Section 3.1

Multiple species

CHAPTER 3.1.19

RABIES (INFECTION WITH RABIES VIRUS AND OTHER LYSSAVIRUSES)

SUMMARY

Rabies is a major zoonosis for which diagnostic techniques have been standardised internationally. As there are neither gross pathognomonic lesions nor specific and constant clinical signs for rabies, confirmatory diagnosis can only be made in the laboratory. Laboratory techniques are preferably undertaken on central nervous system (CNS) tissue removed from the cranium (for example brain stem, Ammon's horn, thalamus, cerebral cortex, cerebellum and medulla oblongata). A composite of CNS samples should be tested and the brain stem is the most important component of the sample. Laboratories should follow appropriate biosafety and containment procedures as determined by biorisk analysis.

Detection and identification of the agent: *Agent detection is preferably undertaken using primary diagnostic tests such as the direct fluorescent antibody (DFA) test, the direct rapid immunohistochemistry test (dRIT), or pan-lyssavirus reverse-transcription polymerase chain reaction (RT-PCR) assays. DFA test, dRIT, and RT-PCR provide a reliable diagnosis in 98–100% of cases for all lyssavirus strains if an appropriate conjugate or primer/probe is used. For a large number of samples, conventional and real-time RT-PCR can provide rapid results in specially equipped laboratories.*

Histological techniques such as Seller's staining (Negri bodies) are no longer recommended for diagnosis.

In cases of inconclusive results from primary diagnostic tests (DFA test, dRIT, or pan-Lyssavirus RT-PCR), further confirmatory tests (molecular tests, cell culture or mouse inoculation tests) on the same sample or repeat primary diagnostic tests on other samples are recommended. Wherever possible, virus isolation in cell culture should replace mouse inoculation tests.

Characterisation of the agent can be carried out in specialised laboratories using monoclonal antibodies, partial and full genome sequencing followed by phylogenetic analysis. Such techniques can distinguish between field and vaccine strains, and identify the geographical origin of the field strains. These very sensitive tests should be conducted and interpreted by well trained expert personnel.

Serological tests: *Virus neutralisation (VN) and enzyme-linked immunosorbent assays (ELISA) are suitable tests for monitoring the antibody response of vaccinated animals in the framework of rabies control. For the purposes of measuring antibody responses to vaccination prior to international animal movement or trade, only VN methods (fluorescent antibody virus neutralisation test and rapid fluorescent focus inhibition test) are acceptable. Serological tests should not be used for primary diagnosis.*

Requirements for vaccines: *For rabies vaccination in animals, inactivated virus (for companion animals and livestock), live attenuated virus (for wildlife and free-roaming dogs), and recombinant*

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vaccines (for wildlife, cats and dogs) are used. Certain vaccines can be categorised in more than one of these groups.
Vaccine manufacturers should make known the characteristics of the product and undertake necessary experiments satisfying minimum requirements established at national and international levels. Before vaccines can receive relevant regulatory approval, 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.

The potency, efficacy and safety of vaccines are established and controlled using tests formulated by recognised pharmacopoeia.

A. INTRODUCTION

Rabies is caused by neurotropic viruses of the genus *Lyssavirus* in the family *Rhabdoviridae* of the order *Mononegavirales* (Kuhn *et al.,* 2021; Walker *et al.,* 2022), and is transmissible to all mammals. Rabies causes 60,000 human fatalities annually, approximately 1 death every 10 minutes. As the viruses are transmissible to humans, all suspect infected human material must be handled under the appropriate safety conditions specified by the World Health Organization (WHO, 2018). Laboratories working with lyssaviruses or suspect animal material must comply with national biocontainment and biosafety regulations as well as following appropriate biosafety and containment procedures as determined by biorisk analysis (see Chapter 1.1.4 *Biosafety and biosecurity: Standard for managing biological risk in the veterinary laboratory and animal facilities*).

Rabies virus (RABV) represents the taxonomic prototype species “Rabies lyssavirus” in the Lyssavirus genus, which includes other genetic and antigenically-related lyssavirus species (ICTV, 2017)[1]. 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 with limited public and animal health implications. However, all lyssaviruses tested cause clinical disease indistinguishable from RABV.

The lyssaviruses have been divided into at least three phylogroups with distinct pathogenicity and immunogenicity (Kuzmin *et al*., 2010). RABV vaccines may not provide adequate cross-protection against all genetically divergent lyssaviruses. Little or no cross-protection with pre-exposure vaccination and with conventional rabies post-exposure prophylaxis was observed against lyssaviruses of phylogroups 2 and 3 (Badrane *et al*., 2001; Brookes *et al*., 2005; Hanlon *et al*., 2005). WHO recommends the preventive immunisation of all staff handling infected or suspect materials (WHO, 2013).

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 not used 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

Table 1. Test methods available for the diagnosis of rabies and their purpose

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Method	Purpose					
	Population freedom from infection	Individual animal freedom from infection prior to movement	Contribute to eradication policies	Confirmation of clinical cases	Prevalence of infection – surveillance	Immune status in individual animals or populations post-vaccination
Detection and identification of the agent						
DFA (antigen detection)	+++	–	+++	+++	+++	–
dRIT (antigen detection)	+++	–	+++	+++	+++	–
RTCIT (virus isolation)	–	–	+++	+++	+++	–
MIT (virus isolation)	–	–	+	+	+	–
Conventional RT-PCR (RNA detection)	+++	–	+++	+++	+++	–
Real-time RT-PCR (RNA detection)	+++	–	+++	+++	+++	–
Detection of immune response						
VN	–	+++	+++	–	–	+++
ELISA	–	–	+++	–	–	+++

Key: +++ = recommended for this purpose; ++ recommended but has limitations; + = suitable in very limited circumstances; – = not appropriate for this purpose.

DFA = direct fluorescent antibody test; dRIT = direct rapid immunohistochemistry test; RTCIT = rabies tissue culture infection test; RT-PCR = reverse-transcription polymerase chain reaction; MIT = mouse inoculation test; VN = virus neutralisation; ELISA = enzyme-linked immunosorbent assay.

1. Detection and 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

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using laboratory tests.
As RABV 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 that vary in their efficiency, specificity and reliability. In animals, 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, RABV antigen is particularly abundant in the thalamus, pons and medulla. It is recommended that a pool of brain tissues, including the brain stem, should be collected and tested (Bingham & van der Merwe, 2002). The most widely used test for rabies diagnosis is the direct fluorescent antibody (DFA) test.

1.1. Collection of brain samples

Precautions should be taken when handling central nervous system tissues from suspected rabies cases. Protective personal equipment (such as gloves, face shield, mask) 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.

Ideally, the brain is collected following the opening of the skull in a necropsy room, and the appropriate samples are collected, preferably brain stem, Ammon's horn, thalamus, cerebral cortex, cerebellum and medulla oblongata. Alternatively, methods of collecting some brain samples without opening the skull can also be applied; these methods are described in Sections B.1.1.1 *Occipital foramen route for brain sampling* and B.1.1.2 *Retro-orbital route for brain sampling*.

1.1.1. Occipital foramen route for brain sampling

A sample containing portions of medulla oblongata, base of the cerebellum, Ammon's horn region and cerebral cortex can be obtained by introduction of an approximately 5 mm in diameter sturdy plastic cylinder (e.g. 1–2 ml truncated syringe, artificial insemination sheath, 2 ml disposable plastic pipette with tip removed, or similar) into the occipital foramen in the direction of the eye (Barrat & Blancou, 1988). Alternatively, a scalpel and thumb forceps can be used to harvest a complete cross-section of brain stem accessed through the foramen magnum, followed by retrieval of portions of cerebellum using a plastic pipette (Patrick *et al.,* 2019; or see Kansas State Veterinary Laboratory for a video of the sampling technique).

1.1.2. 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 an appropriate biopsy needle 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.

1.2. Shipment of samples

Suspect material should be transported by road according to the regulations given in the International Carriage of Dangerous Goods by Road (ADR). For international air transport, Dangerous Goods Regulations of the International Air Transport Association (IATA) should be followed. These regulations are summarised in Chapter 1.1.3 *Transport of biological material*.

If refrigerated/frozen shipment of samples is not possible, other preservation techniques may be used. The choice of the preservative is dependent on the tests to be used for diagnosis:

i) Formalin-fixed specimens

Formalin fixation (10% [w/v] solution in phosphate buffered saline [PBS]) allows testing with DFA test, immunohistochemistry, conventional and real-time RT-PCR, however modifications may be required and tests can be less sensitive compared with using fresh specimens (Warner *et al*., 1997). Formalin fixation inactivates the virus thus preventing virus isolation.

ii) Glycerol/ phosphate buffered saline (PBS)

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For transportation of specimens, infectivity may be extended for several days if diagnostic specimens are kept in a mixture of 50% glycerol in PBS. Glycerol/PBS slows bacterial action and therefore protects against the chemical and biological effects of putrefaction. Due to the fact that RABV is thermo-labile, this method does not prevent a decline in the viral load in the specimen. Under routine transport conditions in regions with high temperatures (above 30°C), this protection may only be effective for a matter of several days. Therefore, whenever possible specimens in glycerol/PBS should be kept refrigerated. As the virus is not inactivated by glycerol/PBS, all laboratory tests can be used to test these specimens.

iii) Preservation for molecular techniques

For molecular techniques, lysis buffers for nucleic acid extraction and RNA preservation buffers impregnated onto filter paper can be used (Picard-Meyer *et al.*, 2007). These buffers preserve RABV RNA and allow transport of specimens at ambient temperature without specific biohazard precautions for detection of viral RNA and further genetic characterisation of RABV strains.

1.3. Laboratory tests

1.3.1. Immunochemical identification of rabies virus antigen

i) Direct fluorescent antibody (DFA) test

The most widely used test for rabies diagnosis is the DFA, which is recommended by both WHO and WOAH. This test is used directly on a brain impression smear. It is also used to confirm the presence of RABV antigen in cell culture or in brain tissue of mice that have been inoculated for diagnosis. The DFA test is highly sensitive and specific (between 96% and 99%), and gives reliable results on fresh specimens in less than 2 hours. Sensitivity depends on the specimen, the degree of autolysis (McElhinney *et al.*, 2014) and the sample type (Barrat & Aubert, 1995).

Impression smears should be prepared from a composite sample of brain tissue, that includes the brain stem and the cerebellum. If the cerebellum is not available, a cross-section of the Ammon's horns may be used. The smears are fixed in 100% high-grade cold acetone (–20°C) for at least 20 minutes or heat-fixed by passing the slide 2–3 times through a flame. They are subsequently air dried and then stained with specific FITC (fluorescein isothiocyanate)-labelled polyclonal or monoclonal anti-rabies antibody conjugate, diluted to working dilution and sufficient to cover the whole smear, for 30 minutes at 37°C in a humid chamber. DFA test slides should then be examined for specific fluorescence using a fluorescence microscope and filter appropriate for the wavelength (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 DFA test slide. Conserved antigenic sites on the nucleocapsid proteins permit identification of all lyssaviruses with modern commercial preparations of polyclonal anti-rabies antibody conjugates used for diagnostic tests on brain tissue, while monoclonal anti-rabies antibody conjugates may have limited sensitivity regarding different lyssaviruses. Fluorescent antibody conjugates, in particular if made locally, should be fully validated for specificity and sensitivity before use.

The DFA test may be applied to glycerol-preserved specimens after a washing step. If the specimen has been preserved in a formalin solution, the DFA test may be used only after the specimen has been treated with a proteolytic enzyme (Warner *et al.*, 1997). However, the DFA test 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 DFA test, or in all cases of human exposure, further tests on the same sample or repeat DFA test on other samples are recommended. This is particularly important where sample autolysis is confirmed or suspected.

a) Test protocol

1) Label four microscope slides: two control slides for impression smears of control brain tissue (one positive and one negative) and two slides for duplicate testing of the appropriate brain regions.

2) Prepare impression smears of control brain tissue (one positive and one negative), as well test samples, by inverting the microscope slide onto the tissue placed on blotting paper. Remove excess tissue by blotting the

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slide onto clear blotting paper. Work with one sample at a time, using fresh blotting paper for each, and process the positive control last.

3) Allow the slides to air dry.

4) Within the biosafety cabinet place all slides into a Coplin jar containing cold acetone (–20°C) for at least 20 minutes.

5) Remove the slides from the Coplin jar and allow to air dry.

6) Prepare FITC-labelled anti-rabies conjugate as directed by the manufacturer.

7) Add conjugate at working dilution to the positive and negative control smears and to all smears of test samples, in sufficient quantity to cover the whole of the smears.

8) Place the slides in a 37°C incubator in a humid chamber for 30 minutes (45 minutes maximum).

9) Remove the slides and wash in 0.1 M PBS 7.2 for 5 minutes in a Coplin jar, then for a further 5 minutes with fresh PBS.

10) Allow the slides to air dry.

11) Mount cover slips on the slides using 50% glycerol/50% PBS solution.

b) Results

The slides are read on a fluorescence microscope capable of excitation at 488 nm (FITC), using an excitation filter with narrow passband windows in the blue spectrum (475–490 nm). The latter filter reduces breakthrough wavelength excitation. Each impression is observed for rabies-specific fluorescence (indicating the presence of viral antigen) at a magnification of 200× or greater. Specific fluorescence is denoted by bright ‘apple’ green fluorescence generally in the peri-nuclear area of cells, or longer ‘string-like’ neurons. Dull green or red/green auto-fluorescent granules should not be counted as positive antigen. Always read the positive control slide first.

Read the sample slide(s). Examine the tissue samples carefully, if necessary keep returning to the positive control for comparison.

A second operator should examine all slides and the diagnoses of both operators should be the same.

ii) Direct rapid immunohistochemistry test (dRIT)

The dRIT can be used as an alternative to DFA in routine rabies diagnosis as it has similar sensitivity and specificity (Lembo *et al.*, 2006). The principle is similar to the DFA except that the dRIT uses streptavidin–biotin peroxidase staining (Coetzer *et al.*, 2014; Madhusudana *et al.*, 2012; Rupprecht *et al.*, 2014). This test can be used in laboratories that do not have access to a fluorescence microscope. Primary antibodies should be fully validated for specificity and sensitivity before use, taking into consideration regional diversity of lyssaviruses.

a) Test protocol

1) Make touch impressions of suspect CNS tissues (including brainstem) on labelled glass microscope slides (always include standard positive and negative controls).

2) Air-dry slides for ~ 5 minutes at room temperature.

3) Immerse slides in 10% buffered formalin at room temperature for 10 minutes in a Coplin jar or other suitable container.

4) Dip-rinse slides several times to wash off any excess fixative in wash buffer (PBS plus 1% Tween 80 – TPBS).

5) Immerse slides in 3% hydrogen peroxide for 10 minutes.

6) Remove excess hydrogen peroxide by dip-rinsing slides in TPBS. Transfer slides to another TPBS rinse. Work with one slide at a time (leave the remaining slides immersed within the TPBS), remove slide, shake off excess buffer, and blot excess buffer from slide edges surrounding the tissue impression.

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7) Add enough primary antibody conjugate (e.g. biotinylated anti-nucleoprotein poly- or monoclonal antibodies) to cover the impression. Incubate for 10 minutes in a "humidity chamber". This may be accomplished by placing slides on a moistened paper towel and covering with the plastic top of a cell culture plate or another simple cover.

8) After incubation shake off excess conjugate. Dip-rinse slides with TPBS. Shake off excess TPBS and blot buffer from slide edges surrounding the impression.

9) Treat each slide with streptavidin–peroxidase complex, adding enough of this reagent to the slide to cover the impression. Incubate in the humidity chamber at room temperature for 10 minutes. After incubation, shake off excess.

10) Dip-rinse slides with TPBS. Shake off excess buffer and blot excess buffer from slide edges surrounding the impression).

11) Incubate slides with amino-ethylcarbizole (AEC) substrate (note, other suitable chromogens may be used). To prepare the AEC stock solution: dissolve one 20 mg tablet of 3-amino 9-ethyl carbizole in 5 ml of N,N, dimethyl formamide in a glass vial or jar. The AEC stock solution should be stored at 4°C for ~ 1–2 months. To prepare the AEC working dilution: add 7 ml of acetate buffer to a 15 ml centrifuge tube using a 10 ml plastic pipette. Add 0.5 ml of AEC stock solution (above) using a 1 ml glass or Pyrex pipette. Add 75 µl of 3% hydrogen peroxide. Filter through a nylon filter (0.45 µm) into a separate 15 ml tube. Once prepared, this mixture is only stable for 2–3 hours, so should be made just prior to use. Add enough of this reagent to the slide to cover the impression, and incubate in the humidity chamber at room temperature for 10 minutes. After incubation, shake off excess substrate.

12) Dip-rinse slides in distilled water.

13) Counterstain with diluted haematoxylin for 2 minutes.

14) Immediately dip-rinse stain from slides with deionised/distilled water. Make a second dip-rinse of slides with fresh deionised/distilled water to ensure removal of excess stain.

15) Transfer slides to fresh distilled water. Working with one slide at a time, shake off excess deionised/distilled water, blot excess from slide edges surrounding the impression, apply water-soluble mounting medium and cover-slip. Do not allow slides to air-dry prior to cover-slipping. If multiple slides are stained, they may stay in the deionised/distilled water rinse before cover-slipping.

16) View slides by light microscopy, using a 20× objective to scan the field thoroughly, and a 40× objective for higher power inspection. Lyssavirus antigens appear as reddish, intra-cytoplasmic inclusions against a blue neuronal background, using AEC and haematoxylin counterstain.

1.3.2. Virus isolation

These tests detect viable (replicating) RABV from specimens using cell cultures or laboratory animals. They should be used as confirmatory tests if the DFA test, dRIT, other antigen detection tests or RT-PCR give inconclusive results. Wherever possible, virus isolation in cell culture should replace the mouse inoculation test (MIT). Cell culture tests are as sensitive as MIT (Robardet *et al.,* 2011; Rudd & Trimarchi, 1989), but are less expensive, give more rapid results and avoid the use of animals. For virus isolation from samples during routine rabies diagnostics, the use of cell culture techniques for isolating RABV should always take precedence over the use of the MIT.

i) Rabies tissue culture infection test (RTCIT)

Neuroblastoma cells e.g. N2a, CCL-131 in the American Type Culture Collection (ATCC) 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 37°C with 0.5% or 5% CO_2 depending on the culture vessel. Cell culture tests may be undertaken in multi-well plastic plates, multi-chambered glass slides, or on glass cover-slips. Additional passages could be considered, including use of T25 flasks, to increase sensitivity; usually three consecutive passages should be conducted to confirm a negative result. Cytotoxicity is a commonly reported factor limiting test robustness. Techniques proposed to reduce cytotoxicity include adding antibiotics, reducing the incubation 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.

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a) Protocol for a 96-well plate

1) 100 µl of clarified brain homogenate (20% w/v in PBS, 0.1 M, pH 7.4) is added to 200 µl of a 2×10^5 cells/ml suspension of 2- to 3-day old cells, freshly prepared from a sub-confluent flask in four wells of a 96-well plate.

2) 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.

3) After a further 72 hours incubation the supernatant is removed by pipette and kept for onward passage if required.

4) The cells are fixed with 80% acetone and stained at 37°C for 30 minutes 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.

b) Protocol for use in 8-chamber slides

1) 50 µl of clarified brain homogenate (20% in a grinding substrate made of PBS, 0.1 M, pH 7.4 with heat-inactivated fetal calf serum) is added to 400 µl of a 10^5 cells/ml suspension, freshly prepared from a sub-confluent flask.

2) After 24 hours incubation at 5% CO_2 and 37°C, the supernatant from each chamber is removed and 400 µl of fresh medium is added to each chamber.

3) After a further 24 hours (or more) of incubation the supernatant is removed, the chamber structure removed, the cell layer dried and fixed with pure high grade 80% cold acetone.

4) The fixed cell layer is then stained with fluorescent antibody at 37°C for 30 minutes according to manufacturers' recommendations.

c) Alternative protocol

1) 500 µl of clarified brain homogenate (20% [w/v] in growth medium [90% DMEM, 1.0% fetal calf serum (FBS) and 2% antibiotics] and centrifuged at approximately 700 ***g*** for 10 minutes) is mixed with 500 µl of 2×10^6 cells/ml freshly prepared from a sub-confluent flask in DEAE-dextran working solution (0.2 ml Dextran-stock solution in 25ml DMEM Dextran-stock solution is 0.50 g DEAE-dextran dissolved in 100 ml PBS [Hanks], sterile filtered).

2) After incubation for 30 minutes at 5% CO_2 and 37°C (agitate cell suspension carefully twice or three times during the incubation period), the cell suspension is gently centrifuged and the cell pellet is resuspended in 10 ml of fresh DMEM.

3) 8 ml of the cell suspension is put in a tissue culture flask (T25) and 2 ml onto a 6- or 24-well plate or petri dish (35/10 mm) to monitor the infection by DFA.

4) After a further 3–4 days incubation, the supernatant is removed by pipette from the monitor plate and discarded, while the T25 flask remains untouched.

5) The monitor plate is fixed in 80% acetone, then stained with fluorescent antibody according to laboratory procedures and observed under an inverse fluorescence microscope.

6) If the monitor plate is negative, cells are trypsinised from the T25 flask, split in a ratio of 1:2 up to 1:4 in fresh DMEM and the cell suspension put into a tissue culture flask (8 ml) and onto a 6- or 24-well plate or petri dish (2 ml).

7) Steps 4–6 are repeated. Three consecutive passages are conducted to confirm a negative result.

Whilst protocols (a) and (b) above only allow consecutive passaging of the supernatant, passaging in protocol (c) is based on splitting of the potentially infected cell monolayer thereby facilitating virus isolation in samples of low viral load.

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ii) Mouse inoculation test (MIT)

Three-to-ten mice, 3- to 4-weeks old (12–14 g), or a litter of 2-day-old new-born mice, are anesthetised and inoculated intracerebrally. The inoculum (0.01 ml for new-born mice or up to 0.03 ml for older mice) is the clarified supernatant of a 10–20% (w/v) homogenate of brain tissue including brainstem (e.g. cortex, Ammon's horn, thalamus, medulla oblongata) in an isotonic buffered solution containing antibiotics. Mice should be anaesthetised for inoculation. The mice are observed daily for 28 days, and every dead mouse is examined for rabies using the DFA or dRIT test. For faster results in new-born mice, it is possible to check one mouse 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 replace 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 the assay can be easily and practicably applied in situations where skills and facilities for other tests (e.g. cell culture) are not available.

1.3.3. Rapid immunochromatographic tests (lateral flow devices)

Commercial rapid immunochromatographic tests, also referred to as lateral flow devices (LFDs), for viral antigen detection are available. Some studies have found that when used under strict adherence to manufacturers' instructions, various available LFDs have shown highly variable results, some with inadequate or even complete lack of diagnostic sensitivity; test kits have also demonstrated inconsistency between batches and based on the type of diagnostic samples used (Eggerbauer *et al.,* 2016; Klein *et al.,* 2020). Field studies showed high sensitivity and specificity for some of those tests, particularly when the manufacturers' instructions were modified, with reported sensitivities compared with DFA ranging between 93% and 100% in field samples (Lechenne *et al.,* 2016; Markotter *et al.,* 2009; Nishizono *et al.,* 2008; Servat *et al.,* 2012). However, further improvements in sensitivity, consistency and validation using appropriate diagnostic samples are still required before LFDs can be recommended by WOAH. If data support the use of LFDs for routine surveillance and establishment of infection, a manufacturer could apply for inclusion of the assay on the WOAH Register of diagnostic kits[2].

Depending on the product-specific sensitivity and specificity, additional confirmation of results should be undertaken using other primary test methods (DFA, RTCIT, RT-PCR). Therefore, standard techniques should be either established or reinforced in parallel for the use of an LFD as a screening tool at a central laboratory. Nevertheless, positive LFD results should be a strong indication for bite victims to seek post-exposure prophylaxis.

1.3.4. 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 DFA and dRIT. 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.

1.3.5. Reverse-transcription polymerase chain reaction (RT-PCR)

RT-PCR assays are sensitive tools for the detection of lyssavirus-derived ribonucleic acid (RNA) within suspect specimens with the advantage that they do not require the presence of live virus.

RT-PCR assays that target the 3' proximal viral gene are considered the most sensitive as the replication cycle of lyssaviruses dictates that the N gene coding viral nucleoprotein is transcribed in the greatest abundance with a transcriptional gradient occurring for downstream genes.

RT-PCR assays should meet the WOAH Standards for validation (see Chapter 1.1.6 *Validation of diagnostic assays for infectious diseases of terrestrial animals*) and should be able to detect a broad spectrum of globally circulating

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RABV strains. Different methods for RNA extraction (manual, commercial conventional column or magnetic bead-based rapid RNA extraction methods) are available and can differ in sensitivity.
RT-PCR assays that have been evaluated in accordance with WOAH Standards (Chapter 1.1.6), have shown similar sensitivity and specificity to the DFA or DRIT, and can ideally detect all known Lyssaviruses and be used as an alternative to DFA or DRIT for routine rabies diagnosis. RT-PCR assays used as primary diagnostics should be conducted from a composite sample of brain tissue that includes brain stem and cerebellum, as stated in Section B.1.1. RT-PCR assays that have reduced sensitivity and specificity or are not able to detect all Lyssaviruses should be considered for confirmatory diagnostics when primary diagnostic assays are indeterminate.

Two reverse-transcriptase polymerase chain reaction (RT-PCR) methods for the detection of lyssavirus RNA in clinical samples are described. The first is a conventional (gel-based) pan-lyssavirus hemi-nested RT-PCR assay (hnRT-PCR). The second is a real-time pan-lyssavirus RT-PCR assay (based on a fluorescent DNA stain). The principal advantages of the hnRT-PCR assay include the applicability to laboratories that only have conventional PCR apparatus and the ability to obtain genetic data from the generated amplicons. The advantages of the fluorescent DNA stain-based assay include increased sensitivity over the conventional assay and a significantly reduced turnaround time. Both assays are based on a one-step approach, which reduces the risks of contamination and during manipulation. The fluorescent DNA stain-based assay is approximately one log more sensitive than the hemi-nested conventional RT-PCR.

There are numerous alternative rabies RT-PCR methodologies that are fit for purpose (for example Freuling *et al.*, 2014; Fischer *et al.*, 2013; Hayman *et al.*, 2011; Suin *et al.*, 2014; Wadhwa *et al.*, 2017; Wakeley *et al.*, 2005). Alternative assays may target different genes with different primers and could also be considered for use where suitable validation data have been obtained (see chapter 1.1.6).

i) Conventional RT-PCR techniques

The use of conventional RT-PCR is of benefit to laboratories that lack real-time RT-PCR platforms or that wish to obtain partial gene sequence data. Further equipment is required to enable electrophoretic resolution of amplicons on a gel. Prior to testing, RNA is extracted from suspect biological samples using a validated method of nucleic acid extraction. The one-step RT-PCR assay combines reverse transcription and first round PCR in a single tube, using a reverse transcriptase to generate a DNA copy of any viral RNA present that then acts as the template for the DNA polymerase to amplify the cDNA template exponentially. It has the added advantage of reducing the turnaround time of the assay. The assay described below is an example of a fully validated, reproducible conventional RT-PCR with a high sensitivity and specificity (modified from Heaton *et al.,* 1997). An optional second round amplification (hemi-nested [hn] PCR) is possible to increase the sensitivity and/or to confirm the specificity of the first round PCR product.

a) Primer sequences

1) JW12 Primer 5’-ATG-TAA-CAC-CYC-TAC-AAT-G-3’

7.5 pmol/µl (first round)

3.5 pmol/µl (second round)

2) JW6UNI Primer 5’-CAR-TTV-GCR-CAC-ATY-TTR-TG-3’

7.5 pmol/µl (first round)

3) JW10UNI Primer 5’-GTC-ATY-ARW-GTR-TGR-TGY-TC-3’

3.5 pmol/µl (second round)

b) Test procedure

Each test must contain positive (PC) and negative (NC) control tissue samples, as well as a no template control (NTC), which are run alongside the test samples.

First round one step (OS) RT-PCR (JW6UNI/12)

In clean room:

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1) Wipe bench with an appropriate disinfectant prior to use or prepare PCR workstation. To prepare, open doors of the PCR workstation and wipe the cabinet surface with an appropriate disinfectant. Place an ice bucket and suitable pipette and tips within the station and close the doors. Switch on UV light for 10 minutes.

2) Obtain the required test reagents. Ensure the enzyme mix is kept on ice. The remaining reagents can be thawed at room temperature.

3) Put the required number of 0.2 ml tubes in a rack and label the tubes clearly with sample identification, denoting that this is the first round reaction. Include PC, NC and NTC.

4) Prepare a JW6UNI/JW12 reaction master mix as detailed below:

First round JW6UNI/12 master mix

Reagent	Volume per reaction (µl)
Molecular grade water	29.0
5× buffer	10.0
dNTPs (10 mM)	2.0
JW12 (7.5 pmol/µl)	3.0
JW6UNI (7.5 pmol/µl)	3.0
Enzyme mix	2.0
Total	49

Keep all reagents on ice, thaw and vortex before using. Allow for pipetting variation by preparing a volume of master mix at least one reaction greater than required.

5) Vortex the prepared master mix thoroughly, centrifuge and dispense 49 µl into each of the 0.2 ml tubes. Close the lids.

6) Transfer the sealed tubes to the ice/cool block in the template room on a tray. Once a tray has been removed it must not be returned to the clean room without decontamination using an appropriate disinfectant

In template room – addition of template

1) Wipe bench with an appropriate disinfectant prior to use.

2) Thaw samples and control RNA (positive and negative controls) on ice.

3) Add 1 µl of test RNA (where possible at concentration of 1µg/µl for extracted samples) below the surface of its allocated master mix tube and mix gently. Discard the tip directly into disinfectant after use. Repeat this process until all samples and controls have been added to their allocated tubes.

4) Press the lids down by hand and seal firmly.

5) Transfer the sealed tubes to the PCR machine and cycle as detailed below: In house validation of cycling parameters is essential to ensure optimisation for local PCR machines.

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Hemi-nested RT-PCR first round cycling parameters:

Temperature	Time	Cycles
50°C	30 minutes	1
95°C	15 minutes	1
94°C 45°C 50°C 72°C	30 seconds 45 seconds 15 seconds 1 minute	45
72°C	7 minutes	1
4°C	∞	n/a

Once complete the resulting amplicons can be visualised on a 1.5–2% agarose gel using standard gel electrophoresis techniques and suitable marker DNA ladders to ensure the appropriate size amplicon has been generated in the positive control samples (for comparison with samples on test). A suitable DNA-detection chemical should be added to the gel and a UV light box used to visualise the products.

Second round OS RT-PCR (JW10/12)

Where no amplicon is generated on the first round reaction, a second round, hemi-nested reaction should be performed.

In clean room:

1) Prepare PCR workstation as described in Section B.1.3.**5**.i.**b**.1.

2) Obtain the required reagents. Ensure enzyme mix is kept on ice. The remaining reagents can be thawed at room temperature.

3) Put the required number of 0.2 ml tubes in a rack and label the tubes clearly with sample identification and denote that this is the second round reaction by labelling with '10/12', or '2'. Label the PCR negative as '–2' or 'NC2' and the no template control as 'NTC2'. This additional negative control must be included in every second round PCR experiment to confirm the master mix is not contaminated.

4) Prepare a JW10UNI/JW12 reaction master mix as detailed below:

Second round JW10UNI/12 master mix

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Reagent	Volume per reaction (µl)
Molecular grade water	22.0
High fidelity Taq polymerase (2×)	25.0
JW12 (3.5 pmol/µl)	1.0
JW10UNI (3.5 pmol/µl)	1.0
Total	49

5) Thaw and vortex all reagents before using. Allow for pipetting variation by preparing a volume of master mix at least one reaction greater than required.

6) Vortex the prepared mastermix thoroughly, centrifuge at 700 ***g*** and dispense 49 µl into each of the 0.2 ml tubes. Seal the tubes.

7) Transfer the sealed tubes to the template room on a disposable tray. Once a tray has been removed it must not be returned to the clean room without appropriate decontamination.

In template room – addition of template:

1) In order to reduce cross contamination, the template may be added within a PCR workstation.

2) To prepare, open the doors of the PCR workstation and wipe the cabinet surface with an appropriate disinfectant. Place an ice bucket, suitable pipette and tips within the station and close the doors. Switch on UV light for 10 minutes.

3) Add 1 µl of undiluted first round PCR product below the surface of the prepared second round master mix to minimise aerosols and mix gently. Discard the tip directly into an appropriate disinfectant after use. Ensure the lid of the PCR tube is sealed firmly. Repeat this step until all first round PCR products and the second round PC, NC and NTC have been added to allocated Second Round master mix tubes. Change gloves regularly and at suitable points to avoid cross-contamination.

4) If using the PCR workstation, remove samples and supplies and switch on the UV for 10 minutes.

5) Run the PCR machine using the following second round cycling parameters. In-house validation of cycling parameters is essential to ensure optimisation for local PCR machines.

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Temperature	Time	Cycles
95°C	15 minutes	1
94°C 45°C 50°C 72°C	30 seconds 10 seconds 15 seconds 1 minute	35
72°C	7 minutes	1
4°C	∞	n/a

Analyse the amplification reactions by electrophoresis on agarose gels using an appropriate DNA ladder.

ii) Real-time RT-PCR techniques

Where capabilities allow, real-time PCR platforms enable a more rapid evaluation of the presence or absence of lyssavirus RNA in suspect samples. The procedure detailed here is an example that uses the same forward primer as the conventional RT-PCR assay described in Heaton *et al.*, 1997. The use of a universal one-step RT-PCR kit that uses a fluorescent DNA stain for the detection of *Lyssavirus* species from clinical specimens has been demonstrated to be both highly sensitive and specific for lyssavirus RNA. Furthermore, by using a fluorescent DNA stain as the detection system it is able to detect all lyssaviruses based on the pan-lyssavirus primer specificity. This method includes a separate RT-PCR assay containing a fluorescent DNA stain for amplification of the internal housekeeping control, mRNA for beta-actin as a template control for RNA extraction.

a) Primer sequences

1) Pan-*lyssavirus*-specific primers (synthesised to 0.05 µmol, HPLC purified) and diluted to 20 pmol/µl:

a) JW12 RT/PCR primer 5'-ATG-TAA-CAC-CYC-TAC-AAT-G-3'

b) N165-146 PCR primer 5'-GCA-GGG-TAY-TTR-TAC-TCA-TA-3'

2) Multispecies Beta Actin primers (synthesised to 0.05 µmole, HPLC purified) and diluted to 20pmol/µl:

a) BatRat Beta-actin intronic primer 5'-CGA-TGA-AGA-TCA-AGA-TCA-TTG-3'

b) BatRat Beta-actin reverse primer 5'- AAG-CAT-TTG-CGG-TGG-AC-3'

b) Test reliability

Instrumentation and equipment are monitored for satisfactory performance and calibrated once a year. Include a calibrated RABV RNA PC on every test run and an internal Beta-actin test may be used as an extraction control. A NC and NTC is included on every test run to confirm the absence of contamination. All test samples should be run at least in duplicate.

c) Test procedure

Gloves and a laboratory coat must be worn at all times.

In clean room/UV cabinet:

1) Prepare PCR workstation as described in Section B.1.3.**5**.i.**b**.1.

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2) Obtain the required reagents. Ensure the enzyme mix is kept on ice, the remaining reagents can be thawed at room temperature.

3) Put the required number of 0.2 ml tubes in a rack and label the tubes clearly with sample identification. Include tubes for PC, NC and NTC.

4) Prepare a reaction master mix as below and keep all reagents on ice. Allow for pipetting variation by preparing at least two extra reaction mixes

Reagent	**Volume per reaction (µl)**
Molecular grade water	7.55
Universal reaction mix containing a fluorescent DNA stain (2×)	10.0
JW12 (20 pmol/µl)	0.6
N165-146 (20 pmol/µl)	1.0
RT enzyme mix	0.25
Total	19.4

5) Prepare a reaction master mix for the β-actin mRNA which assesses the quality in samples extracted from solid tissue. The assay for β-actin must be positive in order to have confidence that RNA was isolated from the starting material.

Reagent	Volume per reaction (µl)
Molecular grade water	7.55
Universal reaction mix containing a fluorescent DNA stain (2X)	10.0
Intronic (20 pmol/µl)	0.6
Reverse (20 pmol/µl)	1.0
RT enzyme mix	0.25
Total	19.4

6) Vortex the prepared master mixes and aliquot 19 µl into each of the relevant wells of a 96 well plate or 8-well strips.

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In template room/UV cabinet – addition of template

1) Prepare PCR workstation as described in Section B.1.3.**5**.i.**b**.1.

2) Thaw samples and control RNA on ice.

3) Add 2 µl of test RNA (where possible at concentration of 0.5–1 µg/µl for extracted samples) below the surface of its allocated master mix tube and mix gently. Discard the tip directly into disinfectant after use. Repeat this process until all samples and controls have been added to their allocated tubes.

4) Press the lids down by hand and seal firmly.

5) Transfer the PCR plate/strips to the real-time machine for thermal cycling.

Setting up of real-time thermal cycler

1) Load the samples into the machine, ensuring that they are orientated the correct way. Ensure that all the lids are firmly sealed, and then close the machine's plate cover and door. Set up the run parameters according to the manufacturer's instructions.

2) Set up the thermal profile as follows:

Temperature	Time	Cycles
50°C	10 minutes	1
95°C	5 minutes	1
95°C 60°C	10 seconds 30 seconds	40
95°C 55°C 55-95°C	1 minutes 1 minute 10 seconds	80

Thermal profiles may be subject to optimisation depending on PCR machines used.

3) In case of an inconclusive assay, an agarose gel can be run to confirm the presence/absence of an amplicon and its approximate size (100 bp).

1.4. Other identification tests

The tests above describe methods to accurately diagnose rabies and to isolate and identify the virus. Characterisation of the virus can provide useful epidemiological information and should be undertaken in specialised laboratories (such as WOAH, WHO or FAO Reference Laboratories). These techniques would include the use of monoclonal antibodies, as well as partial and full genome sequencing followed by phylogenetic analysis. These characterisations enable a distinction to be made between vaccine virus and a field strain of virus, and possibly identify the geographical origin of the latter.

2. Serological tests

The main application of serology for rabies is to determine responses to vaccination in domestic animals, particularly in connection with international travel, or for monitoring mass vaccination campaigns in dogs and other wildlife reservoir species. The measurement of rabies antibodies has typically involved virus neutralisation (VN) tests to detect RABV neutralising antibodies. ELISAs are now also recognised as acceptable tests to detect binding antibodies. A strong but not strict correlation in levels is observed between these two different antibody detection methods. Depending on the nature of the ELISA, there can be variable sensitivity and specificity. In contrast

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to the ELISA, poor quality sera can cause cytotoxicity in VN tests, which could lead to false-positive results. Depending on the intended purpose, both tests are useful for detecting responses to vaccination if appropriate cut-offs are used. However, ELISAs are currently not applicable to international movement of animals or trade.
Serological surveys have also been used to provide information on infection dynamics of lyssaviruses in bats although standardisation of serological tests for bats is still needed.

2.1. Virus neutralisation test in cell culture: fluorescent antibody virus neutralisation test (FAVN)

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

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 WOAH serum of dog origin under the same experimental conditions. The WHO standard[3] for rabies immunoglobulin [human] No. 2, or an internal control calibrated against the international control may also be used to calculate the IU/ml titre of test sera.

Generally, the minimum measurable neutralising VN antibody titre considered to represent a reasonable level of seroconversion is 0.5 IU per ml. The same measure is used in dogs and cats to confirm an adequate response to vaccination prior to international travel. However, within the framework of monitoring mass vaccination campaigns, a single cut-off level of seropositivity may not be universally applicable among different species (Moore *et al.,* 2017).

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).

2.1.1. Viruses and WOAH standard serum

Virus: CVS-11 (previously ATCC reference VR 959) strain, which is available from the ATCC or the WOAH Reference Laboratory for rabies, Nancy, France[4]. Vials are stored at –80°C;

WOAH Standard Serum of dog origin[5] stored at +4°C and diluted to 0.5 IU/ml with sterile deionised or distilled water. This control serum may be used to calibrate an additional internal control that is used for regular FAVN testing.

Negative control serum: A pool of sera from naïve dogs stored at –20°C.

2.1.2. CVS production

i) Cell growth: the BHK-21 C13 cells (ATCC CCL-10) maintained in Dulbecco's modified Eagle's medium (DMEM) or Glasgow modified Eagle's medium (GMEM) with 10% heat-inactivated FCS and antibiotics, 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.

2.1.3. Titration of virus in $TCID_{50}$ (50% tissue culture infective dose)

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

i) 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) The serial dilutions of virus 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) 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) Incubate in a humidified incubator for 3 days at 35.5–37°C in 5% CO_2.

v) The cells are stained using the DFA test, 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, 2018).

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

2.1.4. Test procedure

i) The control plate is used for the titration of CVS (rows 1 to 4), standard sera and negative control serum are used. All other plates are used for the sera to be tested.

ii) Medium is added to the wells as follows: control plate 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. 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:

WOAH 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.

2.1.5. 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. For virus titration, 50 µl is added to wells H1 to H4 (control plate). Next, transfer 50 µl from row to row (control plate, lines 1–4). Discard 50 µl from the last row (control plate, wells A1 to A4). No virus is added to wells A9 to A12 of control plate. The range allowed for the virus dose titre must be between 30 and 300 $TCID_{50}$/50 µl.

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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.

2.1.6. 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.

2.1.7. 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. Use a 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.

ii) Cell and virus controls are read first. For titration of CVS, negative control serum, and WOAH standard serum, titres are calculated according to the Spearman–Kärber method or the neoprobit graphic method (WHO, 2018).

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, 2018).The comparison of the measured titre of the tested sera with that of the 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 positive standard serum.

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

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

Example of conversion:

- log D_{50} of the serum = 2.27
- theoretical titre of positive standard serum 0.5 IU/ml = 0.5 IU/ml
- log D_{50} of positive standard serum = 1.43

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(for the log D_{50} of positive standard, 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:

- RABV: 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 RABV, negative control serum and positive standard serum of dog origin.
- The back titration of the CVS virus, as well as negative control 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.

2.2. Virus neutralisation test in cell culture: the rapid fluorescent focus inhibition test (RFFIT)

2.2.1. 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.

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 calf serum (EMEM-10).

iii) Using standard rabies safety procedures, add 1×10^7 infectious units of CVS-11 RABV (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 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.

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

2.2.2. Titration of seed virus suspension

i) Thaw one aliquot of the seed virus and prepare serial ten-fold 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.

2.2.3. 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.

2.2.4. Titration of stock virus suspension

i) Thaw one aliquot of the stock virus and use this to prepare serial ten-fold 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 or 20 distinct microscopic fields when observed at x100 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 1×10^4 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). For example, a stock virus suspension of this titre (1×10^4 FFD_{50} per 0.1 ml) can then be diluted to $10^{-2.3}$ to obtain a challenge virus containing 50 FFD_{50}. To calculate the working dilution subtract the log of 50 (1.7) from the log of the virus dose (e.g. in the example given 4.0 – 1.7 – 2.3).

2.2.5. Reference sera

A recognised (WHO [see footnote 3], WOAH [see footnote 5]) reference serum standard or a prepared standard verified against a recognised standard diluted to a potency of 2.0 IU/ml should be included in each test. 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.

2.2.6. 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. Five-fold serial dilutions of test sera may be prepared directly in an

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eight-well tissue-culture chamber slide or in a 96 well plate and transferred to the chamber slide wells (final volume 0.1 ml/serial dilution). The RFFIT may be performed as a screen using 2 dilutions or as an endpoint test suing 4 dilutions. Screening dilutions of 1/5 and 1/50 are sufficient for routine evaluation of vaccination efficacy. Endpoint testing is generally performed using dilutions of 1/5, 1/25, 1/125, and 1/625.
For the screening test:

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.

For the endpoint test:

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 0.2 ml of EMEM-10. Discard all but 0.1 ml from the well containing the 1/2.5 dilution (first well).

iii) Transfer 0.05 ml of the 1/2.5 dilution to a third well containing 0.2 ml of EMEM-10. Discard all but 0.1 ml from the well containing the 1/5 dilution (second well).

iv) Transfer 0.05 ml of the 1/2.5 dilution to a fourth well containing 0.2 ml of EMEM-10. Discard all but 0.1 ml from the well containing the 1/25 dilution (third well).

v) Mix the fourth well containing the 1/125 dilution and discard all but 0.1 ml.

2.2.7. Addition of virus

i) Add 0.1 ml of the challenge virus preparation (working dilution) to all serum dilutions.

ii) Prepare a 1/10 and 1/100 back titration of the challenge virus working dilution and add 0.1 ml of each to a slide well.

iii) Mix and incubate at 37°C in a humidified incubator with 0.5% CO_2 for 90 minutes.

2.2.8. Addition of cells

i) During the incubation period, trypsinise a stock culture of 3- to 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.

2.2.9. 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). Note: if using plastic slides use 80% cold acetone.

iii) Leave the slides to dry for 10–30 minutes before adding FITC-conjugated anti-rabies antibody. 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 20–60 minutes at 37°C (optimal time determined by conjugate qualification) and then rinsed in PBS and distilled water, respectively.

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iv) Observe the slides under a fluorescence microscope. Record the number of fields (out of 20 per well) where virus infection of cells is observed.

2.2.10. 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 recognised reference serum 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, 2018).

The following parameters have to be strictly adhered to:

i) RABV; only the CVS-11 strain should be used. Virus: CVS-11 (previously ATCC reference VR 959) strain, which is available from the ATCC or the WOAH Reference Laboratory for rabies, Nancy, France. Vials are stored at –80°C. The back titration should indicate a dose of 30–100 FFD_{50}.

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

iii) The test should be performed only on suitable chamber slides.

iv) Control charts should be used for RABV, naïve serum and positive standard dog serum.

v) The back titration of the CVS virus, as well as the naïve serum and positive standard dog, WOAH Reference Serum must be present on control plate.

vi) Reading method for the test: each chamber slide should contain 25–50 fields and be observed at ×160–200 magnification or 20 fields and be observed at ×100 magnification.

vii) Convert log D_{50} to IU/ml of test sera using the log D_{50} value of the recognised standard serum diluted to a potency of 2.0 IU/ml.

2.3. Enzyme-linked immunosorbent assay (ELISA)

ELISAs provide a rapid serological test that avoids the requirement to handle live RABV. Those tests detect antibodies that can specifically bind to RABV antigens, primarily the RABV glycoprotein and nucleoprotein. None of the available direct, indirect or competitive ELISAs is validated for international animal movement or trade (Wasniewski *et al.,* 2014). However, ELISAs are a useful tool for monitoring rabies vaccination campaigns in wildlife species provided they are properly validated for this purpose. A commercial ELISA has been recommended for monitoring rabies vaccination campaigns in foxes and raccoon dogs (Wasniewski *et al.,* 2016).

3. Quality assurance

Annual 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 WOAH Reference Laboratories. Whenever possible, international accreditation of a laboratory should be considered (see Chapter 1.1.5 *Quality management and veterinary testing laboratories*).

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C. REQUIREMENTS FOR VACCINES

1. General background

The prevention and control of rabies in a country is 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 1.1.8 *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. Varying requirements relating to quality, safety and efficacy apply in particular countries or regions for manufacturers to obtain relevant regulatory approval 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. Internationally and nationally recognised regulations for animal experimentation should be followed in all stages of rabies vaccine development and production.

Virulent RABV 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 1.1.4 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 1.1.8.

Authorised vaccines for the parenteral vaccination of domestic animals and oral vaccines for the immunisation of wild animals and free-roaming dogs are available. These vaccines are frequently used off-label.

Oral rabies vaccination (ORV) has been successfully used to control the disease in certain wildlife reservoir species (Cliquet *et al*., 2012; Freuling *et al.,* 2013). However, because dog-mediated human rabies is a candidate for global elimination, the dog should be considered a main target for rabies elimination; more than 99% of all human cases of rabies are caused by dogs. Countries should assess the need for both ORV of dogs and parenteral vaccination in their rabies control strategy. Parenteral vaccination of dogs should remain the foundation of mass vaccination campaigns. Apart from mass parenteral vaccination (carried out concurrently or sequentially), the use of oral vaccination, especially in free-roaming and inaccessible dogs, taking into account structure and accessibility of the dog population, should represent a complementary measure for the improvement of the overall vaccination coverage in dog rabies control programmes (WHO, 2013). For ORV of dogs, the handout and retrieve model should be used preferably unless the situation requires other means of distribution.

An optimal individual or combination vaccination strategy for both vaccination of wildlife (ORV with or without Trap-Vaccinate-Release) and dogs (Central Point Vaccination, House-to-House vaccination, with or without ORV) should be determined by taking the size of the target species population into account. As regards ORV of wildlife and dogs, oral RABV vaccine bait candidates should be selected based on efficacy and safety profiles. Monitoring of human exposure to oral RABV vaccines and risk management should be undertaken. Under specific circumstances, vaccination of other susceptible companion animals and livestock would be beneficial and should be considered as part of any national vaccination programme.

2. Rabies vaccine for injectable use

2.1. Background

The principal rationale for the use of rabies vaccine is to protect animals and, as a consequence, humans. For injectable rabies vaccination in animals, inactivated virus (for companion animals and livestock), and recombinant vaccines (for cats) are used. As injectable live-attenuated vaccines have been documented to cause vaccine-induced rabies (Bellinger *et al*., 1983; Esh *et al*., 1982) their use should be discontinued.

Rabies virus glycoprotein biotechnology-derived vector vaccines are prepared by inserting non-infectious rabies virus nucleic acid coding for rabies virus glycoprotein into a vector such as avipox for injectable vaccine (WHO, 2018). Alternatively, non-replication competent constructs (e.g. virus like particles [VLPs], replicon vaccines, mRNA vaccines, plasmid vaccines or subunit vaccines etc.) or replication restricted rabies vaccine constructs (e.g. single cell cycle rabies vaccines), may be available for injectable use in the future (see Chapter 1.1.8). While the same efficacy requirements apply in principle to these

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vaccine constructs, additional safety considerations may need to be taken into account. As these vaccines do not contain live rabies virus, animals vaccinated with such vaccines should not be restricted from entry into countries (Taylor *et al.*, 1991).

2.2. Outline of production and minimum requirements for conventional vaccines

2.2.1. Characteristics of the seed

i) Biological characteristics

Any RABV strain considered for vaccine production should protect against any RABV 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). A record of the source of the MSV should be maintained.

Biotechnology-derived vaccines are prepared in appropriate cell lines using a vector expressing the RABV glycoprotein.

ii) Quality criteria

MSVs for vaccine production must be well characterised and proven to be pure and free from all extraneous agents in accordance with Chapter 1.1.9 *Tests for sterility and freedom from contamination of biological materials intended for veterinary use* and those listed by the appropriate regulatory authorities.

The efficacy of the resultant vaccine is assessed by studies on every target species to be vaccinated as recommended in chapter 1.1.8 and Section C.2.3.3 of this chapter.

2.2.2. Method of manufacture

i) Procedures

a) 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 1.1.8).

Cultures are infected with cell-culture-adapted MSV and incubated at the appropriate temperature for a defined period. As RABV 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 is prepared by adsorbing the antigen onto an 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.

a) In cells

The cell lines used for the production of RABV vaccines should be in accordance with chapter 1.1.8.

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b) In embryonated eggs

This method of culture is used for the production of live-attenuated vaccines such as the Flury LEP or the HEP variant strain. Their use should be discontinued as indicated in Section C.2.1 (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 1.1.8. 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.

a) 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-RABV by the immunofluorescence test. The inactivated virus harvest complies if no live virus is detected (European Pharmacopoeia, 2021).

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.

a) Sterility

Tests for sterility and freedom from contamination of biological materials intended for veterinary use may be found in chapter 1.1.9.

b) Safety

Safety tests in target animals are not required by many regulatory authorities for the release of each batch. Where required, standard procedures are generally conducted using fewer animals than are used in the safety tests required for relevant regulatory approval.

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 1.1.8, 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, 2021).

c) 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.

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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 RABV.

d) 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 modified live vaccine. Virus titration should be carried out using cell culture techniques. This allows laboratories to act in accordance with the 3Rs principles (European Commission, 2010).

The potency of inactivated vaccines is tested in mice by a serological test (Krämer *et al*., 2010), or a challenge test (European Pharmacopoeia, 2021; WHO, 2018). 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.2.2.2.iv.d.1 Serological test, and Section C.2.2.2.iv.d.2 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:

1) Serological test

In the serological test, the inactivated vaccine is compared with the standard reference vaccine by measuring the amounts of neutralising anti-RABV-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:

Eight to ten mice, each weighing 18–20 g are used. Each mouse is vaccinated by a subcutaneous, intramuscular or intraperitoneal 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 (see Section B.2 and European Pharmacopoeia, 2021).

The vaccine meets the requirement if the rabies antibody titre of mice immunised with the test vaccine is significantly higher than that obtained with a reference vaccine that gave satisfactory results in the test described in C.2.2.2.iv.d.2 Challenge test.

2) Challenge test

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.

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i) 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.

ii) Preparation of the challenge suspension

A group of mice is inoculated intracerebrally with the CVS strain of RABV; 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 –80°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 anaesthetised and injected intracerebrally with 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 mouse intracerebral lethal dose ($MICLD_{50}$) of the undiluted suspension is calculated.

iii) 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 0.03 ml. Each vaccinated mouse is injected intracerebrally with 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 anaesthetised and injected intracerebrally with 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 develops signs of rabies between day 5 and day 14 after challenge is recorded.

The test is invalid unless:

a) 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;

b) The titration of the challenge suspension shows that 0.03 ml of the suspension contained at least 10 ID_{50};

c) 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;

d) 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 WOAH requirement if the estimated potency is not less than 1 IU in the smallest prescribed dose.

iv) Application of humane end-points

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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 scoring scheme 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:

Score 1: ruffled fur, hunched back;

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

Score 3: shaky movements, trembling, convulsions;

Score 4: signs of paresis or paralysis;

Score 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 score 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, 2021; 9CFR, 2010).

2.3. Requirements for relevant regulatory approval

2.3.1. Manufacturing process

For registration of vaccine, all relevant details concerning manufacture of the vaccine and quality control testing (see Sections C.2.2.1 and C.2.2.2) 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.

2.3.2. Safety requirements

Safety tests for registration of inactivated injectable rabies vaccine are identical to those described in Section C.2.2.2.iv.d.1 and need to be carried out in accordance with VICH 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 is not specified, then a justified multiple of the minimum release potency should be used.

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

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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 RABV, but other safety concerns may be evident (Roess *et al.*, 2012). Specific requirements for safety of this type of vaccine are described in chapter 1.1.8 for biotechnology-derived vaccines.

Tests for reversion to virulence of modified live vaccines (MLV) should be done in accordance with chapter 1.1.8.

i) Precautions and hazards

For adjuvanted vaccines, live attenuated vaccines and biotechnology-derived vaccines, warnings should be provided by manufacturers that medical advice shall be sought in case of self-injection

2.3.3. Efficacy requirements

In herbivores, as a minimum requirement, efficacy can be demonstrated by serology (European Pharmacopoeia, 2021). In other species efficacy is demonstrated by challenge with an appropriate challenge RABV. Test animals shall be uniform and have no neutralising antibodies to rabies as determined by the serum neutralisation tests (see Section B of this chapter).

For challenge tests, challenge-dose finding studies are conducted 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 humanely killed and rabies is confirmed using appropriate diagnostic tests. At the end of the observation period, all surviving animals are humanely 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.

2.3.4. Stability

As described in chapter 1.1.8.

2.3.5. 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

All vaccines currently used for oral vaccination are either MLV or biotechnology-derived vaccines (BDVs). It should be noted that oral rabies vaccine constructs generated using reverse genetics are considered from a regulatory perspective as BDVs although they do not express a foreign gene. While there are numerous oral rabies vaccines for wildlife available (Müller & Freuling, 2020), they are perhaps the most underused of all tools in the fight against dog-mediated rabies (Cliquet *et al.*, 2018; Wallace *et al.*, 2020).

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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 1.1.8). Some of the MLVs have been documented to cause vaccine-derived rabies in target and non-target species (Fehlner-Gardiner *et al.,* 2008, Müller *et al.,* 2009, Hostnik *et al.* 2014, Pfaff *et al.*, 2018).

Requirements for guaranteeing the safety and efficacy of oral vaccines both for the target species and non-target species (especially humans) that might be in contact with baits or a recently vaccinated animal have been developed (European Pharmacopoeia, 2020; WHO, 2007; 2013).

As well as the requirements for oral RABV vaccines as described below, for ORV of wildlife and dogs, appropriate bait configuration and bait delivery systems are also critical and may require adaptation to local circumstances. It may be necessary to reassess efficacy with each significant variation of baits and bait delivery systems.

3.2. Outline of production and minimum requirement for vaccines

In addition to the requirements outlined in chapter 1.1.8, the following specific requirements must be met.

3.2.1. Characteristics of the seed

The seed is a pure preparation of a single immunogenic clonal strain of a highly attenuated MLV or BDV. 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 consensus sequence of the MSV 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.

3.2.2. Method of manufacture

i) Procedure

The MSV is used to infect a suspension or monolayers of an established cell line. Ideally, these cell cultures should 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.

a) Sterility

This test may be done before or after filling the bait. Tests for sterility and freedom from contamination of biological materials intended for veterinary use are described in chapter 1.1.9.

b) Identity

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

c) Batch/serial purity

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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 RABV (for example, street virus, Pasteur strain). Alternatively, genetic characterisation can be used. The vaccine complies with the test if it shows no evidence of contaminating RABV (European Pharmacopoeia, 2021).

d) Safety

Safety tests in target animals are not required by many regulatory authorities for the release of each batch or serial. Where required, standard procedures are generally conducted using fewer animals than are used in the safety tests required for relevant regulatory approval.

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 1.1.8, batch safety testing is to be performed as follows: two healthy seronegative animals of the target species are administered orally with 10 times the field concentration. In addition, a 0.5 ml dose is injected by the intraperitoneal or subcutaneous routes into eight mice. All animals are then observed for at least 14 days. If any intolerable adverse reactions attributable to the products occur in any animals during the observation period, the batch/serial is unsatisfactory.

e) Batch/serial potency

For MLV and BDV, 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 culture. This allows laboratories to act in accordance with the 3Rs principles (European Commission, 2010).

3.3. Requirements for relevant regulatory approval

3.3.1. 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.

3.3.2. Safety requirements

In accordance with chapter 1.1.8, 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 BDV should be done in accordance with chapter 1.1.8.

i) Modified live vaccines (MLV)

a) In target species

For the overdose safety test, a 10 × maximum titre of a field dose is administered, preferably using a syringe, via the oral route to ten 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 and on several additional selected time points within the first week after vaccine administration. Any virus recovered should be characterised. The animals are observed for at least 90 days. Particular attention shall be paid to neurological signs and sudden death and shall be investigated using appropriate tests (see Section B of this chapter). At the

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termination of the study, the brain should be examined for vaccine virus presence using reference tests as described in Section B.1.3.1.

The test is satisfactory if no intolerable 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.

b) In non-target species

A representative group of species including rodents, cats and dogs that are likely to consume the baits should be investigated. At least ten animals of each species should be tested orally with 10 × maximum titre of a field dose and observed for at least 90 days.

As testing wild animals might prove to be difficult and should be kept at a minimum, additional tests using different routes of administration in laboratory rodents (both nude and SCID mice or other immunocompromised animals) 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. After termination of the study, the brain of animals should be examined for RABV antigen using reference tests as described in Section B.1.3.1.

A risk assessment should be undertaken to evaluate directly the safety risk for humans (safety of vaccine) and the risk that humans will come in contact with the vaccine.

ii) Biotechnology-derived vaccines (BDV)

a) In target species

For the overdose safety test, a 10× maximum titre 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 on the first day and on several additional selected time points within the first week after vaccine administration. Any virus recovered should be characterised. The animals should be observed for at least 90 days or according to their known incubation period for the vector used.

The test is satisfactory if no intolerable adverse reactions attributable to the vaccine are observed. Viral RNA and 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.

b) In non-target species

A representative group of species including rodents, cats and dogs 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 10 × maximum titre and observed for at least 90 days or 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 30 days. Animals that die from causes not attributable to the disease caused by the vector are eliminated.

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A risk assessment should be undertaken to evaluate directly the safety risk for humans (safety of vaccine) and the risk that humans will come in contact with the vaccine.

iii) Precautions hazards

The release of oral vaccines into the environment shall comply with the requirements in chapter 1.1.8. 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).

3.3.3. Efficacy requirements

Efficacy of the final product (vaccine bait) (see Section C.3.3.5) shall be demonstrated in each species for which the vaccine use is claimed by the manufacturer. The protection status cannot be checked by serology only; a virulent challenge with an appropriate challenge RABV is necessary. Preferably, a target species adapted RABV strain should be used. The vaccine titre should not be greater than the claimed minimum protective dose.

Test animals at least three months of age should have no rabies specific antibodies to rabies as determined by the serum neutralisation tests (see Section B of this chapter).

For challenge tests, challenge-dose finding studies should be conducted 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 dose. At 180 days after presentation of a vaccine-bait, vaccinates and controls are challenged with the predetermined dose as described above. Animals need to be observed daily for 90 days after challenge and humanely euthanised at the first definitive clinical signs of rabies. The diagnosis of rabies must be confirmed in animals that die or have to be euthanised using appropriate diagnostic tests. At the end of the observation period, all surviving animals are humanely 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.

Once the minimum immunising dose has been established in one species, the efficacy study for additional species can be limited to a study using vaccine-baits. The bait casing may have to be adapted to the new target species (see Section C.3.3.5).

3.3.4. Stability

A minimum of five samples of the final product 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 (European Pharmacopoeia, 2020).

3.3.5. Bait requirements and characteristics

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The bait is an integral part of the product and should ideally meet certain criteria:

i) Designed for and attractive to the target species and adapted to the mode of distribution.

ii) Adapted to the food preferences of the local dog population for ORV;

iii) The attractant should be compatible with bait and vaccine and adherent to the bait, and should remain palatable for a defined period;

iv) Keep its form and shape under a wide range of temperature and weather conditions to protect the vaccine under field conditions;

v) The shape of the bait should allow easy ingestion by all ages and sizes of target species;

vi) Optimise the release of vaccine into the oral cavity and to the target tissues;

vii) Be safe for target and non-target species;

viii) Ingredients should not be harmful, should comply with animal feed standards and should not interfere with vaccine activity;

ix) Allow the incorporation of a biomarker, topical or systemic (for example:

a) surface markers (Rhodamine B, other dyes);

b) tissue markers (Iophenoxic acid, etc.);

c) calciphilic markers such as tetracycline (TC), which should be compatible with other bait components, safe for target and non-target species, detectable in target species for a defined period using technically simple, economical and locally available assay methods, absent or minimally present in subject population. In case of TC, care must be taken that the ratio of TC vs epitetracycline in the final bait formulation should be as high as possible to guarantee biomarker effectiveness.

x) Be economic to produce in standard form, possibly under local conditions;

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

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NB: There are WOAH Reference Laboratories for rabies (please consult the WOAH Web site: https://www.woah.org/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3**).**
Please contact the WOAH Reference Laboratories for any further information on diagnostic tests, reagents and vaccines for rabies

NB: FIRST ADOPTED IN 1989. MOST RECENT UPDATES ADOPTED IN 2023.

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