

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 1.1.2 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 seroconversion 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 1.1.1. Collection and shipment of diagnostic specimens.

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% CO₂. 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% CO₂ 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% CO₂ 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.

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 Expert Committee on Rabies, 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).

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

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 Expert Committee on Biological Standards, 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).

2 National Institute for Biological Standards and Control (NIBSC), Blanche Lane, South Mimms, Potters Bar, Hertfordshire EN6 3QG, United Kingdom (UK).

- **Essential equipment**

Humidified incubator at 35°C/37°C with 5% CO₂; 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 Ca²⁺ and Mg²⁺, 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 3 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 3 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**

- 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 cm² cell culture flask. Cells are collected within a volume of 20–30 ml in cell culture medium with 10% heat-inactivated FCS.
- 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.
- 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.
- 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 TCID₅₀ (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.
- Cell suspension*: the day before titration, a cell suspension containing 10⁵ 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% CO₂.
 - 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% CO₂. 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% CO₂.
- 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 TCID₅₀.

- **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 log₁₀.

		Plate 1: Controls								
		H	G	F	E	D	C	B	A	
Challenge virus standard titration										1
										2
										3
										4
OIE standard serum (0.5 IU/ml)	50 µl						50 µl			5
	50 µl						50 µl			6
	50 µl						50 µl			7
	50 µl						50 µl			8
Serum or internal positive control or WHO Standard serum	50 µl									9
	50 µl									10
	50 µl									11
	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
Serum 1	A	50 µl					50 µl					
	B	50 µl					50 µl					
	C	50 µl					50 µl					
	D	50 µl					50 µl					
Serum 2	E	50 µl					50 µl					
	F	50 µl					50 µl					
	G	50 µl					50 µl					
	H	50 µl					50 µl					

- **Addition of challenge virus standard**

- 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.
- One dilution from this tube is prepared in order to obtain 100 TCID₅₀ 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 TCID₅₀/50 µl.
- Incubate the microplates at $35\text{--}37^{\circ}\text{C}$ in a humid incubator with 5% CO₂ for 1 hour.
- 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.
- Incubate the microplates for 48 hours at $35\text{--}37^{\circ}\text{C}$ in a humid incubator with 5% CO₂.

- **Fixation and staining**

- 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.
- Add 50 µl of the FITC anti-rabies conjugate, at the working dilution, to each well, gently rock the microplates and incubate at $35\text{--}37^{\circ}\text{C}$ 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**

- 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.
- 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).
- Results of titration of CVS (TCID₅₀), naïve serum (D₅₀ [median dose]) and positive standard (D₅₀) 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.
- 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 D₅₀ value of the day or the mean value of the OIE standard serum.

- Formula to convert the log D₅₀ value in IU/ml titre:

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

Example of conversion:

- log D₅₀ of the serum = 2.27
- theoretical titre of OIE serum 0.5 IU/ml = 0.5 IU/ml
- log D₅₀ of OIE serum = 1.43

(for the log D₅₀ 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 D₅₀ in IU/ml, the laboratories should use only the log D₅₀ 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 (CO₂). 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 × 10⁴ 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% CO₂ 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 × 10⁶ 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 × 10⁷ MNA cells with 1 × 10⁷ 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 × 10⁵ 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% CO₂ 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, FFD₅₀). The stock virus suspension should contain at least 1 × 10⁴ FFD₅₀ 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 FFD₅₀.

- **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 FFD₅₀) to all serum dilutions.
- v) Mix and incubate at 35°C in a humidified incubator with 0.5% CO₂ 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% CO₂ 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 $\times 160$ – 200 magnification.
- A minimum of three-to-five-fold dilutions of sera is required.
- For the conversion of log D₅₀ to IU/ml, only the log D₅₀ 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 (~ 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 purposes³. 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 AND DIAGNOSTIC BIOLOGICALS

NB: SECTION C IS “UNDER STUDY”. THIS IS THE LAST ADOPTED VERSION PUBLISHED IN 2008

Rabies vaccines prepared from Pasteur’s original 1885 strain and its derivative strains (Pasteur Virus, Challenge Virus Standard, Pitman-Moore, etc.), and strains isolated more recently (Flury, Street-Alabama-Dufferin [SAD], Vnukovo and Kelev), protect against all strains of genotype 1 isolated so far. Conventional rabies virus vaccines may not provide adequate cross-protection against other lyssaviruses, especially in phylogroup II; there is no protection provided against Mokola virus (Von Teichman *et al.*, 1998) and the recently identified West Caucasian Bat Virus (Hanlon *et al.*, 2005). Cross neutralisation using conventional rabies virus vaccines has been demonstrated against two phylogroup I viruses: EBLV type-1 and EVLV type-2 (Brookes *et al.*, 2005). The principles governing the preparation of inactivated rabies vaccines are identical whether they are to be used in humans or animals, although an adjuvant may be added to vaccines for animal use.

Recombinant vaccine (e.g. vaccinia rabies-glycoprotein recombinant) has also proved to be effective (Brochier *et al.*, 1991; Kieny, 1984). The rabies-glycoprotein recombinant vaccines are not live rabies vaccines. They are prepared by inserting non-infectious rabies nucleic acid into a vector such as vaccinia or canary pox. Since these do not contain live rabies virus, animals vaccinated with rabies-glycoprotein recombinant vaccines should not be restricted from entry into countries that have restrictions on entry of animals vaccinated with live rabies vaccines.

For animals, live and recombinant vaccines are effective by the oral route and can be distributed in baits in order to immunise wild (or domestic) animals.

Guidelines for the production of veterinary vaccines are given in Chapter 1.1.8 Principles of veterinary vaccine production. The guidelines given here and in Chapter 1.1.8 are intended to be general in nature and may be supplemented by national and regional requirements.

Different standards apply to vaccines containing live virus modified by passage in eggs or cell cultures to reduce its virulence for the target animal, and to vaccines prepared from inactivated virus. Both types of vaccine have their advantages and disadvantages (Baer, 1991), but they can both be used to immunise animals for periods of between 1 and 3 years. Live attenuated rabies vaccines are not accepted in some countries. They are not to be relied on to protect previously unvaccinated animals that have been exposed to infection (Blancou *et al.*, 1991). Only in humans has the efficiency of post-exposure prophylaxis with vaccine alone been proven and even in these cases there is an additional strong recommendation to administer anti-rabies immunoglobulin.

All handling of the virus during manufacture and testing of vaccines must conform to the strict safety precautions specified by WHO (WHO Expert Committee on Rabies, 1992; WHO, 1996), the OIE (Chapter 1.1.2) and to national guidelines and regulations.

1. Seed management**a) Characteristics of the seed**

Any strain belonging to serotype 1, which has been proved to protect against field rabies viruses (currently found in the country where the vaccine is to be used), is suitable. The strain of virus used should have well-known biological (e.g. pathogenicity) and antigenic properties (typing by MAbs). If it is to be used as a live vaccine, the master seed virus must be shown not to cause clinical rabies. At least two animals (preferably five to six per group) of each of the species for which the vaccine is intended and, so far as possible, any species that might be in contact with vaccine or vaccinated animals, should be tested. This can be done by

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

inoculating in or adjacent to a major nerve, a dose equivalent to ten times the intended viral titre in one dose of the proposed final product. Animals should be observed for at least 90 days for any adverse effect attributable to the master seed.

b) Method of culture

A master cell stock of the seed virus should be prepared and kept at or below -70°C . Subculture from this stock will be used for vaccine production. Virus multiplication is verified by titration during growth of the seed virus.

c) Validation as a vaccine

Before a vaccine is licensed, evidence of efficacy should be established by the challenge of vaccinated and control animals of each target species. The challenge should be performed at the end of the period after vaccination for which the manufacturer claims maintenance of immunity. Antibody kinetics should also be determined in order to establish the correlation between antibody titre and resistance to challenge.

The efficacy of the produced vaccine is assessed by studies on every target species previously vaccinated as recommended. Protection at the end of the period of immunity is monitored by a measurement of specific neutralising antibodies and by challenge with rabies virus. The experimental conditions of this challenge should mimic the natural conditions of infection. The challenge virus should preferably be prepared from locally isolated strains. In animals vaccinated with inactivated vaccines, the percentage of seroconversion and the mean level of antibody allow a good prognosis for survival to challenge (Aubert, 1992).

The correlation between potency in the target species and antigenic value as estimated in mice should be established (see Section C.4.c below).

For the purposes of licensing a vaccine, safety tests should be conducted in the target species. In the case of live virus vaccines used in oral vaccination campaigns (including recombinant vaccines), safety tests should also be carried out on those other species that live in the area of vaccination and could become exposed to the vaccine (Baer, 1991).

Vaccine stability is ascertained by testing batches after prolonged storage, usually 1–2 years. A process of accelerated ageing, by storage at 37°C for 1 week, is sometimes used. The storage life claimed by the manufacturer is checked by the national licensing authority. In general, it is 12–18 months for fluid vaccines, and possibly 24 months for lyophilised vaccines.

2. Method of manufacture

Whatever method is adopted, close attention should be paid to the quality of the substrate. Eggs should be of SPF origin, and the cell cultures, such as BHK cell lines, should conform to international standards of sterility and innocuity.

During manufacture, the multiplication of the virus in one of the substrates mentioned above is monitored, followed by harvesting at the most appropriate time, usually 4–6 days after inoculation of eggs or cell cultures. The virus harvest is suspended in a buffer solution at a dilution that will provide an optimum antigenicity of the end-product. If required, the suspension is either inactivated or lyophilised. An adjuvant is recommended for vaccines prepared from inactivated virus, as well as for other vaccine antigens that may be incorporated in polyvalent vaccines.

a) In cell cultures

Cultures are infected with cell-culture-adapted strains of rabies virus and incubated at $35\text{--}36^{\circ}\text{C}$. These may then be used as live virus vaccines (as in Flury and SAD vaccines), or as inactivated vaccines after the addition of phenol (Semple vaccine) or some other chemical, such as beta-propiolactone.

Cell culture can also be used to grow the vector viruses (e.g. vaccinia virus) harbouring the gene coding for the expression of rabies virus glycoprotein (Kieny, 1984).

b) In eggs

A modified egg-adapted strain of virus is inoculated into SPF-embryonated chicken eggs, which are then incubated at 38°C for 5–6 days. The virus is harvested in the form of infective embryo tissues, and is usually lyophilised and used as a live vaccine. Examples of such vaccines include those that contain the Flury low egg passage (LEP), or the more desirable high egg passage (HEP) variant strain, which is safer for some animal species such as the cat.

c) In animals

Nervous tissue vaccines prepared in animals are no longer considered safe or effective, and their use should be discontinued.

3. In-process control

This consists of monitoring virus growth to provide an optimum titre and ensure the absence of undesirable microbial contamination.

In live virus vaccines, kinetics of virus growth should be established in order to ensure a final titre of virus correlated to the desired protection in target species.

In inactivated virus vaccines, immunogenic properties of the final product may be evaluated by *in-vitro* techniques (e.g. ELISA, agar gel immunodiffusion, antibody-binding tests or infected cell staining). These evaluations will indicate the best time for harvesting the virus in cell cultures.

4. Batch control

a) Sterility

Tests for sterility and freedom from contamination of biological materials may be found in Chapter 1.1.9.

b) Safety

Safety tests for batches of inactivated virus vaccines are carried out by inoculation of cell culture or intracerebrally into mice to detect viable virus. A suitable safety test for live rabies vaccines should be carried out on each lot of vaccine, in the intended host species. At least three, preferably five to six animals of the intended host species should be given a dose equivalent to ten times the recommended field dose, by the recommended route of administration. The animals should be observed for 180 days for adverse reactions attributable to the vaccine (Council of Europe, 2005).

c) Potency/biological activity

The amount of virus present in live attenuated and recombinant vaccines is determined by titration. Once a correlation has been established between the activity of the vaccine in the target species and virus titres, virus titrations become reliable indicators of vaccine efficacy. This is carried out using cell cultures or by the intracerebral inoculation of unweaned mice (in mice it is only possible with a few attenuated viruses). Recombinant vaccines should be monitored for the expressed rabies protein until assured that expression stability is maintained in the manufacturing process. Titre of the vector can then be used as a reliable indicator of vaccine efficacy.

For inactivated virus vaccines, correlation between potency in the target species and antigenic value as estimated in mice provides a reliable indicator of vaccine activity. The potency of the vaccine is established in the USA by the National Institutes of Health (NIH) test. Elsewhere, the European Pharmacopoeia test is widely adopted.

Groups of at least ten mice, aged 3–4 weeks, are inoculated with single, decreasing doses of vaccine in accordance with the European Pharmacopoeia (Council of Europe, 2005), or with two doses, 1-week apart, according to the NIH test (WHO, 1996). A sufficient number of dilutions of vaccine are compared to estimate the dilution at which 50% of the mice are protected against intracerebral challenge 14 days later (Council of Europe, 2005; WHO, 1996).

A WHO international standard vaccine is available (see footnote 2) for calibration of national standards, so that the results of testing for antigenicity can be expressed in IUs. The test is not valid unless:

- i) For both the vaccine to be examined and the standard preparation, the PD₅₀ (50% protective dose) lies between the largest and smallest doses given to the mice.
- ii) The titration of the challenge virus suspension shows that 0.03 ml of the suspension contained 25 mouse intra-cranial LD₅₀ (MIC LD₅₀). The challenge dose should be in the range 12–50 LD₅₀ for a valid test.

- iii) The confidence interval ($p = 0.95$) for the test should not be less than 25% and not more than 400% of the estimated potency: statistical analysis should show a significant slope and no significant deviations from linearity or parallelism of the dose–response lines.

The vaccine passes the test if the estimated potency is not less than 1 IU per dose, in the smallest prescribed dose.

A simplified test can also be used for the purpose of anticipating which vaccines are likely to be of an antigenic value ≥ 1 IU per dose (Aubert & Blancou, 1982). This test used as a screening test is a good way to reduce the number of mice used in vaccine potency control tests.

d) Duration of immunity

Duration of immunity must be established for the product licence in the target species with a defined vaccination protocol.

e) Stability

The proposed shelf life must be verified by appropriate tests. These experiments include biological and physico–chemical stability tests, and should be performed on a sufficient number of batches of vaccine stored under recommended conditions.

The thermostability of live virus vaccines in liquid form is generally poor. For freeze-dried inactivated virus vaccines, stability is generally granted for 2 years at 4°C.

f) Preservatives

Inactivated virus vaccines may contain preservatives (formalin, merthiolate). The nature and quantity of these preservatives should comply with national control regulations.

5. Tests on the final product

a) Safety

See Section C.4.b.

b) Potency

See Section C.4.c.

6. Oral vaccination

The concept of oral vaccination is unique: as stray or wild animals are out of physical reach, dropping vaccine baits into their environment is the only way to immunise them. In the 1980s and 1990s, the Veterinary Public Health Department of WHO organised several meetings of rabies experts to define the requirements for guaranteeing the safety and efficacy of vaccines both for the target species (red fox, raccoon dog, skunk, dog, etc.) and nontarget species, namely wild rodents and any other wild and domestic species that might be in contact with baits or a recently vaccinated animal (WHO, 1989; WHO Expert Committee on Rabies, 2005).

Several guidelines have been established for the quality criteria that vaccines have to satisfy before marketing; the most precise documents are those produced by WHO, the European Pharmacopoeia and the European Commission (European Commission, 2002; European Directorate for the Quality of Medicines, 2007; WHO Expert Committee on Rabies, 2005). Available oral vaccines have been extensively tested by different routes (cerebral, muscular and oral) in a variety of species: puppies and adults of carnivores, avian species, nonhuman primates, rodents and immunocompromised mice. Nonhuman primates have been added to this list since the discovery in 1992 that the original SAD Bern strain is highly pathogenic for baboons by the oral route (Bingham *et al.*, 1992).

All vaccines currently used for oral vaccination programmes are either modified live-virus vaccines or live recombinant vaccines. At the present time, two oral vaccines are recommended by WHO (WHO Expert Committee on Rabies, 2005): a recombinant vaccine – VRG vaccine, and a highly attenuated vaccine – SAG2.

The production controls are closely related to the ones used for parenteral vaccines. The major differences concern three points:

- i) Safety of the vaccine for man, target and non-target species.

- ii) Efficacy of the protection induced by the vaccine.
- iii) Monitoring of the impact of oral vaccination campaigns in the field.

a) Safety considerations

For oral vaccination, either attenuated rabies strains or live-recombinant vaccines may be used. The vaccine should not induce any adverse signs in target and nontarget species. For vaccines used for dog immunisation, saliva should be checked for the absence of vaccinal virus because of possible contact with humans.

The attenuated rabies virus-based vaccines must achieve the lowest residual pathogenicity for target and nontarget species (European Commission, 2002); this is of utmost importance in the case of oral vaccination of dogs as dogs are often in close contact with humans (WHO Expert Committee on Rabies, 2005).

The recombinant vaccines cannot induce any risk of rabies; the safety controls concern only the possible residual pathogenicity of the recombined parental virus.

b) Protection induced by the vaccine

The protection induced by the vaccine must be tested not only with the virus itself (to determine the minimal vaccinating dose) but also with manufactured baits ready to be used in the field. For foxes for instance, the vaccine should have a minimal titre corresponding to at least ten times the 100% protective dose (obtained with the same vaccine experimentally by direct oral instillation) (Blancou *et al.*, 1986).

The protection status cannot be then checked by serology only; a virulent challenge with the homologous street rabies virus is necessary because of the important implication of cell-mediated immunity in response to oral vaccination (European Directorate for the Quality of Medicines, 2007).

c) Monitoring the impact of oral vaccination

The stability of the vaccine in the field is important. The European Commission stresses the importance of checking the 100% protective dose after 7 days of exposure at 25°C (European Commission, 2002). Each vaccine bait should be tested for stability with a melting point above 40°C, and the blister or sachet containing the vaccine should still be covered by the bait casing after 7 days exposure at 40°C (European Commission, 2002).

Aerial distribution of baits is the only way to perform an homogenous, rapid and sufficient distribution for wildlife vaccination. Quality control measures should be used to monitor different key points of baiting; control of vaccine titre, control of area coverage by air and of baiting density should at least be constantly monitored. The cross border cooperation between neighbouring countries is also needed to avoid any unvaccinated area along the border.

For wildlife in Europe, two campaigns are performed yearly: the spring one aims at vaccinating the young population of the target species, its period should then be fixed according to the biology of the target species. The autumn campaign concerns both adult and young animals. It is generally admitted that four campaigns (i.e. 2 years) should be conducted after the last rabies diagnosis.

The impact of vaccination on the host/vector population is monitored in two different ways:

- Directly by measuring the bait uptake by the wild target species. This supposes that a biomarker (generally tetracycline) is included in the bait casing. The same examination allows the age of animals to be determined.
- Directly by measuring the serological response of target animals. This serological control is better done using validated ELISA techniques (Cliquet *et al.*, 2000; Servat *et al.*, 2007) as they are more robust than seroneutralisation tests when testing poor quality field specimens.
- Indirectly by measuring the incidence of rabies in the vaccinated area. Typing of field isolates should be performed (WHO Expert Committee on Rabies, 2005) either by using MABs or by sequencing positive samples from areas where the target species have been vaccinated with attenuated vaccines to possibly distinguish vaccine and field virus strains.

The first two controls should be performed on specially killed animals to collect good quality samples. Rabies monitoring is more sensitive when performed in found dead or ill animals.

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NB: There are OIE Reference Laboratories for Rabies (see Table in Part 3 of this *Terrestrial Manual* or consult the OIE Web site for the most up-to-date list: www.oie.int).