

## RIFT VALLEY FEVER (INFECTION WITH RIFT VALLEY FEVER VIRUS)

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### SUMMARY

**Description of the disease:** Rift Valley fever (RVF) is a peracute or acute zoonotic disease of domestic ruminants. The virus is confined to the African continent and Arabian Peninsula. It is caused by a single serotype of a mosquito-borne virus of the Bunyaviridae family (genus Phlebovirus). The disease occurs in climatic conditions favouring the breeding of mosquito vectors and is characterised by abortion, neonatal mortality and liver damage. The disease is most severe in sheep, goats and cattle. Older non-pregnant animals, although susceptible to infection, are more resistant to clinical disease. There is considerable variation in the susceptibility to RVF of animals of different species. Camels usually have an inapparent infection with RVF virus (RVFV), but sudden mortality, neonatal mortality and abortion occurs and abortion rates can be as high as in cattle.

Humans are susceptible to RVFV and are infected through contact with infected animal material (body fluids or tissues) or through bites from infected mosquitoes. RVFV has also caused serious infections in laboratory workers and must be handled with biosafety and biocontainment measures. It is recommended that laboratory workers be vaccinated if possible.

**Identification of the agent:** RVFV consists of a single serotype of Phlebovirus that has morphological and physicochemical properties typical of this genus.

Identification of RVFV can be achieved by virus isolation, antigen-detection enzyme-linked immunosorbent assay (ELISA) or immunopathology. Viral RNA can be detected by reverse-transcription polymerase chain reaction.

The virus can be isolated from blood, preferably collected with anticoagulant, during the febrile stage of the disease, or from organs (e.g. liver, spleen and brain tissues) of animals that have died and from the organs of aborted fetuses. Primary isolations are usually made on cell cultures of various types, such as African green monkey kidney (Vero) and baby hamster kidney (BHK) cells. Alternatively, sucking mice may be used for primary virus isolation.

**Serological tests:** Identification of specific antibodies is mostly achieved by ELISA or the virus neutralisation test.

**Requirements for vaccines:** Live attenuated or inactivated vaccines can be used in countries where RVF is endemic or that are at risk of its introduction. These vaccines should preferably be prepared from attenuated strains of RVFV grown in cell culture.

In RVF-free countries, vaccines and diagnostic tests should preferably be limited to those using inactivated virus. Work with live virus should be performed by trained personnel in biocontainment facilities following appropriate biosafety procedures.

There are two OIE Reference Laboratories for RVF (see Table given in Part 4 of this Terrestrial Manual).

### A. INTRODUCTION

Rift Valley fever (RVF) is a peracute or acute, febrile, mosquito-borne, zoonotic disease caused by a virus of the family *Bunyaviridae*, genus *Phlebovirus*. It is usually present in epizootic form over large areas of a country following heavy rains and flooding, and is characterised by high rates of abortion and neonatal mortality, primarily in sheep, goats, cattle and camels. The susceptibility of different species and breeds to RVF may vary

considerably. Some animals may have inapparent infections, while others have severe clinical disease with mortality and abortion. Susceptible, older non-pregnant animals often do not show signs of disease.

Signs of the disease tend to be nonspecific, rendering it difficult to recognise individual cases during epidemics (Coackley *et al.*, 1967; Coetzer, 1982; Coetzer & Barnard, 1977; Easterday, 1965; Gerdes, 2004; Mansfield *et al.*, 2015; Meegan & Bailey, 1989; Swanepoel & Coetzer, 1994; Weiss, 1957); however, the occurrence of numerous abortions and mortalities among young animals, together with disease in humans, is characteristic of RVF. RVF has a short incubation period of about 12–36 hours in lambs. A biphasic fever of up to 41°C may develop, and the body temperature remains elevated until shortly before death. Affected animals are listless, disinclined to move or feed, and may show enlargement of superficial lymph nodes and evidence of abdominal pain. Lambs rarely survive longer than 36 hours after the onset of signs of illness. Animals older than 2 weeks may die peracutely, acutely or may recover or develop an inapparent infection. Some animals may regurgitate ingesta and may show melaena or bloody, foul-smelling diarrhoea and bloodstained mucopurulent nasal discharge. Icterus may sometimes be observed, particularly in cattle. In addition to these signs, adult cattle may show lachrymation, salivation and dysgalactia. In pregnant sheep, the mortality and abortion rates vary from 5% to almost 100% in different outbreaks and between different flocks. The death rate in cattle is usually less than 10%. Camels have been regularly involved in the RVF epidemics in East Africa, Egypt and more recently Mauritania. Clinical disease is usually not seen in adult camels, but sudden deaths, abortion and some early post-natal deaths have been observed. Differential diagnosis includes: bluetongue, Wesselsbron disease, enterotoxemia of sheep, ephemeral fever, brucellosis, vibriosis, trichomonosis, Nairobi sheep disease, heartwater, ovine enzootic abortion, toxic plants, bacterial septicaemias, peste des petits ruminants, anthrax and Schmallenberg disease.

The hepatic lesions of RVF are very similar in all species, varying mainly with the age of the infected individual (Coetzer, 1982). The most severe lesion, occurring in aborted fetuses and newborn lambs, is a moderately to greatly enlarged, soft, friable liver with a yellowish-brown to dark reddish-brown colour with irregular congested patches. Numerous greyish-white necrotic foci are invariably present in the parenchyma, but may not be clearly discernible. In adult sheep, the lesions are less severe and pinpoint reddish to greyish-white necrotic foci are distributed throughout the parenchyma. Haemorrhage and oedema of the wall of the gallbladder are common. Hepatic lesions in lambs are almost invariably accompanied by numerous small haemorrhages in the mucosa of the abomasum. The contents of the small intestine and abomasum can be dark chocolate-brown as a result of the presence of partially digested blood. In all animals, the spleen and peripheral lymph nodes can be enlarged, oedematous and may have petechiae.

Microscopically, hepatic necrosis is the most obvious lesion of RVF in both animals and humans. In fetuses and neonates of cattle and sheep, foci of necrosis consist of dense aggregates of cellular and nuclear debris, some fibrin and a few inflammatory cells. There is a severe lytic necrosis of most hepatocytes and the normal architecture of the liver is lost. In about 50% of affected livers, intranuclear inclusion bodies that are eosinophilic and oval or rod-shaped are found. Mineralisation of necrotic hepatocytes is also seen. In adult animals, hepatic necrosis is less diffuse, and in sheep, icterus is more common than in lambs (Coetzer, 1982; Swanepoel & Coetzer, 1994).

In humans, RVF infections are usually inapparent or associated with a moderate to severe, nonfatal, influenza-like illness (Madani *et al.*, 2003; McIntosh *et al.*, 1980; Meegan, 1981). A minority of patients may develop retinal lesions, encephalitis, or severe hepatic disease with haemorrhagic manifestations, which is generally fatal. RVF virus (RVFV) has caused serious human infections in laboratory workers. Staff should be vaccinated when a vaccine is available. An inactivated vaccine has been developed for human use. However, this vaccine is not licensed and is not commercially available. It has been used experimentally to protect veterinary and laboratory personnel at high risk of exposure to RVF. Further information about the disease and vaccination in humans is available from WHO<sup>1</sup>. RVFV should be handled at an appropriate biosafety and containment level determined by biorisk analysis (see Chapter 1.1.4 *Biosafety and biosecurity: Standard for managing biological risk in the veterinary laboratory and animal facilities*). Particular care needs to be exercised when working with infected animals or when performing post-mortem examinations.

RVFV consists of a single serotype of the *Bunyaviridae* family (genus *Phlebovirus*) and has morphological and physicochemical properties typical of bunyaviruses. The virus is enveloped, spherical and 80–120 nm in diameter. Glycoprotein spikes project through a bilayered lipid envelope. The virus is readily inactivated by lipid solvents and acid conditions below pH 6. RVFV has a three-segmented, single-stranded, negative-sense RNA genome and consists of the following segments: L (large), M (medium) and S (small), each of which is contained in a separate nucleocapsid within the virion. The S segment is an ambisense RNA, i.e. has bi-directional coding (Giorgi, 1991).

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1 <http://www.who.int/mediacentre/factsheets/fs207/en/>

No significant antigenic differences have been demonstrated between RVF isolates and laboratory-passaged strains from many countries, but differences in pathogenicity between genotypes have been shown (Bird *et al.*, 2007; Swanepoel *et al.*, 1986).

RVFV is endemic in many African countries and may involve several countries in the region at the same time or progressively expand geographically over the course of a few years. In addition to Africa, large outbreaks have been observed in the Arabian Peninsula and some Indian Ocean Islands. These generally, but not exclusively, follow the periodic cycles of unusually heavy rainfall, which may occur at intervals of several years, or the flooding of wide areas favouring the proliferation of mosquitoes.

Rainfall facilitates mosquito eggs to hatch. *Aedes* mosquitoes acquire the virus from feeding on infected animals, and may potentially vertically transmit the virus, so that new generations of infected mosquitoes may hatch from their eggs (Linthicum *et al.*, 1985). This provides a potential mechanism for maintaining the virus in nature, as the eggs of these mosquitoes may survive for periods of up to several years in dry conditions. Once livestock is infected, a wide variety of mosquito species may act as the vector for transmission of RVFV and can spread the disease.

Low level RVF activity may take place during inter-epizootic periods. RVF should be suspected when exceptional flooding and subsequent abundant mosquito populations are followed by the occurrence of abortions, together with fatal disease marked by necrosis and haemorrhages in the liver that particularly affect newborn lambs, kids and calves, potentially concurrent with the occurrence of an influenza-like illness in farm workers and people handling raw meat.

During an outbreak, preventive measures to protect workers from infection should be employed when there are suspicions that RVFV-infected animals or animal products are to be handled.

## B. DIAGNOSTIC TECHNIQUES

The collection of specimens and their transport should comply with the recommendations in Chapter 1.1.2 *Collection, submission and storage of diagnostic specimens* and Chapter 1.1.3 *Transport of biological materials* of this *Terrestrial Manual*.

Proper diagnosis should always use a combination of techniques based on history, the purpose of the testing and the stage of the suspected infection. For a definitive interpretation, combined epidemiological, clinical and laboratory information should be evaluated carefully.

All the test methods described below should be validated in each laboratory using them (see Chapter 1.1.6 *Principles and methods of validation of diagnostic assays for infectious diseases*). The OIE Reference Laboratories for RVF should be contacted for technical support. Table 1 provides a general guidance summary on the use of the diagnostic tests methods. More detailed aspects are addressed in the test descriptions that follow.

**Table 1.** Test methods available for diagnosis of Rift Valley fever and their purposes

Method	Purpose					
	Population freedom from infection (unvaccinated animals)	Individual animal freedom from infection prior to movement	Contribute to eradication policies	Confirmation of clinical cases <sup>2</sup>	Prevalence of infection – surveillance	Immune status in individual animals or populations post-vaccination
<b>Agent identification<sup>3</sup></b>						
<b>Virus isolation in cell culture</b>	–	–	–	+++	+	–
<b>Virus isolation in sucking mice</b>	–	–	–	+	+	–

2 Laboratory confirmation of clinical cases should require a combination of at least two positive results from two different diagnostic test methods: either positive for virus or viral RNA and antibodies or positive for IgM and IgG with demonstration of rising titres between paired sera samples collected 2–4 weeks apart. Depending of the stage of the disease, virus or antibodies will be detected.

3 A combination of agent identification methods applied on the same clinical sample is recommended.

Method	Purpose					
	Population freedom from infection (unvaccinated animals)	Individual animal freedom from infection prior to movement	Contribute to eradication policies	Confirmation of clinical cases <sup>2</sup>	Prevalence of infection – surveillance	Immune status in individual animals or populations post-vaccination
RT-PCR	–	–	–	+++	+	–
Antigen detection	–	–	–	++	+	–
Histopathology with immunohistochemistry	–	–	–	++	–	–
Detection of immune response						
ELISA	+++	++	+++	++	+++	+++
PRNT	+++	+++	+++	++	++	+++

Key: +++ = recommended method; ++ = suitable method; + = may be used in some situations, but cost, reliability, or other factors severely limits its application; – = not appropriate for this purpose.  
 Although not all of the tests listed as category +++ or ++ have undergone formal validation, their routine nature and the fact that they have been used widely without dubious results, makes them acceptable.  
 RT-PCR = reverse-transcription polymerase chain reaction;  
 ELISA = enzyme-linked immunosorbent assay; PRNT = plaque reduction neutralisation test.

## 1. Identification of the agent

RVFV may be isolated from serum but preferentially from plasma or blood collected with anticoagulant during the febrile stage of the disease in live animals, or from liver, spleen and brain of animals that have died, or from aborted fetuses. Primary isolation is usually performed in cell cultures of various types or by intracerebral inoculation of sucking mice.

### 1.1. Specimen collection

Using appropriate protective equipment to ensure biosafety of the staff, approximately 5 ml of blood with anticoagulant (preferably ethylene diamine tetra-acetic acid [EDTA]) collected during the febrile stage of the disease, or approximately 1 cm<sup>3</sup> of liver, spleen, brain or abortion products collected post-mortem, should be submitted for virus isolation. The samples should be kept at 0–4°C during transit. If transport to the laboratory is likely to take more than 24 hours, the samples should be frozen and sent on dry ice or frozen cold pack. In the case of a blood sample, plasma should be collected and frozen for transport.

### 1.2. Isolation in cell culture

A variety of cell line monolayers including African green monkey kidney (Vero), baby hamster kidney (BHK) and AP61 mosquito cells (Digoutte *et al.*, 1989) may be used. They are inoculated with 1/10 dilution of the sample and incubated at 37°C for 1 hour (with mosquito cell lines, the incubation should be done at 27°C for 1 hour). It is advisable to also inoculate some cultures with a further 1/100 dilution of the inoculum. This is to avoid the production of defective particles, which follows the use of very high titre virus inoculum. The inoculum is removed and the monolayer is washed with phosphate-buffered saline (PBS) or culture medium. The wash solution is removed, replaced by fresh culture medium and incubated at an appropriate temperature. The cultures are observed for 5–6 days. Mammalian cell lines are preferably used as RVFV induces a consistent cytopathic effect (CPE) characterised by slight rounding of cells followed by destruction of the whole cell sheet within 12–24 hours. Confirmation of virus isolation should be performed preferably by immunostaining or reverse-transcription polymerase chain reaction (RT-PCR).

### 1.3. Isolation in sucking mice

For reasons of animal welfare and biosafety, this method should be avoided if possible. Approximately 1 g of homogenised tissue is suspended 1/10 in cell culture medium or buffered saline, pH 7.5, containing sodium penicillin (1000 International Units [IU]/ml), streptomycin sulphate (1 mg/ml), mycostatin (100 IU/ml), or fungizone (2.5 µg/ml). The suspension is centrifuged at 1000 **g** for

10 minutes and the supernatant fluid is injected intracerebrally into 1- to 5-day-old mice. Sucking mice will either die or be obviously ill by day 2 post-inoculation.

Confirmation of virus isolation should be performed preferably by immunostaining or PCR.

#### 1.4. Reverse-transcription polymerase chain reaction

A rapid diagnosis can also be made by detection of viral RNA (Sall *et al.*, 2001) using validated conventional or real-time RT-PCR (Bird *et al.*, 2007; Drosten *et al.*, 2002; Garcia *et al.*, 2001; Sall *et al.*, 2001). These techniques have been very useful during RVF outbreaks in Africa. They may also be used to detect RVFV RNA in mosquito pools (Jupp *et al.*, 2000).

These techniques should be followed by sequencing of selected samples. Below are proposed protocols for conventional and real-time RT-PCR. For information on specific procedures consult the OIE Reference Laboratories.

##### 1.4.1. Agarose gel-based RT-PCR assay

This procedure is used by the OIE Reference Laboratories. The RT-PCR assay consists of the three successive procedures of (a) extraction of template RNA from the test or control sample followed by (b) RT of the extracted RNA, (c) PCR amplification of the RT product and (d) detection of the PCR products by agarose gel electrophoresis.

###### i) Test procedure

RNA is extracted using an appropriate chemical method according to the procedure recommended by the manufacturer of the commercial kit. When the procedure is completed, retain the extracted RNA samples on ice if the RT step is about to be performed. Otherwise store at  $-20^{\circ}\text{C}$  or  $-70^{\circ}\text{C}$ . For RT-PCR, the protocol from Sall *et al.* (2001) is used. For the first RT-PCR step, NSca (5'-CCT-TAA-CCT-CTA-ATC-AAC-3') and NSng (5'-TA-TCA-TGG-ATT-ACT-TTC-C-3') primers are used.

a) Prepare the PCR mix described below for each sample. It is recommended to prepare the mix in bulk for the number of samples to be tested plus one extra sample.

Nuclease-free water (15.5  $\mu\text{l}$ ); RT-PCR reaction buffer, 5 $\times$  conc (10  $\mu\text{l}$ );  $\text{MgCl}_2$ , 25 mM (1  $\mu\text{l}$ ); dNTPs, 10 mM mixture each of dATP, dCTP, dGTP, dTTP (1  $\mu\text{l}$ ); primer NSca, 10  $\mu\text{M}$  (2.5  $\mu\text{l}$ ); primer NSng 10  $\mu\text{M}$  (2.5  $\mu\text{l}$ ); Enzyme mix, 5 units/ $\mu\text{l}$  (0.25  $\mu\text{l}$ ).

b) Add 40  $\mu\text{l}$  of PCR reaction mix to a well of a PCR plate or to a microcentrifuge tube for each sample to be assayed followed by 10  $\mu\text{l}$  of the RNA (prepared in step i) to give a final reaction volume of 50  $\mu\text{l}$ .

c) Centrifuge the plate or tubes for 1 minute in a suitable centrifuge to mix the contents of each well.

d) Place the plate in a thermal cycler for PCR amplification and run the following programme:

45 $^{\circ}\text{C}$  for 30 minutes: 1 cycle;

95 $^{\circ}\text{C}$  for 2 minutes: 1 cycle;

94 $^{\circ}\text{C}$  for 30 seconds, 44 $^{\circ}\text{C}$  for 30 seconds, 72 $^{\circ}\text{C}$  for 1 minute: 40 cycles;

72 $^{\circ}\text{C}$  for 5 minutes: 1 cycle.

e) Mix a 20  $\mu\text{l}$  aliquot of each PCR reaction product with 4  $\mu\text{l}$  of staining solution and load onto a 1.2% agarose gel. After electrophoresis a positive result is indicated by the presence of a 810 bp (242 bp for Clone 13) band corresponding to RVFV sequence in the NSs coding region of the S segment of the genome.

For the nested RT-PCR step, NS3a (5'-ATG-CTG-GGA-AGT-GAT-GAG-CG-3') and NS2g (5'-GAT-TTG-CAG-AGT-GGT-CGT-C-3') are used.

f) Prepare the PCR mix described below for each sample. It is recommended to prepare the mix in bulk for the number of samples to be tested plus one extra sample.

Nuclease-free water (35.5 µl); RT-PCR reaction buffer, 10x conc (5 µl); MgCl<sub>2</sub>, 25 mM (1.25 µl); dNTPs, 10 mM mixture each of dATP, dCTP, dGTP, dTTP (1 µl); primer NS3a (5'-ATG-CTG-GGA-AGT-GAT-GAG-CG-3'), 10 µM (2.5 µl); primer NS2g (5'-GAT-TTG-CAG-AGT-GGT-CGT-C-3'), 10 µM (2.5 µl); Enzyme mix, 5 units/µl (0.25 µl).

- g) Add 49 µl of PCR reaction mix to a well of a PCR plate or to a microcentrifuge tube for each sample to be assayed followed by 1 µl of the amplicon obtained from RT-PCR reaction with NSca and NSng to give a final reaction volume of 50 µl.
- h) Centrifuge the plate or tubes for 1 minute in a suitable centrifuge to mix the contents of each well.
- i) Place the plate in a thermal cycler for PCR amplification and run the following programme:  
95°C for 2 minutes: 1 cycle;  
94°C for 1 minute, 55°C for 1 minute, 72°C for 1 minute: 25 cycles;  
72°C for 5 minutes: 1 cycle.
- j) Mix a 20 µl aliquot of each PCR reaction product with 4 µl of staining solution and load onto a 1.2% agarose gel. After electrophoresis a positive result is indicated by the presence of a 668 bp (129 bp for Clone 13) band corresponding to RVFV sequence in the NSs coding region of the S segment of the genome.

#### 1.4.2. Real-time RT-PCR assay

The real-time RT-PCR assay can use the same procedures for extraction of total RNA from the test or control sample followed by RT of the extracted RNA as for the conventional procedure. The protocol is adapted from Drosten *et al.* (2002). If commercial kits are used the manufacturers' method should be followed.

- i) Test procedure
  - a) Prepare the PCR mix described below for each sample. Again it is recommended to prepare the mix in bulk for the number of samples to be tested plus one extra sample.  
  
Nuclease-free water (1.4 µl); RT-PCR reaction master mix, 2x conc. (10 µl); real-time PCR forward primer **RVS**: 5'-AAA-GGA-ACA-ATG-GAC-TCT-GGT-CA-3', 10 µM (2 µl); real-time PCR reverse primer **RVA**s: 5'-CAC-TTC-TTA-CTA-CCA-TGT-CCT-CCA-AT-3', 10 µM (2 µl); **RVP**: **FAM** 5'-AAA-GCT-TTG-ATA-TCT-CTC-AGT-GCC-CCA-A-3' **TAMRA** 20 µM (0.2 µl).
  - b) Add 17 µl PCR reaction mix to a well of a real-time PCR plate for each sample to be assayed followed by 3 µl of the prepared RNA to give a final reaction volume of 20 µl.
  - c) Spin the plate for 1 minute in a suitable centrifuge to mix the contents of each well.
  - d) Place the plate in a real-time PCR machine for PCR amplification and run the following programme:  
45°C for 30 minutes: 1 cycle;  
95°C for 5 minutes: 1 cycle;  
95°C for 5 seconds, 57°C for 35 seconds: 45 cycles.
  - e) Interpreting the results: assign a threshold cycle (CT) value to each PCR reaction from the amplification plots (fluorescence signal versus cycle number); different cut-off values may be appropriate for different sample types. The CT values used to assign samples as either RVFV positive or negative should be defined by individual laboratories using appropriate reference material.

#### 1.5. Antigen detection

The antigen detection enzyme-linked immunosorbent assay (ELISA) is an immunocapture test. Samples are tested at different dilutions with appropriate positive and negative controls. This test has been used for human and animal samples during outbreaks in Saudi Arabia and Kenya (Madani *et al.*, 2003; Munyua *et al.*, 2010).

### 1.5.1. Test procedure

The controls and antisera used in the performance of this assay should have been treated to inactivate any RVFV that they may have contained at the time of production. These products are safe, within the limits of our ability to detect viable virus. The material to be tested for the presence of RVF viral antigen is potentially contaminated with viable RVFV or other agents for which a differential determination is being sought. At the very least, good laboratory practices should be used. Samples could be inactivated using appropriate detergent and heat inactivation.

- i) The basic approach is that of a double antibody sandwich capture assay in which the antigen is captured by antibody on a solid phase and then detected by a second antibody. A detection system using horseradish peroxidase (HRPO)–ABTS (2,2'-azino-di-(3-ethyl-benzthiazoline)-6-sulphonic acid) is then applied to determine how much of the detection antibody has been retained on the solid phase of the system.
  - a) Capture (coating) antibody (diluted 1/2000 in PBS [no Tween], pH 7.4 and coated overnight at 4°C; control wells are coated with a similar dilution of normal fluid)

Plates are coated with a specific anti-viral antibody (available in OIE or WHO Reference Laboratories) capable of capturing viral antigen from the test sample. Normal serum is added to rows to serve as controls used to determine the nonspecific background or noise of the system. In this instance it is a hyperimmune mouse ascitic fluid (HMAF) (it could also be monoclonal antibodies) specific for RVF viruses.
  - b) Suspect samples and control antigen (diluted 1/4 and then diluted four-fold, down the plate)

These are added in serum diluent to allow specific viral antigens to bind to the capture antibody. The serum diluent (PBS, 0.01 M, pH 7.4, with or without thiomersal) contains 5% skim milk and 0.1% Tween 20 to reduce nonspecific binding.
  - c) Detection antibody

An antibody, high titered for specific viral antigen, is added to allow detection of the bound viral antigen. In this experiment, it is an anti-RVF hyperimmune rabbit serum (available in OIE or WHO Reference Laboratories) that has a high titre against RVF viruses.
  - d) Anti-rabbit conjugated to HRPO (commercial product)

This is used to detect the rabbit anti-RVF that binds to the antigen.
  - e) Criteria for determining positives

A standard control antigen has been provided and will be run in a standard dilution series. This, in effect, provides a standard curve that will determine the limits of detection of the assay. A group of normal tissues or samples, uncontaminated with antigen, are tested to determine the background of the assay and the limit at which the standard was positive. The values of these normal controls are used to generate the mean and standard deviation of the random background to be expected with negative samples. A sample is considered positive if its optical density (OD) value exceeds the mean plus 3 standard deviations of these normal controls.

### 1.6. Histopathology

Histopathological examination of the liver of affected animals will reveal characteristic cytopathology, and immunostaining will allow the specific identification of RVF viral antigen in tissue (Coetzer, 1982; Swanepoel *et al.*, 1986). This is an important diagnostic tool because liver or other tissue placed in neutral buffered formaldehyde in the field is inactivated (subject to specimen thickness and time of fixation) and does not require a cold chain, which facilitates handling and transport from remote areas.

## 2. Serological tests

Samples collected from animals for antibody testing may contain live virus and appropriate inactivation steps should be put in place. A combination of heat and chemical inactivation has been described (Van Vuren & Paweska, 2010). Immunofluorescence assays are still used, although cross-reactions may occur between RVFV

and other phleboviruses. Techniques such as the agar gel immunodiffusion (AGID), radioimmunoassays, haemagglutination inhibition (HI), and complement fixation are no longer used.

Several assays are available for detection of anti-RVFPV antibodies in a variety of animal species. Currently the most widely used technique is the ELISA for the detection of IgM and IgG. Virus neutralisation tests have been used to detect antibodies against RVFPV in the serum of a variety of species. Neutralisation tests are the most specific diagnostic serological tests, but these tests can only be performed with live virus and are not recommended for use outside endemic areas or in laboratories without appropriate biosecurity facilities and vaccinated personnel. However, alternative neutralisation assays not requiring handling of highly virulent RVFPV and not requiring high containment, are being developed and validated.

## 2.1. Enzyme-linked immunosorbent assay

The ELISA is a reliable and sensitive test to detect antibodies against RVFPV. Both IgG and IgM ELISAs are available for most species. IgM-capture ELISA allows diagnosis of recent infections.

A number of ELISAs using different formats are commercially available and others are under development (Afetine *et al.*, 2007; Cettre-Sossah *et al.*, 2009; Jansen Van Vuren *et al.*, 2007; Madani *et al.*, 2003; Munyua *et al.*, 2010; Paweska *et al.*, 2003; 2005; Van Vuren & Paweska, 2010). They are used routinely in many countries for single case diagnosis, outbreak management, and surveillance.

Below are two such tests used at the OIE Reference Laboratory in South Africa. Both assays use 10% non-fat milk/Tris salt Tween (NFM/TST) as blocking and dilution buffer, and TST buffer (50 mM Tris; 150 mM NaCl; 0.1% Tween 20) as washing buffer (pH 8.0). The reactions are stopped with 2 N H<sub>2</sub>SO<sub>4</sub>.

The recombinant nucleoprotein (rN) of RVFPV is produced and purified as described by Williams *et al.* (2011). Conjugation of the protein to HRPO is performed following the Nakane & Akira Kawaoui (1974) protocol. The rN antigen is stable for up to 1 year at 4°C.

To prepare plates for immediate use, make a checkerboard titration of the capture antibody or antigen against the conjugate in a 96-well ELISA plate to determine the minimum reagent concentration that would give an OD value of 0.5–0.6 when read at 650 nm after an incubation period of 20 minutes. This will determine how the antibody/antigen and conjugate must be diluted for coating of the plates and detection of the antigen/antibody binding in the test.

### 2.1.1. IgM capture ELISA

- i) Test procedure
  - a) Coat each well of the 96-well ELISA plates with 100 µl of the capture antibody (affinity purified rabbit anti-sheep IgM1) diluted to 1 µg/ml in PBS (that is a 1/1000 dilution if so determined by the titration), and incubate overnight at room temperature in a humid chamber.
  - b) Wash the plates three times with wash buffer.
  - c) Block the plates with 300 µl blocking buffer and incubate for 1 hour at 37°C.
  - d) Wash the plates again three times with wash buffer.
  - e) Dilute both control (positive and negative) and test sera 1/100 in blocking buffer and add each serum in a designated well at volumes of 100 µl/well.
  - f) Incubate the plates for 1 hour at 37°C. Avoid drying by putting plates in a humid chamber.
  - g) Following the incubation step, wash the ELISA plates with wash buffer three times.
  - h) Dilute the rN-HRP conjugate 1/6000 and add 100 µl of this in each well. Use blocking buffer as the conjugate control.
  - i) The plates are then incubated for 60 minutes at 37°C.
  - j) The plates are washed, as in step b above. Ready-to-use tetramethyl benzidine (TMB) substrate at 100 µl quantities is then transferred to each well, and the plates allowed to stand at room temperature for a few minutes, until development of a colour change, or OD values of 0.5 when the plates are read at 650 nm. Exposure to direct light should be avoided.
  - k) Stop the reaction with 100 µl stop solution, and read the OD values using an ELISA plate reader at 450 nm.

- l) Interpretation of results: results are expressed as percentage of the positive serum control (PP) using the formula:

$$(\text{mean OD of duplicate test serum})/(\text{mean OD of positive control}) \times 100$$

where positive and negative cut-off values are determined by receiver operating characteristic (ROC) curve analysis.

It should be noted that the cut-off value for an ELISA can be adjusted for different target populations as well as for different diagnostic purposes (Jacobson, 1998). The cut-off values determined by the validation exercise at the OIE Reference Laboratory in South Africa are the following: PP (%) values: negative <4; suspicious 4–5; positive >6.

### 2.1.2. Indirect IgG ELISA

- i) Test procedure
- a) Coat each well of the 96-well ELISA plate with 100 µl of rN diluted in 50 mM of carbonate buffer (pH 9.6) using the dilution ratio determined by prior titration as explained above; incubate overnight at room temperature in a humid chamber.
  - b) Wash the plates three times with approximately 300 µl wash buffer per well.
  - c) Block the plates with approximately 300 µl blocking buffer and incubate for 1 hour at 37°C.
  - d) Wash the plates again three times with nearly 300 µl of wash buffer per well.
  - e) Dilute both control (positive and negative) and test sera 1/100 in blocking buffer.
  - f) Add 100 µl of the diluted sera in designated wells in duplicate.
  - g) Incubate the plates for 1 hour at 37°C. Avoid drying by putting plates in a humid chamber.
  - h) Following the incubation step, wash the ELISA plates with wash buffer three times.
  - i) Dilute protein G-HRP conjugate 1/32,000 in blocking buffer and add 100 µl of the conjugate in each well.
  - j) Incubate for 60 minutes at 37°C.
  - k) The plates are washed, as in step b above. Add 100 µl of ready to use TMB substrate to each well and allow the plates to stand at room temperature for a few minutes, while avoiding exposure to direct light. The plates are read at 650 nm to determine if OD of 0.4–0.6 has been reached.
  - l) Stop the reaction with 100 µl stop solution, and read the plates using ELISA plate reader at 450 nm.
  - m) Interpretation of results: results are expressed as percentage of the positive serum (PP) using the formula:

$$(\text{mean OD of duplicate test serum})/(\text{mean OD of positive control}) \times 100$$

where positive and negative cut-off values are determined by receiver operating characteristic (ROC) curve analysis.

It should be noted that the cut-off value for an ELISA can be adjusted for different target populations as well as for different diagnostic purposes (Jacobson, 1998). The cut-off values determined by the validation exercise at the OIE Reference Laboratory in South Africa are the following: PP values (%): negative <4; suspicious 4–6; positive >7.

### 2.2. Plaque reduction neutralisation test

The plaque reduction neutralisation test (PRNT) may be used to determine the presence of antibodies in naturally infected animals and in vaccinated animals. The test is highly specific and can be used to test serum of any species. It is generally used to measure vaccine efficacy. The Smithburn neurotropic mouse brain strain of highly attenuated RVFV (Smithburn, 1949) or any other, preferably attenuated, RVFV, is used as challenge virus. The virus is stored at –80°C, or 4°C in freeze-dried form.

The PRNT<sub>80</sub> (i.e. 80% reduction) conducted in a cell culture system is generally accepted as the standard assay system for the quantitative determination for neutralisation antibody activity in serum

samples. The PRNT can be run in 6-well, 12-well, or 24-well plastic plates. The following technique is using 12-well and four-fold dilutions of test sera.

### 2.2.1. Test procedure

- i) Inactivate the test sera at a 1:10 (0.050 ml in 0.450 ml) dilution tubes for 30 minutes in a 56°C water bath.
- ii) Make five sequential four-fold dilutions tubes (0.1 ml in 0.3 ml) ending with 1:10,240 in culture media. Include known positive and negative control sera.
- iii) A virus suspension, calculated to yield approximately 100 plaque forming units per 0.1 ml is prepared. Add 0.3 ml of virus suspension to all serum dilution tubes and positive and negative tubes. Cover tubes and refrigerate overnight at 4°C.
- iv) Mark a 12-well plastic plate containing cell monolayers with the identification number of each test sample corresponding to worksheet: virus back-titration, and duplicate wells for dilutions 10, 40, 160, 640, 2560, and 10,240. Place plates in incubator (37°C and 5% CO<sub>2</sub>) until inoculation.
- v) When ready, discard growth media. Starting with the highest dilution of serum-virus mixture (1:10,240) inoculate 0.1 ml (100 µl) into each of the two wells marked 1:10,240 containing confluent cell monolayers. Continue inoculation of wells down to and including 1:10 dilution. Allow inoculated plates to absorb for at least 1 hour at 37°C in a humidified atmosphere with 5% CO<sub>2</sub>. Rock plates every 15 minutes.
- vi) First overlay the nutrient-agarose media. Overlay inoculated cells with 1.5 ml of nutrient-agarose (equal volume of 2 × EBME mixture to melted 2% agarose) and allow to set at room temperature. Invert plates when agarose is solidified and place in a 37°C, 5% CO<sub>2</sub> incubator.
- vii) Twenty-four hours prior to counting plaques, a second overlay (0.75 ml) containing neutral red dye is added to all test wells. Allow the overlay to solidify for 15 minutes at room temperature (keep plates from light). Invert plates and place in 37°C and 5% CO<sub>2</sub> incubator.
- viii) Approximately 24 hours after second overlay, count all plaques in virus controls, panels and positive and negative controls. A titre is expressed as the highest dilution of serum causing an 80% reduction in comparison to virus control count.

Positive control titre will depend on dilution format used in the test proper. If a four-fold dilution of sera is used, positive control titre must be within four-fold (+ or –) of the previously determined positive control titre. The titre that is considered positive for antibodies to RFV is variable depending on the purpose of the test. If the purpose is to measure the immunogenicity of a vaccine, 1/80 or 1/100 is a useful threshold. If it is to check the circulation of RFV in a population, a 1/10 or 1/20 titre in an animal can be considered as positive.

## C. REQUIREMENTS FOR VACCINES

### 1. Background

Currently available RVF vaccines are either live attenuated or inactivated vaccines. National regulatory authorities can advise on availability in a particular country.

*Table 2. Summary of the current RVF vaccine strains*

	<b>Smithburn live attenuated virus vaccines</b>	<b>Clone-13 live attenuated virus vaccine</b>	<b>Inactivated virus vaccines</b>	<b>TSI–GSD–200 inactivated human vaccine (presently not available)</b>
<b>Origin of the isolate</b>	Mosquito isolate, Uganda, 1948	Human isolate, 1974	Field strains (South Africa and Egypt) used	Mosquito isolate, Uganda, 1944
<b>Attenuation</b>	More than 200 passages in murine brain	Natural deletion in NSs gene	Not applicable	Not applicable

	<b>Smithburn live attenuated virus vaccines</b>	<b>Clone-13 live attenuated virus vaccine</b>	<b>Inactivated virus vaccines</b>	<b>TSI-GSD-200 inactivated human vaccine (presently not available)</b>
<b>Production substrate</b>	BHK cell line	Vero cell line	BHK cell line	Diploid fetal rhesus lung cell line
<b>Target</b>	livestock	livestock	livestock	human
<b>DIVA policy</b>	No	No	No	Not applicable

### 1.1. The live attenuated Smithburn RVF vaccine

The vaccine virus is derived from Smithburn's original neurotropic strain. This strain is not lethal to adult mice inoculated intraperitoneally and is safe for use in all breeds of cattle, sheep and goats (Barnard, 1979; Smithburn, 1949). However, it may cause fetal abnormalities or abortion in pregnant animals. The Smithburn RVF vaccine has been used for decades in the control of RVF in Eastern and Southern Africa and in the Middle East, and is still used to date in different endemic region.

### 1.2. The Clone 13 RVF vaccine

Clone 13 is a naturally attenuated strain characterised by a large deletion of the gene encoding for the main virulence factor, the NSs (Muller *et al.*, 1995). The risk of reversion is considered unlikely. No abortion or side effects have been seen in experimental vaccine trials (Dungu *et al.*, 2010; Hunter & Bouloy, 2001). It was recently introduced in South Africa for use in sheep and cattle using a single injection regimen.

### 1.3. The inactivated RVF vaccine

The currently produced formalin-inactivated vaccines derived from a field strain of RVFV adapted to growth in cell culture (Barnard, 1979; Barnard & Botha, 1977). These vaccines are currently adjuvanted in aluminium hydroxide. However inactivated RVF vaccines require a booster 3–6 months following initial vaccination, followed by yearly boosters. Inactivated RVF vaccine is also used in outbreak situations, and in pregnant animals as the attenuated Smithburn vaccine is not suitable for this group.

### 1.4. Inactivated experimental human vaccine

Formerly produced by the Salk Institute (USA), the inactivated experimental human vaccine is no longer available (Meadors *et al.*, 1986).

Many other candidate vaccines are either being developed and evaluated in target animals or are in an early stage of development (Food and Agriculture Organization of the United Nations [FAO], 2011; Morrill *et al.*, 1997a; 1997b).

There are a number of product characteristics that it would be preferable to have in an effective and safe RVF vaccine, which should be used to define a target product profile. Elements of a target product profile for a RVF vaccine should preferably meet recommendation 2 of the report of the FAO meeting, 2011 (FAO, 2011) and as indicated below.

The main purpose of a RVF vaccine is to prevent epizootics and epidemics in species of economic interest (susceptible livestock species [ruminants] and, potentially, camelids), and limit the impact on animal and public health (Mansfield *et al.*, 2015). In addition to the potential economic impact, it could also have some implications in international movements of animals. It is relevant to distinguish specific requirements for endemic regions and regions free of the disease.

### 1.5. Endemic region

The objective is the prevention and control of epizootics and epidemics in endemic areas and to contribute to the improvement of livestock production in endemic areas. In order of priorities, characteristics of the vaccines are:

- i) preferably one dose, resulting in a long-lasting immunity of at least 1 year;
- ii) preferably a life-long immunity after a limited number of doses.

## 1.6. Free or non-endemic region

Vaccines would be used either for the prevention of, or the response to an introduction of the virus. The expected characteristics of the vaccines are: safe with a quick onset of protective immunity and protection in animals of all ages and physiological status. Although DIVA (detection of infection in vaccinated animals) is an important property of any future vaccine, a requirement for DIVA should not hinder or block the development or licensing of an effective RVF vaccine.

In all cases, the vaccines should be:

- i) Safe for the staff involved in the production of the vaccines and for the users, safe to all physiological stages of animals, and with minimal risk of introduction into the environment (potential vectors);
- ii) Protective in multispecies and if possible in all susceptible species of economic importance, to prevent infection and transmission;
- iii) Cost effective for producers and users, preferentially with a single-dose vaccination;
- iv) Easy to use (e.g. needle-free delivery), suitable for stockpiling (e.g. a vaccine bank) and quick availability.

Staff handling virulent RVFV should preferably work in high containment facilities and be vaccinated, if vaccines are available, to minimise the risk of infection.

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

In the following description of vaccine production, information is given on live vaccine production adjacent to information on inactivated vaccine production.

## 2. Outline of production and minimum requirements for conventional vaccines

### 2.1. Characteristics of the seed virus

#### 2.1.1. Biological characteristics of the master seed virus

The exact source of the isolate should be recorded and should include the type of material from which the virus was derived. The *in-vitro* passage history of the virus and details of the ingredients should be recorded in accordance with chapter 1.1.8. The master seed virus (MSV) should be tested for identity, purity (freedom from adventitious agents) and safety. Characterisation of the MSV should be done using biological or genetic parameters, as relevant.

Assuming adequate immunogenicity and for obvious safety reasons, it is highly recommended that attenuated virus strains be used for the production of inactivated vaccines. The number of virus passages from the MSV stock to the final product should not exceed five (European Pharmacopoeia, 2012).

#### 2.1.2. Quality criteria

The purity of the MSV and cells to be used for vaccine production must be maintained during the process. The seed virus should be free from adventitious agents, bacteria and *Mycoplasma*, using tests known to be sensitive for detection of these microorganisms (see Chapter 1.1.9 *Tests for sterility and freedom from contamination of biological materials intended for veterinary use*). The aliquot to be tested should be representative of a titre adequate for vaccine production, but not such a high titre that hyperimmune antisera are unable to neutralise seed virus during purity testing. Seed virus is neutralised with monospecific antiserum or monoclonal antibody against a RVFV different from the seed virus, and the virus/antibody mixture is cultured on several types of cell line monolayers. Neutralised cultures should be passaged and tested for adventitious viruses that may have infected the cells or virus seed during previous passages. As an example, bovine viral diarrhoea virus (BVDV) is a potential contaminant introduced through the use of fetal bovine serum in cell culture systems. A cell line highly permissive for BVDV types 1 and 2 is recommended as one of the cell lines chosen for evaluation of the MSV. Products of bovine origin should be obtained from countries with negligible bovine spongiform encephalopathy risk.

### 2.1.3. Validation as a vaccine strain

The vaccine derived from the MSV must be shown to be satisfactory with respect to safety and efficacy for the species for which it is intended.

## 2.2. Method of manufacture

### 2.2.1. Procedure

#### i) Live vaccines

Production of vaccines should be performed according to Chapter 1.1.8. Virus seed is produced in cell culture, see Table 2 above. The number of passages from the MSV should be restricted to five. The dose of virus used to inoculate cell culture should be kept to a minimum to reduce the potential for defective interfering viral particles. When the virus has reached its appropriate titre, as determined by CPE or other approved technique, the harvest can be clarified. Generally, the vaccine is freeze-dried, preferably in the presence of a suitable stabiliser.

#### ii) Inactivated vaccines

Antigens used in inactivated vaccines are generally prepared in a similar way to live vaccines. The virus present in the virus maintenance medium is inactivated using a validated inactivation method then can be eventually concentrated or purified and formulated with a suitable adjuvant.

Where a virulent RVFV is used for inactivated vaccine production, staff handling the live virus should be vaccinated, if vaccines are available, and the facilities and practices should conform to a high containment level minimising the risk of infection of the staff and release into the environment.

### 2.2.2. Requirements for ingredients

Cell lines used for cell culture should be demonstrated free of extraneous agents. All animal origin products used in the production and maintenance of cells (i.e. trypsin, fetal bovine sera) and growth of virus should be free of extraneous agents, with special attention paid to the presence of BVDV.

### 2.2.3. In-process controls

Yield can be assessed using antigenic mass or infectivity assays. Sterility of antigens should be checked throughout the process.

A validated inactivation control method is used to assure complete inactivation of the bulk material of each batch. For inactivated vaccines, samples taken at regular timed intervals during inactivation, then inoculated into a susceptible cell line (as used for production), should indicate a complete loss of titre by 2/3 of the total duration of the inactivation process.

For tests in cell cultures, not less than 150 cm<sup>2</sup> of cell culture monolayer is inoculated with 1.0 ml of inactivated harvest. The product complies with the test if no evidence of the presence of any live virus or other micro-organism is observed.

At the end of the production, antigen content is measured to establish that minimum bulk titres or antigenic mass have been achieved.

### 2.2.4. Final product batch tests

#### i) Sterility

The final products should be tested for absence of bacteria, *Mycoplasma* and fungal contamination (see chapter 1.1.9).

#### ii) Identity

The bulk live attenuated virus or the inactivated antigen as well as the final formulated product (freeze-dried or liquid) should undergo identity testing before release to demonstrate that the relevant RVF strain is present.

iii) Safety

The final product batch safety test is designed to detect any abnormal local or systemic adverse reactions.

Each of at least two healthy sero-negative target animals should be inoculated by the recommended route of administration with the recommended dose of vaccine. The animals are observed for local and systemic reactions to vaccination for no fewer than 14 days. Any adverse reaction attributable to the vaccine should be assessed and may prevent acceptance of the batch. If the potency test is performed in the target species, observation of the safety during this test can also be considered as an alternative to the batch safety test described here.

iv) Batch potency

For live vaccines, potency is based on live virus titre. For batch release of inactivated vaccines, indirect tests can be used for practicability and animal welfare considerations, as long as correlation has been validated to the percentage of protection in the target animal. Frequently indirect potency tests include antibody titration after vaccination of suitable species. Alternative methods (antigen mass) could be used if suitably validated.

v) Moisture content

The moisture content of the lyophilised attenuated vaccine should not exceed 5%.

### 2.3. Requirements for authorisation/registration/licensing

#### 2.3.1. Manufacturing process

For registration of vaccine, all relevant details concerning manufacture of the vaccine and quality control testing (see Section C.2.2.1 to C.2.2.4 of this chapter) should be submitted to the Regulatory Authorities. This information shall be provided from three consecutive vaccine batches with a volume not less than 1/3 of the typical industrial batch volume.

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

#### 2.3.2. Safety requirements

For the purpose of gaining regulatory approval, the following safety tests should be performed satisfactorily. In addition of these tests, the vaccines should be tested for safety in the field (see chapter 1.1.8 on field tests [safety and efficacy]).

i) Live vaccines

Vaccines should be tested for any pathogenic effects in each of the target species claimed on the label.

a) Safety test (overdose) in young animals

Carry out the test for each recommended route of application using in each young target animal not older than the minimum age recommended for vaccination. Use vaccine virus at the least attenuated passage level that will be present in a batch of the vaccine.

Use no fewer than eight healthy young target animals without antibodies against RVFV. Administer to each animal a quantity of the vaccine virus equivalent to not less than 10 times the maximum virus titre likely to be contained in 1 dose of the vaccine. Observe the animals daily for at least 14 days. The body temperature of each vaccinated animal is measured on at least the 3 days preceding administration of the vaccine, at the time of administration, 4 hours after and then daily for at least 14 days. The vaccine complies with the test if the average body temperature increase for all animals does not exceed 1.5°C, no animal shows a temperature rise greater than 1.5°C for a period exceeding 3 consecutive days, and no animal shows notable signs of disease or dies from causes attributable to the vaccine.

b) Safety test in pregnant animals

Safety at different stages of gestation should be demonstrated if the product is to be used in pregnant animals.

Carry out the test with vaccination by a recommended route using a number of seronegative animals of the same age and origin sufficient to give a desired level of statistical certainty regarding the probability of side effects. Eight animals should be tested in each trimester of gestation (i.e. 24 animals in total) noting that the teratogenic risk of RVF is highest in the first two thirds of gestation (Botros *et al.*, 2006; Hunter *et al.*, 2002). Use vaccine virus at the least attenuated passage level that will be present in a batch of the vaccine.

Administer to each group a quantity of the vaccine virus equivalent to not less than the maximum virus titre likely to be contained in 1 dose of the vaccine. Clinical observation of animals is carried out daily until parturition. Blood samples should be taken from newborn animals before ingestion of colostrum.

The test is invalid if the vaccinated animals do not seroconvert before parturition. The vaccine virus complies with the test if no abnormalities in the gestation or in the animals are noted. No animal shows notable signs of disease or dies from causes attributable to the vaccine.

Vaccine virus must not be present in blood samples from newborn animals.

c) Non-transmissibility

This test should be performed in the most susceptible species livestock for RVF, generally regarded as sheep.

Use a group of no fewer than 12 healthy lambs, at the minimum age recommended for vaccination and of the same origin, and that do not have antibodies against RVFV. Use vaccine virus at the lowest passage level that will be present between the MSV and a batch of the vaccine. Administer by a recommended route to no fewer than six lambs a quantity of the vaccine virus equivalent to not less than the maximum virus titre likely to be contained in 1 dose of the vaccine.

Maintain no fewer than six lambs as contact controls. The mixing of vaccinated lambs and contact lambs is done 24 hours after vaccination.

After 45 days, euthanise all lambs. Carry out appropriate tests on the lambs to detect antibodies against RVF virus and on the control lambs to detect RVFV in the spleen and liver. The vaccine complies with the test if antibodies are found in all vaccinated lambs and if no antibodies and no virus are found in the control lambs.

d) Reversion-to-virulence

This study is carried out using the master seed lot; the most susceptible species, age and route of inoculation should be used. If the quantity of the master seed lot sufficient for performing the test is not available, the lowest passage material used for the production that is available in sufficient quantity may be used. At the time of inoculation, the animals in all groups are of an age suitable for recovery of the strain. Serial passages are carried out in target animals using five groups of animals, unless there is justification to carry out more passages or unless the strain disappears from the test animal sooner. *In-vitro* propagation may not be used to expand the passage inoculum.

The passages are carried out using animals most appropriate to the potential risk being assessed.

Initially, the vaccine is administered by the recommended route most likely to lead to reversion-to-virulence, using an initial inoculum containing the maximum release titre. After this, no fewer than four further serial passages through animals of the target species are undertaken. The passages are undertaken by the route of administration most likely to lead to reversion-to-virulence. If the properties of the strain allow sequential passage via natural spreading, this method may be used, otherwise passage of the virus is carried out and the virus that is recovered at the final passage

is tested for increase in virulence. For the first four groups, a minimum of two animals is used. The last group consists of a minimum of eight animals. At each passage, the presence of living vaccine-derived virus in the material used for passage is demonstrated. Care must be taken to avoid contamination by virus from previous passages. When the virus is not recovered from any intermediate *in-vivo* passage, the passage is repeated in ten animals using *in-vivo* passaged material from the last passage in which the virus was recovered. The virus recovered is used as the inoculum for the next passage. If the vaccine virus is not recovered, the experiment is considered to be completed with the conclusion that the vaccine virus does not show an increase in virulence.

General clinical observations are made during the study. Animals in the last group are observed for 21 days unless otherwise justified. These observations include all relevant parameters typical for the disease that could indicate increase in virulence. Compare the clinical signs and other relevant parameters with those observed in the animals used in the test for safety of the administration of 1 dose. If the last group of animals shows no evidence of an increase in virulence, further testing is not required. Otherwise, material used for the first passage and the virus recovered at the final passage level are used in a separate experiment using at least eight animals per group, to compare directly the clinical signs and other relevant parameters. This study is carried out using the route of administration that was used for previous passages. An alternative route of administration may be used if justified.

Unless otherwise justified and authorised, the product complies with the test if no animal dies or shows signs attributable to the vaccine strain and no indication of increased virulence is observed in the animals of the last group.

e) Environmental considerations

A risk assessment should be prepared where potential spread or risk of live vaccines to non-target species or spread by vector is considered.

f) Precautions (hazards)

Modified live virus vaccines may pose a hazard to the vaccinator depending on the strain and level of attenuation of the virus. Manufacturers should provide adequate warnings that medical advice should be sought in case of self-injection of vaccine.

ii) Inactivated vaccines

a) Safety test (of one dose and a repeated dose)

For the purposes of gaining regulatory approval, a trial batch of inactivated vaccine should be tested for local and systemic safety by each recommended route of administration in an *in-vivo* test in eight animals of each target species. Single dose and repeat dose tests using vaccines formulated to contain the maximum permitted payload should be conducted. The repeat dose test should correspond to the primary vaccination schedule (e.g. two injections) plus the first revaccination (i.e. a total of three injections). The animals are observed for local and systemic reactions to vaccination for no fewer than 14 days after each injection. Any undue reaction attributable to the vaccine should be assessed and may prevent acceptance of the vaccine.

b) Safety test in pregnant animals

Safety at different stages of gestation should be demonstrated if the product is to be used in pregnant animals.

Carry out the test with vaccination by a recommended route using no fewer than 16 healthy animals of the same age and origin and without antibodies against RVFV: eight in the first third of gestation and eight in the second third (periods of time where the teratogenic risk of RVF is the highest [Botros, 2006; Hunter, 2002]).

Administer to each group a quantity of the vaccine equivalent to not less than the maximum antigen mass likely to be contained in 1 dose of the vaccine. Clinical observation of animals is carried out daily until parturition.

The test is invalid if the vaccinated animals do not seroconvert before parturition. The vaccine complies with the test if no abnormalities in the gestation or in the animals are noted, and no animal shows notable signs of disease or dies from causes attributable to the vaccine.

c) Precautions (hazards)

Inactivated RVFV vaccines present no danger to vaccinators, although accidental inoculation may result in an adverse reaction caused by the adjuvant and secondary components of the vaccine. Manufacturers should provide adequate warnings that medical advice should be sought in case of self-injection of vaccine.

### 2.3.3. Efficacy requirements

Vaccine efficacy is estimated in vaccinated animals directly by evaluating resistance to live virus challenge using a controlled host animal vaccination–challenge study. In situations in which a host animal vaccination–challenge study is not possible, the elicitation of virus neutralising antibody by vaccination is considered an indication of efficacy, as neutralising antibody is considered to be protective; however, the minimum protective titre will vary with the type of neutralisation assay used and the virus used. Protective titre can be estimated by conducting a host animal vaccination–challenge study along with neutralising antibody measurements, thereby tying titre to efficacy. In general, a successful test in lamb is considered to be sufficient evidence of the quality of a vaccine to endorse its use in other species. Under circumstances where a vaccine is produced for use primarily in a species other than sheep, it may be more appropriate to test the efficacy of the vaccine in that same species. However, except for cattle, efficacy tests in other target species, such as goats or camelids have not yet been developed.

i) Immunogenicity study in young animals

The following test is applicable to sheep. For other species, appropriate modifications could be made.

A test is carried out for each route and method of administration recommended for vaccination using in each case lambs of the minimum age to be recommended. The quantity of vaccine to be administered to each lamb for a live vaccine is not greater than the minimum virus titre to be stated on the label and the virus is at the most attenuated passage level that will be present in a batch of vaccine. For inactivated vaccines, a minimum antigenic dose should be used according to the recommended vaccination schedule.

For the test use no fewer than 16 lambs without antibodies against RVF.

For live vaccine, collect sera from the lambs before vaccination, 7 and 14 days after vaccination and just before challenge. For inactivated vaccine, collect sera from the lambs before the first and second injection of the primo vaccination and at the time of the challenge.

Vaccinate no fewer than eight lambs, according to the recommended schedule. Maintain no fewer than eight lambs as controls. For live vaccines, challenge each lamb after 20–22 days by an appropriate route with a virulent RVFV. In the case of inactivated vaccines, challenge each lamb 14 days after completion of primo vaccination. Observe the lambs at least daily for 14 days after challenge and monitor for clinical signs and viral load by virus isolation and quantitative RT-PCR in blood.

The test is invalid if antibodies against RVFV in the sera of the control animals indicate that there was intercurrent infection with the virus during the test.

The vaccine complies with the test if, during the observation period after challenge, there is a significant reduction in duration and titre of viraemia, and a notable reduction in clinical signs (if the challenge virus used produces such signs) in vaccinated lambs compared with controls.

ii) Immunogenicity test in pregnant animals

Unless otherwise prescribed in a specific country monograph, immunogenicity should be tested in pregnant animals. The following test is applicable to sheep. For other species, appropriate modifications could be made.

A test is carried out for each route and method of administration recommended for vaccination using, in each case, pregnant ewes of the minimum age to be recommended. The quantity of vaccine to be administered to each ewe for a live vaccine is not greater than the minimum virus titre to be stated on the label and the virus is at the most attenuated passage level that will be present in a batch of vaccine. For inactivated vaccines, a minimum antigenic dose should be used according to the recommended vaccination schedule.

For the test use no fewer than 8 pregnant ewes without antibodies against RVF.

For live vaccine, collect sera from the ewes before vaccination, 7 and 14 days after vaccination and just before challenge. For inactivated vaccine, collect sera from the lambs before the first and second injection of the primo vaccination and at the time of the challenge.

Vaccinate no fewer than eight ewes, according to the recommended schedule. Maintain no fewer than eight ewes as controls. For live vaccines, challenge each ewe after 20–22 days by an appropriate route with a virulent RVFV. In the case of inactivated vaccines, challenge each ewe 14 days after completion of primo vaccination. Observe the ewes at least daily for 14 days after challenge and monitor for clinical signs and viral load by virus isolation and quantitative RT-PCR in blood.

The test is invalid if antibodies against RVFV in the sera of the control animals indicate that there was intercurrent infection with the virus during the test.

The vaccine complies with the test if, during the observation period after challenge, there is a significant reduction in duration and titre of viraemia, and a notable reduction in clinical signs (if the challenge virus used produces such signs) in vaccinated ewes compared with controls

#### **2.3.4. Vaccines permitting a DIVA strategy (detection of infection in vaccinated animals)**

There is currently no DIVA strategy available for the existing RVF vaccines.

#### **2.3.5. Duration of immunity**

As part of the authorisation procedure the manufacturer should 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. The duration of immunity should be at least 1 year, with the vaccine to be administered at the start of the mosquito season.

#### **2.3.6. Stability**

The stability of all vaccines should be demonstrated as part of the shelf-life determination studies for authorisation.

The period of validity of a batch of lyophilised RVF vaccine or a batch of liquid inactivated vaccine should not be less than 1 year.

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**NB:** There are OIE Reference Laboratories for Rift Valley fever  
(see Table in Part 4 of this *Terrestrial Manual* or consult the OIE Web site for the most up-to-date list:  
<http://www.oie.int/en/scientific-expertise/reference-laboratories/list-of-laboratories/>)

Please contact the OIE Reference Laboratories for any further information on  
diagnostic tests, reagents and vaccines for Rift Valley fever

**NB:** FIRST ADOPTED IN 1989; MOST RECENT UPDATES ADOPTED IN 2016.