Miss Flury transmitted by a dog
		
		136 passages in 1 day-old-chicks
		
		40/50 passages in embryonated eggs: low egg passage (LEP)
		
		220/227 passages in embryonated eggs: high egg passage (HEP)
			CTN: China from a dog (1956)

Rabies virus vaccines may not provide adequate cross-protection against all lyssaviruses. For example, there is no protection provided against phylogroups 2 and 3, which include Mokola virus (Von Teichman *et al.,* 1998), Lagos bat virus (Markotter *et al.,* 2008), West Caucasian bat virus (Hanlon *et al.,* 2005) and Shimoni bat virus (Kuzmin *et al.,* 2010).

Rabies is not a candidate for global eradication (Rupprecht *et al.,* 2008). From a global public health perspective however, the dog should be considered a main target for rabies elimination as it is the principal reservoir. The disease can also be successfully controlled in certain wild carnivores, such as red foxes and raccoon dogs (Cliquet *et al.,* 2012). Apart from dogs, other companion animals (such as cats, ferrets, etc.) and livestock pose a risk for human exposure and would benefit from inclusion in any national vaccination programme. Additionally, vaccination of livestock is recommended as it secures livelihoods in many parts of the world.

Rabies vaccines are formulated for their specific purpose and application either by the injectable or oral route for the immunisation of domestic animals or wildlife.

Rabies vaccines are either produced in eggs or cell culture. Nerve-tissue vaccines prepared in animals are no longer considered safe or effective for use in humans (WHO, 2005) and their use should be discontinued in animals.

2. Rabies vaccine for injectable use

2.1. Background

The principal rationale for the use of injectable rabies vaccine is to protect human health through the prevention and control of rabies in animals, particularly dogs. Injection ensures that the immunogen is delivered to the target species. Vaccination is also used to protect endangered species and livestock.

Live-attenuated vaccines of Flury strain (LEP or HEP) or SAD origin, have been widely used for injection in domestic animals. However, several of these products have been documented to cause rabies in vaccinated animals, and injectable use should be discontinued (Bellinger *et al.,* 1983; Esh *et al.,* 1982; Fehlner-Gardiner *et al.,* 2008).

The rabies virus glycoprotein biotechnology-derived vector vaccines are not live rabies virus vaccines (WHO, 1996). They are prepared by inserting non-infectious rabies virus nucleic acid coding for rabies virus glycoprotein into a vector such as avipox for injectable vaccine. As these do not contain live rabies virus, animals vaccinated with rabies virus glycoprotein vaccines should not be restricted from entry into countries that have restrictions on entry of animals vaccinated with live rabies virus vaccines (Taylor *et al.,* 1991). However, the ERA 333 strain used in Eastern Europe is a live rabies virus vaccine expressing a manipulated rabies virus glycoprotein.

2.2. Outline of production and minimum requirements for conventional vaccines

a) Characteristics of the seed

i) *Biological characteristics*

Any rabies virus strain considered for vaccine production should protect against any rabies virus variant of phylogroup 1. Selection of master seed viruses (MSVs) should ideally be based on the ease of growth in culture, virus yield, stability and antigenic spectrum (Wu *et al.,* 2011) OIE Reference Laboratories for Rabies may be able to provide virus isolates as possible candidates for vaccine development. A record of the source of the MSV should be maintained*.*

Biotechnology-derived vaccines are prepared in appropriate non-tumorigenic cell lines using a vector expressing the rabies virus glycoprotein.

ii) *Quality criteria*

Only MSV that has been established as pure (free from extraneous agents) and immunogenic shall be used for preparing the seed virus for vaccine production. If it is to be used as a live attenuated vaccine, the MSV must be shown not to cause clinical rabies in target animals when injected.

iii) *Validation as a vaccine strain*

MSVs must be well characterised and proven to be pure and free from all extraneous agents in accordance with Chapter 1.1.7 *Tests for sterility and freedom from contamination of biological materials* and those listed by the appropriate licensing authorities.

The efficacy of the resultant vaccine is assessed by studies on every target species previously vaccinated as recommended in chapter 1.1.6 and Section C.2.1.c.iii of this chapter.

b) Method of manufacture

i) Procedures

In cell culture

The virus is used to infect a suspension or monolayers of an established cell line. Such cell culture should be proven to be free from contaminating microorganisms (see chapter 1.1.6).

Cultures are infected with cell-culture-adapted strains of virus and incubated at the appropriate temperature for a defined period. As rabies virus does not normally cause cytopathic effect, this allows several harvests from the same culture. This material is processed and used to formulate vaccine. For inactivated (killed) vaccine the virus is inactivated by addition of an inactivant of the first order, usually β-propiolactone (BPL) or ethyleneimine (EI) in the form of binary ethyleneimine (BEI). It is important that the necessary safety precautions for working with inactivants are fully observed. Other inactivants, such as formalin or phenic acid, should not be used. The inactivant is added to a virus suspension to achieve a predetermined final concentration. Inactivation must be duly validated and documented to show the inactivation kinetics and the results of the inactivation controls. The time period for inactivant treatment and temperature used for inactivation must be validated for the actual conditions and equipment used during industrial production.

Inactivated rabies vaccines are usually formulated as liquid or freeze dried. The liquid vaccine, which is most commonly used, is prepared by adsorbing the antigen onto the adjuvant, for example aluminium hydroxide gel.

ii) *Requirements for media and substrates*

The final blend may include antifoam, phenol red dye (if permitted by the country requiring vaccine), lactalbumin hydrolysate, tryptose phosphate broth, amino acids, vitamins and buffer salts. Saponin or other polysaccharides as adjuvant could be incorporated in rabies vaccines for ruminants. Addition of preservatives is recommended for multi-dose vials. The freeze-dried vaccines should be reconstituted before injection with the appropriate solvent.

In cells

The cell lines used for the production of rabies virus vaccines should be in accordance with chapter 1.1.6.

In embryonated eggs

This method of culture is used for the production of live-attenuated vaccine that contains the Flury LEP or the HEP variant strain. Their use should be discontinued as indicated in Section 2.1 above (Tao *et al.,* 2010; Wachendörfer *et al.,* 1982).

iii) *In-process control*

During the production process, tests are undertaken at different times before constitution of the final blend, which allows the consistency of production to be verified as in accordance with chapter 1.1.6. Tests for infectivity, sterility and inactivation are fundamental in-process controls. The formulation of the final product can be standardised using additional tests to measure viral integrity after storage, antigenic mass and glycoprotein content.

Inactivation test

Inactivation is verified using a test for residual live virus. For this, the inactivated harvest is inoculated into the same type of cell culture as that used in the production of the vaccine or a cell culture shown to be at least as sensitive. The quantity of inactivated virus harvest used is equivalent to not less than 25 doses of the vaccine. After incubation for 4 days, a subculture is made using trypsinised cells; after incubation for a further 4 days, the cultures are examined for residual live-rabies virus by the immunofluorescence test. The inactivated virus harvest complies if no live virus is detected European Pharmacopoeia, 2012b).

iv) *Final product batch/serial tests*

After combining all of the ingredients the final blend contains the definite vaccine formulation. Filling of the final blend into vials is the last step in the production process for a batch/serial. This final batch/serial undergoes the tests described below.

Sterility

Tests for sterility and freedom from contamination of biological materials may be found in chapter 1.1.7.

Safety

Unless consistent safety of the product is demonstrated and approved in the registration dossier, and the production process is approved for consistency in accordance with the standard requirements referred to in chapter 1.1.6, batch safety testing is to be performed.

This final product batch/serial safety test is conducted to detect any abnormal local or systemic adverse reactions. For the purposes of batch/serial release, each of at least two healthy seronegative target animals is inoculated by the recommended route a minimum of a double dose of the vaccine. The animals are observed at least daily for 14 days. The vaccine complies with the test if no animal shows adverse reactions or dies of causes attributable to the vaccine (European Pharmacopoeia, 2012b).

Residual live virus

The test is carried out using a pool of the contents of five containers.

For vaccines that do not contain an adjuvant, a suitable amplification test for residual live virus is carried out using the same type of cell culture as that used in the production of the vaccine or a cell culture shown to be at least as sensitive. The vaccine complies with the test if no live virus is detected.

For vaccines that contain an adjuvant, 0.03 ml of a pool of at least five times the smallest stated dose is injected intracerebrally into each of no fewer than ten mice, each weighing 11–15 g. To avoid interference of any microbial preservative or the adjuvant, the vaccine may be diluted more than 10 times before injection. In this case, or if the vaccine strain is pathogenic only for suckling mice, the test is carried out on 1- to 4-day-old mice. The animals are observed for 21 days. If more than two animals die during the first 48 hours, the test is repeated. The vaccine complies with the test if, from the day 3 to day 21 post-injection, the animals show no signs of rabies and immunofluorescence test carried out on the brains of the animals show no indication of the presence of rabies virus.

Batch/serial potency

For live attenuated and current biotechnology-derived vaccines, virus titrations are reliable indicators of vaccine potency once a relationship has been established between the level of protection conferred by the vaccine in the target species and titres of the modified live vaccine. Virus titration should be carried out using cell cultures. This allows laboratories to act in accordance with the provisions of the European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes.

The potency of inactivated vaccines is tested in mice by a serological test (Krämer *et al.,* 2010), or a challenge test (European Pharmacopoeia, 2012b; WHO, 1996). For inactivated virus vaccines, an *in-vitro* agent identification test has been reported (Stokes *et al.,* 2012).

It is not necessary to carry out the potency tests described in Section C.2.1.b.iv.a *Serological test*, and Section C.2.1.b.iv.b *Challenge test*, for each batch/serial of vaccine produced, provided that at least one of these tests has been carried out on a previous batch/serial of vaccine and this batch/serial has been demonstrated to meet the minimum potency requirements. Under these circumstances, an alternative validated method may be used to establish batch/serial potency, the criteria for acceptance being set with reference to the batch/serial of vaccine that has given satisfactory results in either the serological test or the challenge test as described below:

a) In the serological test, the test vaccine is compared with the standard reference vaccine by measuring the amounts of neutralising anti-rabies virus-specific antibodies in mouse serum. The test vaccine passes if it induces more antibodies than the standard reference vaccine. The test should be performed as follows:

    Five mice, each weighing 18–20 g are used. Each mouse is vaccinated by a subcutaneous or intramuscular route using 1/5 of the recommended dose volume. Blood samples are taken 14 days after the injection and the sera are tested individually for rabies antibodies using the FAVN test or the RFFIT (European Pharmacopoeia, 2012b).

    The vaccine meets the requirement if the average or median antibody titre is equal to or higher than that obtained with a batch/serial of vaccine that gave satisfactory results in the test described in Section C.2.1.b.iv.b: *Challenge test*.

b) In the challenge test, the test vaccine is compared with the reference vaccine by measuring the protection conferred on mice. The test vaccine passes if it induces more protection than the reference vaccine.

    According to the European Pharmacopoeia, the test described below uses a parallel-line model with at least 3 points for the vaccine to be examined and the reference preparation.

    Selection and distribution of the test animals

    Healthy female mice about 4 weeks old, preferably in a range of 18–20 g live weight and from the same stock should be used in the test. The mice should be distributed into at least ten groups of no fewer than ten mice.

    Preparation of the challenge suspension

    A group of mice is inoculated intracerebrally with the CVS strain of rabies virus; when the mice show signs of rabies, they are killed, the brains are removed and a homogenate of the brain tissue is prepared in a suitable diluent. Gross particulate matter is separated by centrifugation and the supernatant is used as challenge suspension. The suspension is distributed in small volumes in ampoules that are sealed and stored at a temperature below –60°C. One ampoule of the suspension is thawed and serial dilutions are made in a suitable diluent. Each dilution is allocated to a group of mice and each mouse is injected intracerebrally with 0.025–0.03 ml of the dilution allocated to its group. The animals are observed at least daily for 14 days and the number in each group that develop signs of rabies between day 5 and day 14 is recorded. The median infectious dose (ID_{50}) of the undiluted suspension is calculated.

    Determination of potency of the vaccine to be examined

    At least three serial dilutions of the vaccine are prepared for examination along with three similar dilutions of the reference preparation. The dilutions are prepared such that those containing the largest quantity of vaccine may be expected to protect more than 50% of the animals into which they are injected and those containing the smallest quantities of vaccine may be expected to protect less than 50% of the animals into which they are injected.

    Each dilution is allocated to a different group of mice and each mouse is injected by the intraperitoneal route with 0.5 ml of the dilution allocated to its group. A suspension of the challenge virus is prepared 14 days after the injection such that, on the basis of the preliminary titration, it contains about 50 ID_{50} in each 0.025–0.03 ml. Each vaccinated mouse is injected intracerebrally with 0.025–0.03 ml of this suspension.

    Three suitable serial dilutions of the challenge suspension are prepared. The challenge suspension and the three dilutions are allocated, one to each of four groups of ten unvaccinated mice. Each mouse is injected intracerebrally with 0.025–0.03 ml of the suspension of the dilution allocated to its group (Stokes *et al.*, 2012). The animals in each group are observed at least daily for 14 days. The test is invalid if more than two mice of any group succumb within the first 4 days after challenge. The number in each group that develop signs of rabies between day 5 and day 14 after challenge is recorded.

The test is invalid unless:

- for both the vaccine being examined and the reference preparation, the 50% protective dose lies between the smallest and the largest dose given to the mice;

- the titration of the challenge suspension shows that 0.03 ml of the suspension contained between 10 and 50 ID_{50};

- the confidence limits (p = 0.95) are not less than 25% and not more than 400% of the estimated potency; when this validity criterion is not met, the lower limit of the estimated potency must be at least 1 IU in the smallest prescribed dose;

- statistical analysis shows a significant slope (p = 0.95) and no significant deviations from linearity or parallelism of the dose–response curves (p = 0.99).

The vaccine meets the OIE requirement if the estimated potency is not less than 1 IU in the smallest prescribed dose.

Application of alternative end-points

Once a laboratory has established the above assay for routine use, the lethal end-point is replaced by an observation of clinical signs and the application of an end-point earlier than death to reduce animal suffering. The following is given as an example.

The progress of rabies infection in mice following intracerebral injection can be represented by five stages defined by typical clinical signs:

Stage 1: ruffled fur, hunched back;

Stage 2: slow movements, loss of alertness (circular movements may also occur);

Stage 3: shaky movements, trembling, convulsions;

Stage 4: signs of paresis or paralysis;

Stage 5: moribund state.

Mice are observed at least twice daily from day 4 after challenge. Clinical signs are recorded at each observation. Experience has shown that using stage 3 as an end-point yields assay results equivalent to those found when a lethal end-point is used. This must be verified by each laboratory by scoring a suitable number of assays using both clinical signs and the lethal end-point.

The potency test of the National Institute of Health (NIH test), as described in the US Code of Federal Regulations (9CFR), is similar to the European test, except that a second injection of vaccine is performed one week after the first injection. Reading and calculation are identical (European Pharmacopoeia, 2012a; 9CFR, 2010).

c) Requirements for authorisation/licensing/registration

i) *Manufacturing process*

For registration of vaccine, all relevant details concerning manufacture of the vaccine and quality control testing (see Section C.2.1.a and b) should be submitted to the authorities. This information shall be provided from three consecutive vaccine batches/serials with a volume not less than 1/3 of the typical industrial batch/serial volume.

ii) *Safety requirements*

Safety tests for registration of inactivated injectable rabies vaccine are identical to those described in Section C.2.1.b.iv *Safety* and need to be carried out in accordance with VICH[4] Guideline 44 Section 2.1.2, as outlined here.

For vaccines that require a single life-time dose or primary vaccination series only, the primary vaccination regimen should be used. For vaccines that require a single dose or primary vaccination series followed by booster vaccination, the primary vaccination regimen and an additional dose should be used. For convenience, the recommended intervals between administrations may be shortened to an interval of at least 14 days. Evaluation of the one or repeat dose testing should be conducted using either a pilot or production batch containing the maximum release potency or, in the case where maximum release potency to be licensed is not specified, then a justified multiple of the minimum release potency should be used.

---

4   VICH: International Cooperation on Harmonisation of Technical Requirements for Registration of Veterinary Products

In general, eight animals per group should be used unless otherwise justified. For each target species, the most sensitive class, age and sex proposed on the label should be used. Seronegative animals should be used. In cases where seronegative animals are not available, the use of alternatives should be justified.

If multiple routes and methods of administration are specified for the product concerned, administration by all routes is recommended. If one route of administration has been shown to cause the most severe effects, this single route may be selected as the only one for use in the study. Special attention shall be paid to the site of injection, especially for cats. Site recommendations should be followed.

Biotechnology-derived injectable vaccines do not shed virulent rabies virus, but other safety concerns may be evident (Roess *et al.,* 2012). Specific requirements for safety of this type of vaccine are described in chapter 1.1.6 for biotechnology-derived vaccines.

Tests for reversion to virulence of modified live vaccines (MLV) should be done in accordance with chapter 1.1.6.

Precautions and hazards

Current injectable rabies vaccines are innocuous if inactivated, not adjuvanted and present no toxic hazard to vaccinators. For adjuvanted vaccines, live attenuated vaccines and biotechnology-derived vaccines, warnings shall be provided by manufacturers that medical advice shall be sought in case of self-injection

iii) *Efficacy requirements*

In herbivores, as a minimum requirement, efficacy can be demonstrated by serology (European Pharmacopoeia, 2012a). In other species efficacy is demonstrated by challenge.

Test animals shall be uniform and have no neutralising antibodies to rabies as determined by the serum neutralisation tests that are prescribed tests for international trade (see Section B of this chapter).

For challenge tests, a challenge virus is prepared to determine the dose and route that is sufficient to induce clinical signs of rabies in at least 80% of unvaccinated control animals. As soon as clinical signs of rabies are observed, animals are killed and rabies is confirmed using the diagnostic tests described in Section B of this chapter.

For efficacy tests in vaccinated animals, such as dogs, 25 or more animals shall be used as vaccinates. The vaccine formulation used for the efficacy trial is the minimum to be used for routine production. Ten or more additional animals shall be added as controls. At the end of the period claimed for duration of immunity, vaccinates and controls are challenged with the predetermined dose as described above. Animals are observed at least daily for 90 days after challenge. As soon as clinical signs of rabies are observed, animals are killed and rabies is confirmed using appropriate diagnostic tests. At the end of the observation period, all surviving animals are killed and their brains are tested using the diagnostic tests described in Section B of this chapter.

Requirements for acceptance in challenge tests shall be death due to rabies in at least 80% of the control animals while at least 22 of 25 or 26 of 30 or a statistically equivalent number of the vaccinates remain free of rabies for a period of 90 days.

iv) *Stability*

As described in chapter 1.1.6.

v) *Duration of immunity*

As part of the authorisation procedure the manufacturer should be required to demonstrate the *duration of immunity* of a given vaccine by either challenge or the use of a validated alternative test, such as serology at the end of the claimed period of protection.

3. Rabies vaccines for oral use

3.1. Background

The concept of oral vaccination of rabies in animals was developed in the 1970s as a result of the work of George Baer in the USA (Baer *et al.,* 1991), continued by Franz Steck in Switzerland (Steck *et al.,* 1982). All vaccines currently used for oral vaccination programmes are either MLV or biotechnology-derived vaccines (BDVs), which are constructed by insertion of a rabies virus glycoprotein gene into a viral vector. Pox viruses (e.g. vaccinia) and adenoviruses are the vectors most commonly used. Immunisation occurs by uptake of the oral vaccine through

the lymphoid tissue of the oral mucosa and tonsils, where expression of viral proteins stimulates the immune system. The majority of vaccine viruses are destroyed by the gastric environment in the stomachs of vaccinates. The accessibility of feral and wild animals for parenteral vaccination is problematic, and oral vaccination offers an effective solution. Of paramount consideration for oral vaccine use is safety, not only for the target animals, but for the environment and other species, including humans, who may come in contact with the vaccine (see chapter 1.1.6).

The Veterinary Public Health Department of WHO was instrumental in defining the requirements for guaranteeing the safety and efficacy of oral vaccines both for the target species and non-target species (especially humans) who might be in contact with baits or a recently vaccinated animal (WHO, 1989; 2005; 2007).

3.2. Outline of production and minimum requirement for vaccines

In addition to the requirements outlined in chapter 1.1.6, the following specific requirements must be met.

a)   Characteristics of the seed

The seed is a preparation of a suitable immunogenic strain of a highly attenuated rabies virus or a glycoprotein (G) vectored virus. The history of the MSV, its immunogenic properties, safety and absence of reversion to virulence shall be well characterised, including the presence of genetic markers for MLV. A full genome sequence of the seed virus should be submitted to the regulatory authority and deposited in a public database for verification of identity and genetic stability. For biotechnology-derived MSV, additional information on recombination should be considered, as a theoretical risk exists for the potential of genetic transfer and exchange with other viruses.

b)   Method of manufacture

i)   *Procedure*

The seed virus is used to infect a suspension or monolayers of an established cell line. These cell cultures should be proven to be non-tumorigenic and free from contaminating microorganisms.

ii)   *In-process control*

During the production process, tests are undertaken at different times before constitution of the final blend, which allow the consistency of production to be verified. Tests for infectivity and sterility are fundamental in process controls.

iii)   *Final product batch/serial tests*

After combining all of the ingredients the final blend contains the definitive formulation that is either used in a freeze-dried or in liquid form. Filling the final blend into sachets/capsules to be included in baits or filling directly into the bait is the last step of production of a batch/serial. This final batch/serial undergoes the tests described below:

Sterility

This test may be done before or after filling the bait. Tests for sterility and freedom from contamination with biological materials are described in chapter 1.1.7.

Identity

The identity of the immunogen is tested using rabies anti-serum monospecific of the glycoprotein G for biotechnology-derived vaccine, and for MLV a test is carried out to demonstrate the presence of the genetic marker.

Batch/serial purity

For MLV, 1 in 10 and 1 in 1000 dilutions of the vaccine are inoculated into susceptible cell cultures. The dilutions are incubated at 37°C. After 2, 4 and 6 days, the cells are stained with a panel of monoclonal antibodies that do not react with the vaccine strain but that react with other strains of rabies vaccine (for example, street virus, Pasteur strain). The vaccine complies with the test if it shows no evidence of contaminating rabies virus (European Pharmacopoeia, 2012b).

Safety

Unless consistent safety of the product is demonstrated and approved in the registration dossier, and the production process is approved for consistency in accordance with the standard requirements referred to in chapter 1.1.6, batch safety testing is to be performed as follows: two healthy dogs are

administered orally with ten doses and are observed for 14 days. In addition, a 0.5 ml dose is injected by the intraperitoneal or subcutaneous routes into eight mice, which are then observed for 14 days. If any adverse reactions attributable to the products occur in any animals during the observation period, the batch/serial is unsatisfactory.

Batch/serial potency

For live attenuated and biotechnology-derived vaccines, virus titrations are reliable indicators of vaccine potency once a relationship has been established between the level of protection conferred by the vaccine in the target species and titres of the vaccine. Virus titration should be carried out using cell cultures. This allows laboratories to act in accordance with the provisions of the European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes. The potency of a biotechnology-derived vaccine can also be determined by measuring seroconversion in vaccinated animals. More than 80% of vaccinates should develop a titre of >0.5 IU/ml.

c) Requirements for authorisation/registration/licensing

i) *Manufacturing process*

For registration of a vaccine, all relevant details concerning manufacture of the vaccine and quality control testing should be submitted to the Competent Authority. This information shall be provided from three consecutive vaccine batch/serials with a volume not less than 1/3 of the typical industrial batch/serial volume.

The in-process controls are part of the manufacturing process.

ii) *Safety requirements*

In accordance with chapter 1.1.6, safety tests are required in each species for which the product is indicated. For purposes of this class of product, only the overdose and reversion-to-virulence safety tests are required.

Tests for reversion to virulence of MLVs and safety testing of biotechnology-derived vaccines should be done in accordance with chapter 1.1.6.

Modified live vaccines (*MLV*)

- *In target species*

  For the overdose safety test, a 10 × dose of the vaccine suspension is administered, preferably using a syringe, via the oral route to ten young animals (less than 6 months of age for wild animals and less than 10 weeks for dogs), that are free of rabies antibodies. After administration, the possibility of excretion of vaccine virus in the saliva of the animals described above should be assessed by taking swabs several times within the first day after oral immunisation and then daily over 7 days. Any virus recovered should be characterised. The animals are observed for 180 days. Particular attention shall be paid to neurological signs and sudden death and shall be investigated using the prescribed tests for international trade (see Section B of this chapter). At the termination of the study, the brain should be examined for vaccine virus presence.

  The test is satisfactory if no adverse reactions attributable to the vaccine are observed and if no virus is detected in the brain. Virus recovered in swabs should be the vaccine strain and be consistent temporally and quantitatively with limited viral replication.

- *In non-target species*

  A representative group of species that are susceptible to rabies and likely to consume the baits should be investigated. At least ten animals of each species should be tested orally with a field dose of vaccine and observed according to their known incubation period for rabies.

  As testing wild animals might prove to be difficult and should be kept at a minimum, additional tests in laboratory rodents are recommended. Rodents (i.e. mice and rats) should be tested using at least 20 animals per test, inoculated orally with the amount of vaccine strain equivalent to one maximum oral dose. The same number of contact animals should be used for investigation of virus transmission. All animals should be observed daily for at least 30 days. Animals that die from causes not attributable to rabies are eliminated.

  Safety studies should also be undertaken in non-human primates.

Biotechnology-derived vaccines (BDV)

- *In target species*

    For the overdose safety test, a 10× dose of the vaccine suspension is administered, preferably using a syringe, via the oral route to ten animals that are free of rabies antibodies. After administration, the possibility of excretion of vaccine virus in the saliva of these animals should be assessed by taking swabs several times within the first day after oral immunisation and then daily within 7 days. Any virus recovered should be characterised. The animals should be observed for 14 days.

    The test is satisfactory if no adverse reactions attributable to the vaccine are observed. Virus recovered from swabs should be the vaccine strain and be consistent temporally and quantitatively with limited viral replication. For vaccine intended for use in dogs, absence of the virus should be demonstrated 4 days post-immunisation.

- *In non-target species*

    A representative group of species that are susceptible to the virus vector and likely to consume the baits should be investigated. At least ten animals of each species should be tested orally with a field dose of vaccine and observed according to their known incubation period for the vector used.

    As testing wild animals might prove to be difficult and should be kept to a minimum, additional tests in laboratory animals susceptible to the vector are recommended. Laboratory animals should be tested using at least 20 animals per test, inoculated orally with the amount of vaccine strain equivalent to one maximum oral dose. The same number of contact animals should be used for investigation of virus transmission. All animals should be observed daily for at least 14 days. Animals that die from causes not attributable to the disease caused by the vector are eliminated.

    If the viral vector is infectious for humans, safety studies should also be undertaken in non-human primates and in immunocompromised mice.

    The product should also comply with the section in chapter 1.1.6 on licensing of products derived through biotechnology (under revision).

Precautions hazards

The release of oral vaccines into the environment shall comply with the requirements in chapter 1.1.6 (under revision). Current oral rabies vaccines are innocuous when presented in bait form and present no toxic hazard to vaccinators. For leaks from ruptured sachets containing vaccines, warnings shall be provided by manufacturers that medical advice shall be sought in the event of inadvertent contact, especially when contact is with mucosal membranes, skin or skin abrasions.

Prior to initiating vaccination campaigns, public health officials should be informed and public education provided, particularly not to touch baits or be in contact with animals that have recently consumed baits.

Public Health information with respect to the risk of oral vaccines in specific human population groups is provided by WHO (2005).

iii) *Efficacy requirements*

Efficacy shall be demonstrated in each species for which the vaccine use is claimed by the manufacturer, using in a first step a direct oral instillation and the virulent virus in challenge tests.

Test animals shall be uniform and have no neutralising antibodies to rabies as determined by the serum neutralisation tests that are prescribed tests for international trade (see Section B of this chapter).

For challenge tests, a challenge virus is prepared to determine the dose and route that is sufficient to induce clinical signs of rabies in at least 80% of unvaccinated control animals for each target species. As soon as clinical signs of rabies are observed, animals are killed and rabies is confirmed using the diagnostic tests described in Section B of this chapter.

For efficacy tests in vaccinated animals, at least 25 animals shall be used as vaccinates. The titre of the vaccine virus that is used in the efficacy test establishes the minimum immunising infectious dose. The volume must not be greater than the volume to be included into the bait. Ten or more additional animals shall be added as controls. After 30 days, vaccinates and controls are challenged with the predetermined dose as described above. Animals are observed daily for 90 days after challenge. As

soon as clinical signs of rabies are observed, animals are killed and rabies is confirmed using appropriate diagnostic tests. At the end of the observation period, all surviving animals are killed and their brain tissues tested using the virus identification tests described in Section B of this chapter.

Requirements for acceptance in challenge tests shall be death due to rabies in at least 80% of the control animals while at least 22 of 25, 26 of 30 or a statistically equivalent number of the vaccinates remain free of rabies for a period of 90 days.

In a second step, efficacy studies should be conducted using oral vaccine with manufactured baits ready to be used in the field. The vaccine should have a minimal titre corresponding to at least ten times the protective dose obtained with the same vaccine experimentally by direct oral instillation (Blancou *et al.,* 1986).

The protection status cannot be checked by serology only; a virulent challenge with the appropriate challenge rabies virus is necessary. The same challenge conditions and requirements as above apply except that the challenge virus will be administered 180 days after vaccination instead of 30 days.

Once the minimum immunising dose has been established in one species, the efficacy study for additional species can be limited to a study using baits. The bait is an integral part of the product and should ideally meet certain criteria:

- Designed for and attractive to the target species and adapted to the mode of distribution. It should not be attractive to humans;

- Keep its form and shape under a wide range of temperature and weather conditions;

- Allow the incorporation of a marker, topical or systemic;

- Ingredients are non-harmful, comply with animal feed standards and should not interfere with vaccine activity;

- Feature a labelling system with a public warning and identification of the product.

iv) *Stability*

A minimum of five samples of the final product ready to be used are incubated at 25°C for 5 days. The vaccine is titrated three times. The mean virus titre must be at least the minimum virus titre stated on the label or as approved for end of shelf life. The bait is heated at 40°C for 1 hour, and the bait casing complies with the test if it remains in its original shape and adheres to the vaccine container.

REFERENCES

BADRANE H., BAHLOUL C., PERRIN P. & TORDO N. (2001). Evidence of two Lyssavirus phylogroups with distinct pathogenicity and immunogenicity. *J. Virol.*, **75**, 3268–3276.

BAER G.M. (1991). The Natural History of Rabies, Second Edition. CRC Press, Boca Raton, Florida, USA, 620 pp.

BARRAT J. (1992). Experimental Diagnosis of Rabies. Adaptations to Field and Tropical Conditions. Proceedings of the International Conference on Epidemiology, Control and Prevention of Rabies in Eastern and Southern Africa. Lusaka, Zambia, 2–5 June 1992, 72–83.

BARRAT J. & AUBERT M.F.A. (1995). Diagnostic de la rage animale en France de 1991 à 1993, bilan de CNEVA laboratoire d'études sur la rage et la pathologie des animaux sauvages en France. *Revue Méd. Vét.,* **146**, 561–566.

BARRAT J. & BLANCOU J. (1988). Technique simplifiée de prélèvement, de conditionnement et d'expédition de matière cérébrale pour le diagnostic de rage. Doc. WHO/Rab. Res./88.27.

BELLINGER D.A., CHANG J., BUNN T.O., PICK J.R., MURPHY M. & RAHIJA R. (1983). Rabies induced in a cat by high-egg-passage Flury strain vaccine. *J. Am. Vet. Med. Assoc.*, **183**, 997–998.

BINGHAM J. & VAN DER MERWE M. (2002). Distribution of rabies antigen in infected brain material: determining the reliability of different regions of the brain for the rabies fluorescent antibody test. J. *Virol. Methods*, **101**, 85–94.

BLANCOU J., SCHNEIDER L.G., WANDELER A. & PASTORET P.P. (1986). Vaccination du renard roux (*Vulpes vulpes*) contre la rage par voie orale. Bilan d'essais en station expérimentale. *Rev. Ecol.* (*Terre Vie*), **40**, 249–255.

BOURHY H., KISSI B. & TORDO N. (1993). Molecular diversity of the lyssavirus genus. *Virology*, **194**, 70–81.

BROOKES S.M., PARSONS G., JOHNSON N., MCELHINNEY L.M. & FOOKS A.R. (2005). Rabies human diploid cell vaccine elicits cross-neutralising and cross-protecting immune responses against European and Australian bat lyssaviruses. *Vaccine*, **23**, 4101–4109.

CLIQUET F., AUBERT M. & SAGNE L. (1998). Development of a fluorescent antibody virus neutralisation test (FAVN test) for the quantitation of rabies-neutralising antibody. *J. Immunol. Methods*, **212**, 79–87.

CLIQUET F., ROBARDET E., MUST K., LAINE M., PEIK K., PICARD-MEYER E., GUIOT A.L. & NIIN E. (2012). Eliminating rabies in Estonia. *PLoS Negl. Trop. Dis.*, **6** (2):e1535. doi: 10.1371/journal.pntd.0001535. Epub 2012 Feb 28.

CODE OF FEDERAL REGULATIONS (OF THE UNITED STATES OF AMERICA) (9CFR) (2010). Rabies Vaccine, Killed Virus, 9 CFR 113.209.

ESH J.B., CUNNINGHAM J.G. & WIKTOR T.J. (1982). Vaccine-induced rabies in four cats. *J. Am. Vet. Med. Assoc.*, **180**, 1336–1339.

EUROPEAN PHARMACOPOEIA 7.0. (2012a). Monograph 0451: Rabies vaccine (live, oral) for foxes. European Directorate for the Quality of Medicines and HealthCare (EDQM), Council of Europe, Strasbourg, France.

EUROPEAN PHARMACOPOEIA 7.0. (2012b). Monograph 0723: Rabies vaccine (inactivated) for veterinary use. European Directorate for the Quality of Medicines and HealthCare (EDQM), Council of Europe, Strasbourg, France.

FEHLNER-GARDINER C., NADIN-DAVIS S., ARMSTRONG J., MULDOON F., BACHMANN P. & WANDELER A. (2008). Era vaccine-derived cases of rabies in wildlife and domestic animals in Ontario, Canada, 1989–2004. J. *Wildl. Dis.*, **44** (1), 71–85.

FOOKS A.R., JOHNSON N., FREULING C.M., WAKELEY P.R., BANYARD A.C., MCELHINNEY L.M., MARSTON D.A., DASTJERDI A., WRIGHT E., WEISS R.A. & MULLER T. (2009). Emerging technologies for the detection of rabies virus: challenges and hopes in the 21st century. *PLoS Negl. Trop. Dis.*, **3**, e530.

GEUE L., SCHARES S., SCHNICK C., KLIEMT J., BECKERT A., FREULING C., CONRATHS F.J., HOFFMANN B., ZANONI R., MARSTON D., MCELHINNEY L., JOHNSON N., FOOKS A.R., TORDO N. & MÜLLER T. (2008). Genetic characterisation of attenuated SAD rabies virus strains used for oral vaccination of wildlife. *Vaccine*, **26**, 3227–3235.

HANLON C.A., KUZMIN I.V., BLANTON J.D., WELDON W.C., MANANGAN J.S. & RUPPRECHT C.E. (2005). Efficacy of rabies biologics against new lyssaviruses from Eurasia. *Virus Res.*, **111**, 44–54.

HOOPER D.C., MORIMOTO K., BETTE M., Weihe E., Koprowski H. & Dietzschold B. (1998). Collaboration of antibody and inflammation in clearance of rabies virus from the central nervous system. *J. Virol.*, **72**, 3711–3719.

ICTV (INTERNATIONAL COMMITTEE ON TAXONOMY OF VIRUSES): http://www.ictvonline.org/virusTaxonomy.asp?bhcp=1

KANG B., OH J., LEE C., PARK B.K., PARK Y., HONG K., LEE K., CHO B. & SONG D. (2007). Evaluation of a rapid immunodiagnostic test kit for rabies virus. *J. Virol. Methods,* **145**, 30–36.

KRÄMER B., BRUCKNER L., DAAS A. & MILNE C. (2010). Collaborative study for validation of a serological potency assay for rabies vaccine (inactivated) for veterinary use. *Pharmeur. Bio. Sci. Notes*, **2**, 37–55.

KUZMIN I.V., MAYER A.E., NIEZGODA M, MARKOTTER W., AGWANDA B., BREIMAN R.F. & RUPPRECHT C.E. (2010). Shimoni bat virus, a new representative of the Lyssavirus genus. *Virus Res.*, **149**, 197–210.

LEMBO T., NIEZGODA M., VELASCO-VILLA A., CLEAVELAND S., ERNEST E. & RUPPRECHT C.E. (2006). Evaluation of a direct, rapid immunohistochemical test for rabies diagnosis. *Emerg. Infect. Dis.,* **12** (2), 310–313.

LYNG J. (1994). Calibration of a replacement preparation for the international standard for rabies immunoglobulin. *Biologicals*, **22** (3), 249–255.

MARKOTTER W., KUZMIN I., RUPPRECHT C.E. & NEL L.H. (2008). Phylogeny of Lagos bat virus: challenges for lyssavirus taxonomy. *Virus Res.*, **135**, 10–21.

MONTANO HIROSE J.A., BOURHY H. & SUREAU P. (1991). Retro-orbital route for brain specimen collection for rabies diagnosis. *Vet. Rec.*, **129**, 291–292.

Müller T., Bätza, H.-J., Beckert A., Bunzenthal C., Cox J.H., Freuling C., Fooks A.R., Frost J., Geue L., Hoeflechner A., Marston D., Neubert A., Neubert L., Revilla-Fernández S., Vanek E., Vos A., Wodak E., Zimmer K. & Mettenleiter T.C. (2009). Analysis of vaccine-virus-associated rabies cases in red foxes (*Vulpes vulpes*) after oral rabies vaccination campaigns in Germany and Austria. *Arch. Virol.*, **154** (7), 1081–1091.

Pastoret P.P., Brochier A., Aguilar-Setién A. & Blancou J. (1997). Chapter 18 Examples of vaccination, Part 2: Vaccination against rabies. *In:* Veterinary Vaccinology, Pastoret, P.-P., Blancou J., Vannier P. & Verschueren, eds. Elsevier Science, BV, Amsterdam, the Netherlands, pp 616–628.

Picard-Meyer E., Barrat J. & Cliquet F. (2007). Use of filter paper (FTA) technology for sampling, recovery and molecular characterisation of rabies viruses. *J. Virol. Methods*, **140**, 174–182.

Roess A.A., Rea N., Lederman E., Dato V., Chipman R., Slate D., Reynolds M.G., Damon I.K. & Rupprecht C.E. (2012). National surveillance for human and pet contact with oral rabies vaccine baits, 2001–2009. *J. Am. Vet. Med. Assoc.*, **240**, 163–168.

Rudd R.J. & Trimachi C.V. (1989). Development and evaluation of an *in vitro* virus isolation procedure as a replacement for the mouse inoculation test in rabies diagnosis. *J. Clin. Microbiol.*, **27**, 2522–2528.

Rupprecht C.E., Barrett J., Briggs D., Cliquet F., Fooks A.R., Lumlertdacha B., Meslin F.X., Müler T., Nel L.H., Schneider C., Tordo N. & Wandeler A.I. (2008). Can rabies be eradicated? *Dev. Biol.* (Basel), **131**, 95–121.

Servat A., Feyssaguet M., Blanchard I., Morize J.L., Schereffer J.L., Boué F. & Cliquet F. (2007). A quantitative indirect ELISA to monitor the effectiveness of rabies vaccination in domestic and wild carnivores. *J. Immunol. Methods*, **318**, 1–10.

Smith J.S., Yager P.A. & Baer G.C. (1973). A rapid reproducible test for determining rabies neutralizing antibody. *Bull. WHO*, **48**, 535–541.

Steck F., Wandeler A.I., Bichsel P., Capt S. & Schneider L. (1982). Oral immunisation of foxes against rabies: a field study. *Zentralblatt für Veterinärmedizin*, Reihe **B 29**, 372–396.

Stokes W., McFarland R., Kulpa-Eddy J., Gatewood D., Levis R., Halder M., Pulle G., Kojima H., Casey W., Gaydamaka A., Miller T., Brown K., Lewis C., Chapsal J.M., Bruckner L., Gairola S., Kamphuis E., Rupprecht C.E., Wunderli P., McElhinney L., De Mattia F., Gamoh K., Hill R., Reed D., Doelling V., Johnson N., Allen D., Rinckel L. & Jones B. (2012). Report on the international workshop on alternative methods for human and veterinary rabies vaccine testing: State of the science and planning the way forward. *Biologicals*, **40**, 369–381.

Tao L., Ge J., Wang X., Zhai H., Hua T., Zhao B., Kong D., Yang C., Chen H. & Bu Z. (2010). Molecular basis of neurovirulence of flury rabies virus vaccine strains: importance of the polymerase and the glycoprotein R333Q mutation. *J. Virol.*, **84**, 8926–8936.

Taylor J., Trimarchi C., Weinberg R., Languet B., Guillemin F., Desmettre P. & Paoletti E. (1991). Efficacy studies on a canarypox-rabies recombinant virus. *Vaccine*, **9**, 190–193.

Von Teichman B.F., De Koker W.C., Bosch S.J., Bishop G.C., Meridith C.D. & Bingham J. (1998). Mokola virus infection: description of recent South African cases and a review of the virus epidemiology. *J. S. Afr. Vet. Assoc.,* **69**, 169–171.

Wachendôrfer G., Kiefert C. & Frost J.W. (1982). Safety tests with Flury HEP strain 675 in wild-living European mammals. *Comp. Immunol. Microbiol. Infect. Dis.*, **5** (1–3), 177–180.

Warner C.K., Whitfield S.G., Fekadu M. & Ho H. (1997). Procedures for reproducible detection of rabies virus antigen mRNA and genome *in situ* in formalin-fixed tissues. *J. Virol. Methods*, **67**, 5–12.

World Health Organization (1989). Report of WHO Consultation on requirements and criteria for field trials on oral rabies vaccination of dogs and wild carnivores, Geneva, 1–2 March 1989, Doc. WHO/Rab.Res./89.32.

World Health Organization (1985). World Health Organisation Expert Committee on Biological Standards, Thirty-Fifth Report; WHO Technical Report Series No. 725. WHO, Geneva, Switzerland.

World Health Organisation (1996). Laboratory Techniques in Rabies, Fourth Edition, Meslin F.-X., Kaplan M.M. & Koprowski H., eds. WHO, Geneva, Switzerland.

WORLD HEALTH ORGANIZATION (2005). World Health Organisation Expert Committee on Rabies, Eighth Report; WHO Technical Report Series, **931**. WHO, Geneva, Switzerland, 1–87.

WORLD HEALTH ORGANIZATION (2007). Oral Vaccination of Dogs against Rabies. WHO, Geneva, Switzerland.

WU X., SMITH T.G. & RUPPRECHT C.E. (2011). From brain passage to cell adaptation: the road of human rabies vaccine development. *Exp. Rev. Vaccines*, **10**, 1597–1608.

XU G., WEBER P., HU Q., XUE H., AUDRY L., LI C., WU J. & BOURHY H. (2007). A simple sandwich ELISA (WELYSSA) for the detection of lyssavirus nucleocapsid in rabies suspected specimens using mouse monoclonal antibodies. *Biologicals*, **35**, 297–302.

*

*   *

NB: There are OIE Reference Laboratories for Rabies
(see Table in Part 4 of this *Terrestrial Manual* or consult the OIE web site for the most up-to-date list:
http://www.oie.int/en/our-scientific-expertise/reference-laboratories/list-of-laboratories/).
Please contact the OIE Reference Laboratories for any further information on
diagnostic tests, reagents and vaccines for rabies