CHAPTER 2.1.5.

FOOT AND MOUTH DISEASE

SUMMARY

Foot and mouth disease (FMD) is the most contagious disease of mammals and has a great potential for causing severe economic loss in susceptible cloven-hoofed animals. There are seven serotypes of FMD virus (FMDV), namely, O, A, C, SAT 1, SAT 2, SAT 3 and Asia 1. Infection with one serotype does not confer immunity against another. FMD cannot be differentiated clinically from other vesicular diseases, such as swine vesicular disease, vesicular stomatitis and vesicular exanthema. Laboratory diagnosis of any suspected FMD case is therefore a matter of urgency.

Typical cases of FMD are characterised by a vesicular condition of the feet, buccal mucosa and, in females, the mammary glands. Clinical signs can vary from mild to severe, and fatalities may occur, especially in young animals. In some species the infection may be subclinical, e.g. African buffalo (Syncerus caffer). The preferred tissue for diagnosis is epithelium from unruptured or freshly ruptured vesicles or vesicular fluid. Where collecting this is not possible, blood and/or oesophageal–pharyngeal fluid samples taken by probang cup in ruminants or throat swabs from pigs provide an alternative source of virus. Myocardial tissue or blood can be submitted from fatal cases, but vesicles are again preferable if present.

It is vital that samples from suspected cases be transported under secure conditions and according to international regulations. They should only be dispatched to authorised laboratories.

Diagnosis of FMD is by virus isolation or by the demonstration of FMD viral antigen or nucleic acid in samples of tissue or fluid. Detection of virus-specific antibody can also be used for diagnosis, and antibodies to viral nonstructural proteins (NSPs) can be used as indicators of infection, irrespective of vaccination status.

Identification of the agent: *The demonstration of FMD viral antigen or nucleic acid is sufficient for a positive diagnosis. Due to the highly contagious nature and economic importance of FMD, the laboratory diagnosis and serotype identification of the virus should be done in a laboratory that meets the OIE requirements for Containment Group 4 pathogens.*

Enzyme-linked immunosorbent assays (ELISA) can be used to detect FMD viral antigens and for serotyping. Lateral flow devices (LFD) are also becoming more readily available and can also be used to detect FMD viral antigens. The ELISA has replaced complement fixation (CF) in most laboratories as it is more specific and sensitive and it is not affected by pro- or anti-complement factors. If the sample is inadequate or the diagnosis remains uncertain, sample materials can be tested by reverse transcription polymerase chain reaction (RT-PCR) and/or virus isolation using susceptible cell cultures or 2–7-day old unweaned mice to amplify any nucleic acid or live virus that may be present. The cultures should preferably be of primary bovine (calf) thyroid, but pig, lamb or calf kidney cells, or cell lines of comparable sensitivity may be used. Once a cytopathic effect (CPE) is complete in the cultures, the fluids can be tested for FMDV using ELISA, CF or RT-PCR. Similar tests can be performed on homogenised suspensions of the dissected musculo-skeletal tissues of any mice that die.

Serological tests: *The demonstration of specific antibodies to structural proteins in nonvaccinated animals is indicative of prior infection with FMDV. This is particularly useful in mild cases or where epithelial tissue cannot be collected. Tests for antibodies to some NSPs of FMDV are useful in providing evidence of previous or current viral replication in the host, irrespective of vaccination status. NSPs, unlike structural proteins, are highly conserved and therefore are not serotype specific and as a consequence, the detection of these antibodies is not serotype restricted.*

Virus neutralisation tests (VNTs) and ELISAs for antibodies to structural proteins are used as serotype-specific serological tests. VNTs depend on tissue cultures and are therefore more prone to variability than ELISAs; they are also slower and subject to contamination. ELISAs for detection of antibodies have the advantage of being faster, and are not dependent on cell cultures. The ELISA can be performed with inactivated antigens, thus requiring less restrictive biocontainment facilities.

Requirements for vaccines and diagnostic biologicals: *Inactivated virus vaccines of varying composition are available commercially. Typically, virus is used to infect a suspension or monolayer cell culture and the resulting preparation is clarified, inactivated with ethyleneimine and blended with adjuvant. Many FMD vaccines are multivalent to provide protection against the different serotypes likely to be encountered in a given field situation.*

The finished vaccine must be shown to be free from residual live virus. This is most effectively done using in-vitro *tests on concentrated inactivated virus preparations prior to formulation of the vaccine and freedom from live virus is subsequently confirmed during* in-vivo *and/or* in-vitro *tests on the finished product. Challenge tests are also conducted in vaccinated cattle to establish a PD_{50} (50% protective dose) value or protection against generalised foot infection (PGP), although a serological test is considered to be satisfactory where a valid correlation between the observed protection, and the specific antibody response has been established.*

FMD vaccine production facilities should also meet the OIE requirements for Containment Group 4 pathogens.

Diagnostic and reference reagents are available from the OIE Reference Laboratories for FMD or the FAO (Food and Agriculture Organization of the United Nations) World Reference Laboratory for FMD. The Institute for Animal Health Pirbright Laboratory has dual designations as both the FAO World Reference Laboratory and as an OIE Reference Laboratory for FMD.

A. INTRODUCTION

Foot and mouth disease (FMD) is caused by a virus of the genus Aphthovirus, family Picornaviridae. There are seven serotypes of FMD virus (FMDV), namely O, A, C, SAT 1, SAT 2, SAT 3, and Asia 1, that infect cloven-hoofed animals. Infection with any one serotype does not confer immunity against another. Within serotypes, many strains can be identified by biochemical and immunological tests.

Of the domesticated species, cattle, pigs, sheep, goats and water buffalo (*Bubalus bubalis*) are susceptible to FMD (Food and Agricultural Organization of the United Nations [FAO]; 1984). Many species of cloven-hoofed wildlife may become infected, and the virus has occasionally been recovered from other species as well. Amongst the camelidae, Bactrian camels and new world camelids have been shown to be susceptible (Larska *et al*., 2009). In Africa, SAT serotypes of FMD viruses are often maintained by African buffalo (*Syncerus caffer*). There is periodic spillover of infection into livestock or sympatric cloven-hoofed wildlife. Elsewhere in the world cattle are usually the main reservoir for FMD viruses, although in some instances the viruses involved appear to be specifically adapted to pigs The pig-adapted Cathay strain of type O FMDV apparently does not infect large ruminants in the field or experimentally and requires cells of porcine origin for primary isolation. Small ruminants can play an important role in the spread of FMDV, but it is not clear whether the virus can be maintained in these species for long periods in the absence of infection of cattle. Strains of FMDV that infect cattle have been isolated from wild pigs, antelope and deer. The evidence indicates that, in the past, infection of deer was derived from contact, direct or indirect, with infected domestic animals, and that apart from African buffalo, wildlife has not, so far, been shown to be able to maintain FMD viruses independently for more than a few months.

Infection of susceptible animals with FMDV can lead to the appearance of vesicles on the feet, in and around the oral cavity, and on the mammary glands of females. The vesicles rupture and then heal whilst coronary band lesions may give rise to growth arrest lines that grow down the side of the hoof. The age of lesions can be estimated from these changes as they provide an indicator of the time since infection occurred (UK Ministry of Agriculture, Fisheries and Food; 1986). Mastitis is a common sequel of FMD in dairy cattle. Vesicles can also occur at other sites, such as inside the nostrils and at pressure points on the limbs – especially in pigs. The severity of clinical signs varies with the strain of virus, the exposure dose, the age and breed of animal, the host species and the immunity of the animal. The signs can range from a mild or inapparent infection to one that is severe. Death may result in some cases. Mortality from a multifocal myocarditis is most commonly seen in young animals: myositis may also occur in other sites.

On premises with a history of sudden death in young cloven-hoofed livestock, close examination of adult animals may often reveal the presence of vesicular lesions if FMD is involved. The presence of vesicles in fatal cases is variable.

In animals with a history of vesicular disease, the detection of FMDV in samples of vesicular fluid, epithelial tissue, oesophageal–pharyngeal (OP) sample, milk, or blood is sufficient to establish a diagnosis. Diagnosis may also be established by the detection of FMDV in the blood, heart or other organs of fatal cases. A myocarditis may be seen macroscopically (the so-called "tiger heart") in a proportion of fatal cases.

FMD viruses may occur in all the secretions and excretions of acutely infected animals, including expired air. Transmission is generally effected by direct contact between infected and susceptible animals or, more rarely, indirect exposure of susceptible animals to the excretions and secretions of acutely infected animals or uncooked meat products. Following recovery from the acute stage of infection, infectious virus disappears with the exception of low levels that may persist in the oropharynx of some ruminants. Live virus or viral RNA may continue to be recovered from oropharyngeal fluids and cells collected with a probang cup. FMD virus has also been shown to persist in a nonreplicative form in lymph nodes (Juleff *et al*., 2008). Animals in which the virus persists in the oropharynx for more than 28 days after infection are referred to as carriers. Pigs do not become carriers. Circumstantial evidence indicates, particularly in the African buffalo, that carriers are able, on rare occasions, to transmit the infection to susceptible domestic animals with which they come in close contact: the mechanism involved is unknown. The carrier state in cattle usually does not persist for more than 6 months, although in a small proportion, it may last up to 3 years. In African buffalo, individual animals have been shown to harbour the virus for at least 5 years, but it is probably not a lifelong phenomenon. Within a herd of buffalo, the virus may be maintained for 24 years or longer. Sheep and goats do not usually carry FMD viruses for more than a few months, whilst there is little information on the duration of the carrier state in Asian buffalo species and subspecies.

Because of the highly contagious nature and economic importance of FMD, the laboratory diagnosis and serotype identification of the virus should be done in a facility that meets the requirements for Containment Group 4 pathogens as outlined in Chapter 1.1.2 Biosafety and biosecurity in the veterinary microbiology laboratory and animal facilities. Countries lacking access to such a specialised national or regional laboratory should send specimens to an OIE FMD Reference Laboratory. Vaccine production facilities should also meet the requirements for Containment Group 4 pathogens.

Diagnostic and standard reagents are available in kit form or as individual items from OIE Reference Laboratories for FMD. The use of inactivated antigens in the enzyme-linked immunosorbent assay (ELISA), as controls in the antigen-detection test or to react with test sera in the liquid-phase blocking or solid-phase competitive ELISA, reduces the disease security risk involved compared with the use of live virus. Reagents are supplied freeze-dried or in glycerol or nonglycerinated but frozen and can remain stable at temperatures between +1°C and +8°C, –30°C and –5°C and –90°C and –50°C, respectively, for many years. The International Atomic Energy Agency has produced a manual that includes a recommended test and quality control protocols. There are a number of commercially available diagnostic test kits, for the detection of virus antigens or antibodies.

B. DIAGNOSTIC TECHNIQUES

For laboratory diagnosis, the tissue of choice is epithelium or vesicular fluid. Ideally, at least 1 g of epithelial tissue should be collected from an unruptured or recently ruptured vesicle, usually from the tongue, buccal mucosa or feet. To avoid injury to personnel collecting the samples, as well as for animal welfare reasons, it is recommended that animals be sedated before any samples are obtained.

Epithelial samples should be placed in a transport medium composed of equal amounts of glycerol and 0.04 M phosphate buffer, pH 7.2–7.6, preferably with added antibiotics (penicillin [1000 International Units (IU)], neomycin sulphate [100 IU], polymyxin B sulphate [50 IU], mycostatin [100 IU]). If 0.04 M phosphate buffer is not available, tissue culture medium or phosphate-buffered saline (PBS) can be used instead, but it is important that the final pH of the glycerol/buffer mixture be in the range pH 7.2–7.6. FMDV is extremely labile in low pH and buffering of the transport media is critical for successful sample collection. Samples should be kept refrigerated or on ice until received by the laboratory.

Where epithelial tissue is not available from ruminant animals, for example in advanced or convalescent cases, or where infection is suspected in the absence of clinical signs, samples of OP fluid can be collected by means of a probang (sputum) cup (or in pigs by swabbing the throat) for submission to a laboratory for virus isolation or reverse-transcription polymerase chain reaction (RT-PCR). Viraemia may also be detected by examining serum samples by means of RT-PCR or virus isolation. For the collection of throat swabs from pigs, the animal should be held on its back in a wooden cradle with the neck extended. Holding a swab in a suitable instrument, such as an artery forceps, the swab is pushed to the back of the mouth and into the pharynx.

Before the collection of OP samples from cattle or large ruminants (e.g. buffalo), 2 ml transport fluid (composed of 0.08 M phosphate buffer containing 0.01% bovine serum albumin, 0.002% phenol red, antibiotics [1000 units/ml penicillin, 100 units/ml mycostatin, 100 units/ml neomycin, and 50 units/ml polymyxin], and adjusted to pH 7.2) should be added to a container of around 5 ml capacity capable of withstanding freezing above dry ice (solid carbon dioxide) or liquid nitrogen (Kitching & Donaldson, 1987).

An OP sample is collected by inserting a probang over the tongue into the oro-pharyngeal area and then passing it vigorously backwards and forwards 5–10 times between the first portion of the oesophagus and the back of the pharynx. The purpose is to collect oro-pharyngeal fluid and especially superficial epithelial cells from these areas, including the proximal part of the oesophagus, the walls of the pharynx, the tonsillar crypts and the surfaces of the soft palate. If the sample does not contain adequate cellular debris the actions may be repeated.

After collection of OP fluid by probang, the contents of the cup should be poured into a wide-necked transparent bottle of around 20 ml capacity. The fluid is examined, and should contain some visible cellular material. This fluid is then added to an approximately equal volume of transport fluid, ensuring that cellular material is transferred; the mixture is shaken gently and should have a final pH of around pH 7.6. Samples contaminated with ruminal contents may be unsuitable for culture. Samples seen to contain blood are not entirely satisfactory. Repeat sampling can be done after the mouth and throat of the animal have been rinsed with water or PBS. Where several animals are to be sampled, the probang must be cleaned and disinfected between each animal. This is done by washing the probang in tap water, immersing it in a suitable disinfectant (e.g. 0.5% [w/v] citric acid in tap water) and then rinsing off all the disinfectant with water before sampling the next animal.

OP samples from small ruminants are collected by putting 2 ml of transport fluid into a wide-necked bottle of about 20 ml capacity and, after collection, rinsing the probang cup in this transport fluid to discharge the OP sample. This is then transferred to a container of about 5 ml capacity for transport. The small container should be capable of withstanding freezing above dry ice or liquid nitrogen (Kitching & Donaldson, 1987).

Samples of OP fluid should be refrigerated or frozen immediately after collection. If they are to remain in transit for more than a few hours, they should preferably be frozen by being placed either above dry ice or liquid nitrogen. Before freezing, the containers should be carefully sealed using airtight screw caps or silicone. This is particularly important when using dry ice, as introduction of CO_2 into the OP sample will lower its pH, inactivating any FMDV that may be in the samples. Glass containers should not be used because there is a risk that they will explode on defrosting in the event of liquid nitrogen leaking into them. Samples should reach the laboratory in a frozen state or, if this is not feasible, maintained under reliable cold chain conditions during transit.

Special precautions are required when sending perishable suspect FMD material both within and between countries. The International Air Transport Association (IATA), Dangerous Goods Regulations (DGR) has explicit requirements for packaging and shipment of diagnostic specimens by all commercial means of transport. These are summarised in Chapter 1.1.1 Collection and shipment of diagnostic specimens. Forms and guidance on sample submission and specifications for manufacture of probang cups can be found on the website of the Pirbright OIE Reference Laboratory at http://www.wrlfmd.org/. Procedures for collection and shipment of field samples for the diagnosis of vesicular diseases and its differential diagnosis can be found at the Pan-American FMD OIE Reference Laboratory at http://www.panaftosa.org.br

1. Identification of the agent

A range of sample types, including epithelium, OP samples and serum, may be examined by virus isolation or RT-PCR. By contrast, ELISA CF and the lateral flow device are suited to the examination of epithelial suspensions, vesicular fluids or cell culture supernatants, but are insufficiently sensitive for the direct examination of OP samples or serum.

a) Virus isolation

The epithelium sample should be taken from the PBS/glycerol, blotted dry on absorbent paper to reduce the glycerol content, which is toxic for cell cultures, and weighed. A suspension should be prepared by grinding the sample in sterile sand in a sterile pestle and mortar with a small volume of tissue culture medium and antibiotics. Further medium should be added until a final volume of nine times that of the epithelial sample has been added, giving a 10% suspension. This is clarified on a bench centrifuge at 2000 *g* for 10 minutes. Once clarified, such suspensions of field samples suspected to contain FMDV are inoculated onto cell cultures or into unweaned mice. Sensitive cell culture systems include primary bovine (calf) thyroid cells and primary pig, calf or lamb kidney cells. Established cell lines, such as BHK-21 (baby hamster kidney) and IB-RS-2 cells, may also be used but are generally less sensitive than primary cells for detecting low amounts of infectivity. The sensitivity of any cells used should be tested with standard preparations of FMDV. The use of IB-RS-2 cells aids the differentiation of swine vesicular disease virus (SVDV) from FMDV (as SVDV will only grow in cells of porcine origin) and is often essential for the isolation of porcinophilic strains, such as O Cathay. The cell cultures should be examined for cytopathic effect (CPE) for 48 hours. If no CPE is detected,

the cells should be frozen and thawed, used to inoculate fresh cultures and examined for CPE for another 48 hours. Unweaned mice are an alternative to cell cultures and should be 2–7 days old and of selected inbred strains. Some field viruses may require several passages before they become adapted to mice (Skinner, 1960). In the case of OP fluids, pretreatment with an equal volume of chloro-fluoro-carbons may improve the rate of virus detection by releasing virus from immune complexes.

b)   Immunological methods

- **Enzyme-linked immunosorbent assay**

The preferred procedure for the detection of FMD viral antigen and identification of viral serotype is the ELISA (Ferris & Donaldson, 1992; Roeder & Le Blanc Smith, 1987). This is an indirect sandwich test in which different rows in multiwell plates are coated with rabbit antisera to each of the seven serotypes of FMDV. These are the 'capture' sera. Test sample suspensions are added to each of the rows, and appropriate controls are also included. Guinea-pig antisera to each of the serotypes of FMDV are added next, followed by rabbit anti-guinea-pig serum conjugated to an enzyme. Extensive washing is carried out between each stage to remove unbound reagents. A colour reaction on the addition of enzyme substrate and chromogen indicates a positive reaction. With strong positive reactions, this will be evident to the naked eye, but results can also be read spectrophotometrically at an appropriate wavelength. In this case, an absorbance reading greater than 0.1 above background indicates a positive reaction; the serotype of FMDV can also be identified. Values close to 0.1 should be confirmed by retesting or by amplification of the antigen by tissue culture passage and testing the supernatant once a CPE has developed. A suitable protocol is given below. Other protocols are available with slightly different formats and interpretation criteria (Alonso *et al*., 1993).

Depending on the species affected and the geographical origin of samples, it may be appropriate to simultaneously test for SVDV or vesicular stomatitis virus (VSV). Ideally a complete differential diagnosis should be undertaken in all vesicular conditions.

Rabbit antiserum to the 146S antigen of each of the seven serotypes of FMDV (plus SVDV or VSV if required) is used as a trapping antibody at a predetermined optimal concentration in carbonate/bicarbonate buffer, pH 9.6.

Control antigens are prepared from selected strains of each of the seven types of FMDV (plus SVDV or VSV if appropriate) grown on monolayer cultures of BHK-21 cells (IB-RS-2 cells for SVD or VSV). The unpurified supernatants are used and pretitrated on ELISA plates. The final dilution chosen is that which gives an absorbance at the top of the linear region of the titration curve (optical density approximately 2.0), so that the five-fold dilutions of the control antigens used in the test give two additional lower optical density readings from which the titration curve can be derived. PBS containing 0.05% Tween 20 and phenol red indicator is used as a diluent (PBST).

Guinea-pig antisera prepared by inoculating guinea-pigs with 146S antigen of one of the seven serotypes of FMDV (plus SVDV if required) and preblocked with normal bovine serum (NBS) is used as the detecting antibody. Predetermined optimal concentrations are prepared in PBS containing 0.05% Tween 20, and 5% dried, nonfat skimmed milk (PBSTM).

Rabbit (or sheep) anti-guinea-pig immunoglobulin conjugated to horseradish peroxidase and preblocked with NBS is used at a predetermined optimum concentration in PBSTM. As an alternative to guinea-pig or rabbit antisera, suitable monoclonal antibodies (MAbs) can be used coated to the ELISA plates as capture antibody or peroxidase-conjugated as detecting antibody.

- **Test procedure**

i)   ELISA plates are coated with 50 µl/well rabbit antiviral sera in 0.05 M carbonate/bicarbonate buffer, pH 9.6. Rows A to H receive, respectively, antisera to serotypes O, A, C, SAT 1, SAT 2, SAT 3, Asia 1 and SVDV or VSV (optional).

ii)   Leave overnight at 4°C in a stationary position or place on an orbital shaker set at 100–120 revolutions per minute in a 37°C incubator for 1 hour.

iii) Prepare test sample suspension (10% original sample suspension or undiluted clarified cell culture supernatant fluid).

iv)   The ELISA plates are washed five times in PBS.

v)   On each plate, load wells of columns 4, 8 and 12 with 50 µl PBST. Additionally, add 50 µl of PBST to wells 1, 2 and 3 of rows A to H on plate 1. To well 1 of row A of plate 1 add 12.5 µl of control antigen type O, to well 1 of row B add 12.5 µl of control antigen type A; continue in this manner for control antigen of types C, SAT 1, SAT 2, SAT 3, Asia 1 and SVDV or VS (if appropriate) in order to well 1,

rows C to H. Mix diluent in well 1 of rows A to H and transfer 12.5 µl from well 1 to 2 (rows A to H), mix and transfer 12.5 µl from well 2 to 3, mix and discard 12.5 µl from well 3 (rows A to H) (this gives a five-fold dilution series of each control antigen). It is only necessary to change pipette tips on the micropipette between antigens. The remainder of the plate can be loaded with the test sample(s). Add 50 µl of sample one to wells 5, 6 and 7 of rows A to H, the second sample is placed similarly in columns 9, 10 and 11, rows A to H.

If more than two samples are to be tested at the same time, the other ELISA plates should be used as follows:

Dispense 50 µl of the PBST to the wells (rows A to H) of columns 4, 8 and 12 (buffer control columns). Note that the control antigens are not required on these plates. These test samples may be added in 50 µl volumes in rows A to H to columns 1, 2, 3; 5, 6, 7; 9, 10, 11, respectively.

vi)   Cover with lids and place on an orbital shaker at 37°C for 1 hour.

vii)   Wash the plates by flooding with PBS – wash three times as before and empty residual wash fluid. Blot the plates dry.

viii)   Transfer 50 µl volumes of each guinea-pig serum dilution to each plate well in the appropriate order, e.g. rows A to H receive, respectively, antisera to serotypes O, A, C, SAT 1, SAT 2, SAT 3, Asia 1 and SVDV or VSV (optional).

ix)   Cover the plates with lids and replace on the orbital shaker. Incubate at 37°C for 1 hour.

x)   The plates are washed again three times, and 50 µl of rabbit anti-guinea-pig immunoglobulin conjugated to horseradish peroxidase is added to each well. The plates are incubated at 37°C for 1 hour on a rotary shaker.

xi)   The plates are washed again three times, and 50 µl of substrate solution, containing 0.05% % H_2O_2 plus orthophenylene diamine or a suitable alternative chromogen, is added to each well.

xii)   The reaction is stopped after 15 minutes by the addition of 50 µl of 1.25 M sulphuric acid. The plates are read at 492 nm on a spectrophotometer linked to a computer.

- **Lateral flow device test**

There are commercially available lateral flow devices (LFD) (Ferris *et al*., 2009), but the OIE has not yet received a validation dossier for these tests. As soon as a dossier is received, the manufacturer could apply for inclusion on the OIE test register.

- **Complement fixation test (an alternative test for international trade)**

In general, the ELISA is preferable to the complement fixation test (CFT) because it is more sensitive and it is not affected by pro- or anti-complementary factors (Ferris & Dawson, 1988). If ELISA reagents are not available, or if subtyping is pursued, the CFT may be performed as follows:

The CF50% protocol in tubes used widely in South America for typing, subtyping and for establishing serological relationships (r values) is performed as follows: 0.2 ml antiserum to each FMD serotype diluted at a predetermined optimal dilution in veronal buffer diluent (VBD) or borate-saline solution (BSS) is placed in separate tubes. To these, 0.2 ml of test sample suspension is added, followed by 0.2 ml of a complement dilution containing 4 units of complement. The test system is incubated at 37°C for 30 minutes prior to the addition of 0.4 ml 2% standardised sheep red blood cells (SRBC) in VBD or BSS sensitised with rabbit anti-SRBC. The reagents are incubated at 37°C for further 30 minutes and the tubes are subsequently centrifuged and read. Samples with less than 50% haemolysis are considered positive (3B).

Other protocols performed in microplates are available and are performed as follows: antisera to each of the seven types of FMDV are diluted in VBD in 1.5-fold dilution steps from an initial 1/16 dilution to leave 25 µl of successive antiserum dilutions in U-shaped wells across a microtitre plate. To these are added 50 µl of 3 units of complement, followed by 25 µl of test sample suspension(s). The test system is incubated at 37°C for 1 hour prior to the addition of 25 µl of 1.4% SRBC in VBD sensitised with 5 units of rabbit anti-SRBC. The reagents are incubated at 37°C for a further 30 minutes and the plates are subsequently centrifuged and read. Appropriate controls for the test suspension(s), antisera, cells and complement are included. CF titres are expressed as the reciprocal of the serum dilution producing 50% haemolysis. A CF titre ≥36 is considered to be a positive reaction. Titre values of 24 should be confirmed by retesting an antigen that has been amplified through tissue culture passage.

c)   Nucleic acid recognition methods

RT-PCR can be used to amplify genome fragments of FMDV in diagnostic materials including epithelium, milk, serum and OP samples. RT combined with real-time PCR has a sensitivity comparable to that of virus isolation and automated procedures enhance sample throughput (Reid *et al.,* 2003). Serotyping primers have also been developed (Vangrysperre & De Clercq, 1996). Simplified RT-PCR systems for potential field-use are under development (Callahan *et al.,* 2002).

•   **Agarose gel-based RT-PCR assay**

The procedure used at the OIE Reference Laboratory at Pirbright is described (Reid *et al.,* 2000). The RT-PCR assay consists of the three successive procedures of (i) extraction of template RNA from the test or control sample followed by (ii) RT of the extracted RNA, (iii) PCR amplification of the RT product and (iv) detection of the PCR products by agarose gel electrophoresis.

•   **Test procedure**

i)   Add 200 µl of test sample to 1 ml of TRIzol® Reagent in a sterile tube. Store at –70°C until required for RNA extraction.

ii)   Transfer 1 ml of the solution from i) into a fresh, sterile tube containing 200 µl of chloroform. Vortex mix for about 10–15 seconds and leave at room temperature for 3 minutes.

iii)   Centrifuge for 15 minutes at 20,000 *g*.

iv)   Transfer 500 µl of the aqueous phase into a fresh, sterile tube containing 1 µl of glycogen (20 mg/ml) and add 500 µl of iso-propyl-alcohol (propan-2-ol). Vortex mix for a few seconds.

v)   Leave at room temperature for 10 minutes then centrifuge for 10 minutes at 20,000 *g*.

vi)   Discard the supernatant fluid from each tube and add 1 ml of 70% ethanol. Vortex mix for a few seconds.

vii)   Centrifuge for 10 minutes at 20,000 *g*.

viii) Carefully remove the supernatant fluid from each tube taking care not to dislodge or lose any pellet at the bottom of the tube.

ix)   Air dry each tube at room temperature for 2–3 minutes.

x)   Resuspend each pellet by adding 20 µl of nuclease-free water to the tube.

xi)   Keep the extracted RNA samples on ice if the RT step is about to be performed. Otherwise store at –70°C.

xii)   For each sample to be assayed, add 2 µl of random hexamers (20 µg/ml) and 5 µl of nuclease-free water into a sterile 0.5 ml microcentrifuge tube. It is recommended to prepare the dilution in bulk for the total number of samples to be assayed but allowing for one extra sample.

xiii) Add 5 µl of RNA from the extraction procedure described above to give a volume of 12 µl in each tube. Mix by gently pipetting up and down.

xiv)   Incubate at 70°C for 5 minutes.

xv)   Cool at room temperature for 10 minutes.

xvi)   During the 10-minute incubation period, prepare the RT reaction mixture described below for each sample. Prepare the reaction mixture in bulk in a sterile 1.5 ml microcentrifuge tube for the number of samples to be assayed plus one extra sample.

       First strand buffer, 5× conc. (4 µl); bovine serum albumin (acetylated), 1 mg/ml (2 µl); dNTPs, 10 mM mixture each of dATP, dCTP, dGTP, dTTP (1 µl); DTT, 1 M (0.2 µl); Moloney Murine Reverse Transcriptase, 200 U/µl (1 µl).

xvii) Add 8 µl reaction mix to the 12 µl of random primer/RNA mix. Mix by gently pipetting.

xviii) Incubate at 37°C for 45 minutes.

xix)   Keep the RT products on ice if the PCR amplification step is about to be performed, otherwise store at –20°C.

xx)   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 µl); PCR reaction buffer, 10× conc (5 µl); $MgCl_2$, 50 mM (1.5 µl); dNTPs, 10 mM mixture each of dATP, dCTP, dGTP, dTTP (1 µl); primer 1, 10 pmol/µl (1 µl); primer 2, 10 pmol/µl (1 µl); *Taq* Polymerase, 5 units/µl (0.5 µl).

xxi) Add 45 µl of PCR reaction mix to a well of a PCR plate or to a microcentifuge tube for each sample to be assayed followed by 5 µl of the RT product to give a final reaction volume of 50 µl.

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

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

94°C for 5 minutes: 1 cycle;

94°C for 1 minute, 55°C for 1 minute, 72°C for 2 minutes: 30 cycles;

72°C for 7 minutes: 1 cycle.

xxiv) Mix a 20 µl aliquot of each PCR reaction product with 4 µl of staining solution and load onto a 1.5% agarose gel. After electrophoresis a positive result is indicated by the presence of a 328 bp band corresponding to FMDV sequence in the 5' untranslated region of the genome.

- **Stock solutions**

i) Nuclease-free water, TRIzol® Reagent, chloroform, glycogen, iso-propyl-alcohol (propan-2-ol), ethanol, random hexanucleotide primers, First strand buffer, BSA (acetylated), dNTPs, DTT, Moloney Murine Reverse Transcriptase, PCR reaction buffer (10×), $MgCl_2$ and *Taq* Polymerase are commercially available.

ii) Primers at a concentration of 10 pmol/µl: Primer 1 sequence 5'-GCCTG-GTCTT-TCCAG-GTCT-3' (positive strand); Primer 2 sequence 5'-CCAGT-CCCCT-TCTCA-GATC-3' (negative strand).

- **Real-time RT-PCR assay**

The real-time RT-PCR assay can use the same procedures of extraction of total RNA from the test or control sample followed by RT of the extracted RNA as for the conventional agarose gel-based procedure. Automated extraction of total nucleic acid from samples followed by automated pipetting programmes for the RT and PCR steps (Reid *et al.,* 2003) can be used as an alternative to the manual procedures described above. PCR amplification of the RT product is performed by a different procedure. A one-step method for combining the RT and PCR steps has also been described (Shaw *et al.,* 2007). Detection of the PCR products in agarose gels is not required following real-time amplification.

i) Take the RT products from step xix (see above).

ii) 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 (6 µl); PCR reaction master mix, 2× conc. (12.5 µl); real-time PCR forward primer, 10 pmol/µl (2.25 µl); real-time PCR reverse primer, 10 pmol/µl (2.25 µl); TaqMan® probe, 5 pmol/µl (1 µl).

iii) Add 24 µl PCR reaction mix to a well of a real-time PCR plate for each sample to be assayed followed by 1 µl of the RT product to give a final reaction volume of 25 µl.

iv) Spin the plate for 1 minute in a suitable centrifuge to mix the contents of each well.

v) Place the plate in a real-time PCR machine for PCR amplification and run the following programme:

50°C for 2 minutes: 1 cycle;

95°C for 10 minutes: 1 cycle;

95°C for 15 seconds, 60°C for 1 minute: 50 cycles.

vi) *Reading the results:* Assign a threshold cycle (CT) value to each PCR reaction from the amplification plots (a plot of the fluorescence signal versus cycle number; different cut-off values may be appropriate for different sample types; Parida *et al.,* 2007). The CT values used to assign samples as either FMDV positive or negative should be defined by individual laboratories using appropriate reference material. For example at the OIE Reference Laboratory at Pirbright, negative test samples and negative controls should have a CT value at >50.0. Positive test samples and positive control samples should have a CT value <40. Samples with CT values falling within the range 40–50 are designated "borderline" and can be retested. Strong positive FMD samples have a CT value below 20.0 (Reid *et al.,* 2001).

- **Stock solutions for real-time PCR assay**

i) Nuclease-free water and real-time PCR reaction master mixes are available from commercial suppliers.

ii) Either of the two following primers and probe sets can be used for real-time PCR of FMDV:

5'UTR (Reid *et al.,* 2001) Forward primer: CACYT YAAGR TGACA YTGRT ACTGG TAC; Reverse primer: CAGAT YCCRA GTGWC ICITG TTA and TaqMan® probe: CCTCG GGGTA CCTGA AGGGC ATCC.

3D (Callahan *et al.,* 2002) Forward primer: ACTGG GTTTT ACAAA CCTGT GA; Reverse primer: GCGAG TCCTG CCACG GA and TaqMan® probe: TCCTT TGCAC GCCGT GGGAC.

- **Molecular epidemiology**

The molecular epidemiology of FMD is based on the comparison of genetic differences between viruses. Dendrograms showing the genomic relationship between vaccine and field strains for all seven serotypes based on sequences derived from the 1D gene (encoding the VP1 viral protein) have been published (Knowles & Samuel, 2003; see also http://www.wrlfmd.org/). Comparison of whole genome sequences can provide further discrimination between closely related viruses and help to recreate the transmission pathways between farms within outbreaks (Cottam *et al.,* 2008). RT-PCR amplification of FMDV RNA, followed by nucleotide sequencing, is the current preferred option for generating the sequence data to perform these comparisons. Many laboratories have developed techniques for performing these studies, and reference laboratories hold databases containing over 3000 partial sequences.

The recommended method for VP1 analysis is to:

i) Extract FMDV RNA directly from epithelial suspensions or from a low cell culture passage.

ii) Perform an RT-PCR of the complete 1D gene (or if only part of the 1D gene, then the 3' end of the gene is more useful).

iii) Determine the nucleotide sequence of the PCR product (or at least 170 nucleotides [preferably 420 for the SAT types] at the 3' end of the gene).

A protocol, complete with primer sequences, is available from the OIE Reference Laboratories on request or can be downloaded from the following World Wide Web URLs:

http://www.wrlfmd.org/

http://bvs.panaftosa.org.br/textoc/SerManDid17.pdf

2. Serological tests

Serological tests for FMD are performed in support of four main purposes namely: 1) to certify individual animals prior to import or export (i.e. for trade); 2) to confirm suspected cases of FMD; 3) to substantiate absence of infection; 4) to demonstrate the efficacy of vaccination. For substantiating freedom from infection, different approaches are required according to whether the population has been vaccinated or not and if vaccination has been used, whether this has been applied as an emergency application or as part of an ongoing programme of vaccination. Different tests and different interpretations of test results will be appropriate according to the above-mentioned purposes and the validation of the selected procedure must take account of the purpose. For example, test cut-offs may be set at a different threshold for herd-based serosurveillance than is appropriate for certifying freedom from infection for individual animals for the purposes of international trade.

Serological tests for FMD are of two types; those that detect antibodies to viral structural proteins (SP) and those that detect antibodies to viral nonstructural proteins (NSPs).

The SP tests are serotype-specific and detect antibodies elicited by vaccination and infection; examples are the virus neutralisation test (VNT) (Golding *et al.,* 1976), the solid-phase competition ELISA (SPCE; Brocchi *et al.*, 1990; Chenard *et al.*, 2003; Mackay *et al.*, 2001; Paiba *et al.*, 2004) and the liquid-phase blocking ELISA (LPBE; Hamblin *et al.*, 1986; 1987). These tests are serotype-specific and are highly sensitive, providing that the virus or antigen used in the test is closely matched to the strain circulating in the field. They are the prescribed tests for trade and are appropriate for confirming previous or ongoing infection in nonvaccinated animals as well as for monitoring the immunity conferred by vaccination in the field. The VNT requires cell culture facilities, the use of live virus and takes 2–3 days to provide results. The ELISAs are blocking- or competition-based assays that use serotype-specific polyclonal antibodies (PAbs) or MAbs, are quicker to perform and are not dependent on tissue culture systems and the use of live viruses. Low titre false-positive reactions can be expected in a small proportion of the sera in either ELISA. An approach combining screening by ELISA and confirming the positives by the VNT minimises the occurrence of false-positive results. Reference sera to standardise FMD SP serological tests for some serotypes and subtypes are available from the Reference Laboratory at Pirbright.

The detection of antibody to the NSPs of FMDV can be used to identify past or present infection with any of the seven serotypes of the virus, whether or not the animal has also been vaccinated. Therefore the tests can be

used to confirm suspected cases of FMD and to detect viral activity or to substantiate freedom from infection on a population basis. For certifying animals for trade, the tests have the advantage over SP methods that the serotype of virus does not have to be known. However, there is experimental evidence that some cattle, vaccinated and subsequently challenged with live virus and confirmed persistently infected, may not be detected in some anti-NSP tests, causing false-negative results (Brocchi *et al*., 2006). These assays measure antibody to NSPs using antigens produced by recombinant techniques in a variety of in-vitro expression systems. Antibody to the polyproteins 3AB or 3ABC are generally considered to be the most reliable indicators of infection (Mackay *et al*., 1997). In animals seropositive for antibody to 3AB or 3ABC, antibody to one or more of the other NSPs can aid in the final interpretation of the test (Bergmann *et al*., 2000; Mackay *et al*., 1997). However, lack of vaccine purity may affect diagnostic specificity as the presence of NSPs in some vaccine preparations may result in misclassification in animals that have been repeatedly vaccinated. Procedures for evaluating vaccine purity are covered in Section D of this chapter.

International standard sera for testing of cattle have been developed and are available from the OIE Reference Laboratories in Brazil and UK (Campos *et al*., 2008). In the future, standard sera will also be made available for sheep and pigs. Bovine serum panels have also been established to compare the sensitivity of NSP tests (Parida *et al*., 2007).

a) Virus neutralisation test (a prescribed test for international trade)

The quantitative VN microtest for FMD antibody is performed with IB-RS-2, BHK-21, lamb or pig kidney cells in flat-bottomed tissue-culture grade microtitre plates.

Stock virus is grown in cell monolayers and stored at –20°C after the addition of 50% glycerol. (Virus has been found to be stable under these conditions for at least 1 year.) The sera are inactivated at 56°C for 30 minutes before testing. The control standard serum is 21-day convalescent or post-vaccination serum. A suitable medium is Eagle's complete medium/LYH (Hank's balanced salt solution with yeast lactalbumin hydrolysate) with hepes buffer and antibiotics.

The test is an equal volume test in 50 µl amounts.

- **Test procedure**

i) Starting from a 1/4 dilution, sera are diluted in a twofold, dilution series across the plate, using at least two rows of wells per serum, preferably four rows, and a volume of 50 µl.

ii) Previously titrated virus is added; each 50 µl unit volume of virus suspension should contain about 100 $TCID_{50}$ (50% tissue culture infective dose) within an accepted range (e.g. 32–320 $TCID_{50}$).

iii) Controls include a standard antiserum of known titre, a cell control, a medium control, and a virus titration used to calculate the actual virus titre used in the test.

iv) Incubate at 37°C for 1 hour with the plates covered.

v) A cell suspension at 10^6 cells/ml is made up in medium containing 10% bovine serum (specific antibody negative) for cell growth. A volume of 50 µl of cell suspension is added to each well.

vi) Plates are sealed with pressure-sensitive tape and incubated at 37°C for 2–3 days. Alternatively, the plates may be covered with loosely fitting lids and incubated in an atmosphere of 3–5% carbon dioxide at 37°C for 2–3 days.

vii) Microscope readings may be feasible after 48 hours. The plates are finally fixed and stained routinely on the third day. Fixation is effected with 10% formol/saline for 30 minutes. For staining, the plates are immersed in 0.05% methylene blue in 10% formalin for 30 minutes. An alternative fixative/stain solution is naphthalene blue black solution (0.4% [w/v] naphthalene blue black, 8% [w/v] citric acid in saline). The plates are rinsed in tap water.

viii) Positive wells (where the virus has been neutralised and the cells remain intact) are seen to contain blue-stained cells sheets; the negative wells (where virus has not been neutralised) are empty. Titres are expressed as the final dilution of serum present in the serum/virus mixture where 50% of wells are protected (Kärber, 1931). The test is considered to be valid when the amount of virus used per well is in the range $\log_{10}$ 1.5–2.5 $TCID_{50}$, and the positive standard serum is within twofold of its expected titre.

ix) Interpretation of tests can vary between laboratories in regard to the negative/positive cut-off threshold. Laboratories should establish their own criteria by reference to standard reagents that can be obtained from the OIE Reference Laboratory at Pirbright. In general, a titre of 1/45 or more of the final serum dilution in the serum/virus mixture is regarded as positive. A titre of less than 1/16 is considered to be negative. For certification of individual animals for the purposes of international trade, titres of 1/16 to 1/32 are considered to be doubtful, and further serum samples may be requested for testing; results

are considered to be positive if the second sample has a titre of 1/16 or greater. For the purposes of herd-based serosurveillance as part of a statistically valid serological survey, a cut-off of 1/45 may be appropriate. Cut-off titres for evaluating immunological protection afforded by vaccination have to be established from experience of potency test results with the relevant vaccine and target species.

b) Solid-phase competition enzyme-linked immunosorbent assay (a prescribed test for international trade)

The method described (Paiba *et al.,* 2004) can be used for the detection of antibodies against each of the seven serotypes of FMDV. As an alternative to guinea-pig or rabbit antisera, suitable MAbs can be used coated to the ELISA plates as capture antibody or peroxidase-conjugated as detecting antibody (Brocchi *et al.,* 1990). A commercial kit is available for serotype O with a different format but similar performance characteristics (Chenard *et al.,* 2003).

Rabbit antiserum to the 146S antigen of one of the seven types of FMDV is used as the trapping antibody at a predetermined optimal concentration in carbonate/bicarbonate buffer, pH 9.6.

Antigens are prepared by inactivating viruses propagated in cell culture with ethyleneimine using the procedures described for vaccine manufacture. The final dilution chosen is that which, after addition of an equal volume of diluent, gives an absorbance on the upper part of the linear region of the titration curve (optical density approximately 1.5). PBS containing 0.05% Tween 20, 10% NBS and 5% normal rabbit serum and phenol red indicator is used as a diluent (blocking buffer).

Guinea-pig antisera, prepared by inoculating guinea-pigs with 146S antigen of one of the seven serotypes and preblocking with NBS, is used as the detecting antibody. Predetermined optimal concentrations are prepared in blocking buffer PBS containing 0.05% Tween 20, and 5% dried, nonfat skimmed milk (PBSTM).

Rabbit (or sheep) anti-guinea-pig immunoglobulin conjugated to horseradish peroxidase and preblocked with NBS is used as conjugate at a predetermined optimum concentration in PBSTM blocking buffer.

Test sera are diluted in PBST blocking buffer.

The solid-phase competitive ELISA is more specific but as sensitive as the liquid-phase blocking ELISA (Mackay *et al.,* 2001; Paiba *et al.,* 2004). Methods have been described for the development of secondary and working standard sera (Goris & De Clercq, 2005a) and for charting assay performance (Goris & De Clercq, 2005b).

- **Test procedure**

i)   ELISA plates are coated with 50 µl/well rabbit antiserum homologous to the antigen being used, diluted in carbonate/bicarbonate buffer, pH 9.6, and left overnight in a humid chamber at 4°C.

ii)   The ELISA plates are washed three times with PBS.

iii)   Then 50 µl of the FMDV antigen diluted in blocking buffer is added to each well of the ELISA plates. (Blocking buffer: 0.05% [w/v] Tween 20, 10% [v/v] NBS, 5% [v/v] normal rabbit serum.) The plates are covered and placed on an orbital shaker at 37°C for 1 hour, with continuous shaking.

iv)   After washing three times with PBS, 40 µl of blocking buffer is added to each well, followed by 10 µl of test sera (or control sera), giving an initial serum dilution of 1/5.

v)   Immediately 50 µl of guinea-pig anti-FMDV antiserum diluted in blocking buffer is added, giving a final serum dilution of 1/10.

vi)   The plates are covered and incubated on an orbital shaker at 37°C for 1 hour.

vii)   After washing three times with PBS, 50 µl of anti-guinea-pig Immunoglobulin conjugate (preblocked by incubation for 1 hour at room temperature with an equal volume of NBS) diluted in blocking buffer is added. The plates are covered and incubated for 1 hour at 37°C on an orbital shaker.

viii) After washing three times with PBS, 50 µl of substrate solution, containing 0.05% H_2O_2 plus orthophenylene diamine or a suitable alternative chromogen, is added to each well.

ix)   The reaction is stopped after 10 minutes by the addition of 50 µl of 1 M sulphuric acid. The plates are read at 492 nm on a spectrophotometer linked to a computer.

x)   *Controls:* On each plate two wells are used for conjugate control (no guinea-pig serum), four wells each for strong and weak positive sera, two wells for negative sera, and four wells for 0% competition (no test sera).

xi)   *Interpretation of the results:* A percentage of inhibition is calculated for each well, either manually or using a suitable computer programme (100 – [optical density of each test or control value/mean optical density of the 0% competition] × 100%), representing the competition between the test sera and the

guinea-pig anti-FMDV antisera for the FMDV antigen on the ELISA plate. Laboratories should validate the assay in terms of the cut-off value above which sera should be considered positive in relation to (i) the particular serotypes and strains of virus under investigation (ii) the purpose of testing (iii) the population under test, using the methods described in Chapter 1.1.4 Principles of validation of diagnostic assays for infectious diseases. At the OIE Reference Laboratory at Pirbright, for serotype O, for all species, for the purposes of demonstrating freedom from infection in a naïve population, greater than 60% inhibition is considered positive (Paiba *et al.,* 2004). For maximum sensitivity, for example when certifying individual animals for international trade, an inconclusive range may be set between 40 and 60%.

c) Liquid-phase blocking enzyme-linked immunosorbent assay (a prescribed test for international trade)

Antigens are prepared from selected strains of FMDV grown on monolayers of BHK-21 cells. The unpurified supernatants are used and pretitrated in a twofold dilution series but without serum. The final dilution chosen is that which, after addition of an equal volume of diluent (see below), gives an absorbance on the upper part of the linear region of the titration curve (optical density approximately 1.5). PBS containing 0.05% Tween 20 and phenol red indicator is used as a diluent (PBST). The other reagents used in the test are the same as those in the solid-phase blocking ELISA. An example of the test procedure is described below. Temperature and incubation times can vary depending on the protocol.

- **Test procedure**

i) ELISA plates are coated with 50 µl/well rabbit antiserum homologous to the antigen being used and left overnight in a humid chamber at room temperature.

ii) The ELISA plates are washed three times with PBS.

iii) In U-bottomed multiwell plates (carrier plates) 50 µl of a duplicate, twofold series of each test serum is prepared, starting at 1/8. To each well, 50 µl of a constant dose of viral antigen that is homologous to the rabbit antisera used to coat the plates is added and the mixtures are left overnight at 4°C, or incubated at 37°C for 1 hour. The addition of the antigen increases the final serum dilution to 1/16.

iv) Then 50 µl of serum/antigen mixtures is transferred from the carrier plates to the rabbit-serum coated ELISA plates and the plates are incubated at 37°C for 1 hour on a rotary shaker.

v) After washing, 50 µl of guinea-pig antiserum homologous to the viral antigen used in the previous step (iv) (preblocked with NBS and diluted in PBST containing 5% skimmed milk powder) is added to each well. The plates are then incubated at 37°C for 1 hour on a rotary shaker.

vi) The plates are washed and 50 µl of rabbit anti-guinea-pig immunoglobulin conjugated to horseradish peroxidase (preblocked with NBS and diluted in PBST containing 5% skimmed milk powder) is added to each well. The plates are incubated at 37°C for 1 hour on a rotary shaker.

vii) The plates are washed again three times and 50 µl of substrate solution, containing 0.05% H_2O_2 plus orthophenylene diamine or a suitable alternative chromogen, is added to each well.

viii) The reaction is stopped after 15 minutes by the addition of 50 µl of 1 M sulphuric acid. The plates are read at 492 nm on a spectrophotometer linked to a computer.

ix) Controls: A minimum of four wells each of strong positive, weak positive and negative bovine reference sera at a final dilution of 1/32 should be included on each plate together with an equivalent number of reaction (antigen) control wells containing antigen in diluent alone without serum. For end-point titration tests, duplicate twofold dilution series of positive and negative homologous bovine reference sera should be included on at least one plate of every run.

x) *Interpretation of the results:* Antibody titres are expressed as the 50% end-point titre, i.e. the dilution at which the reaction of the test sera results in an optical density equal to 50% inhibition of the median optical density of the reaction (antigen) control wells (Kärber, 1931). The median is calculated as the mean of two mid-values of the reaction control wells, eliminating from the calculation the highest and lowest values (alternatively, the mean value can be used after setting suitable tolerance limits to control for inter-well variation). In general sera with titres greater than or equal to 1/90 are considered to be positive. A titre of less than 1/40 is considered to be negative. For certification of individual animals for the purposes of international trade, titres of greater than 1/40, but less than 1/90 are considered to be doubtful, and further serum samples may be requested for testing; results are considered to be positive if the second sample has a titre of 1/40 or greater. For the purposes of herd-based serosurveillance as part of a statistically valid serological survey, a cut-off of 1/90 may be appropriate. Cut-off titres for evaluating immunological protection afforded by vaccination have to be established from experience of potency test results with the relevant vaccine and target species.

d) Nonstructural protein (NSP) antibody tests

Antibody to expressed recombinant FMDV NSPs (e.g. 3A, 3B, 2B, 2C, 3ABC) can be measured by different ELISA formats or immunoblotting. These ELISAs either use purified antigens absorbed directly to microplates or use PAbs or MAbs to trap specific antigens from semi-purified preparations (Bergmann *et al*., 2000; De Diego *et al*., 1997; Mackay *et al*., 1997; Sorensen *et al*., 1998). The index screening method used in Panaftosa is described in detail below. Other indirect and competitive ELISAs detecting bovine antibodies to 3ABC have been shown to have equivalent diagnostic performance characteristics (Brocchi *et al*., 2006). This same study corroborates preliminary data from Panaftosa that suggests that the diagnostic performance characteristics of these tests are similar in cattle, sheep and pigs.

- **Indirect enzyme-linked immunosorbent assay**

  - **Preparation of recombinant antigens (see Section B.2.d Enzyme-linked immunoelectrotransfer blot assay below)**

  - **Test procedure**

  i)   Microplates are coated overnight at 4°C with 1 µg/ml of the fusion antigen 3ABC in carbonate/ bicarbonate buffer, pH 9.6 (100 µl per well). Antigen 3ABC was expressed and purified as indicated for the EITB (enzyme-linked immunoelectrotransfer blot) tests (Neizert *et al.,* 1991).

  ii) The plates are washed six times with PBS, pH 7.2, supplemented with 0.05% Tween 20 (PBST).

  iii) Test sera (100 µl per well) are added in a 1/20 dilution in blocking buffer consisting of PBS, 0.05% Tween 20, 5% nonfat dry milk, 10% equine sera and 0.1% *Escherichia coli* lysate. Each plate includes a set of strong and weak positive and negative controls calibrated against the International Standard Sera described below.

  iv) The plates are incubated for 30 minutes at 37°C and washed six times in PBST.

  v)   Horseradish-peroxidase-conjugated rabbit anti-species IgG is diluted optimally in the blocking buffer, added at 100 µl per well and the plates are incubated for 30 minutes at 37°C.

  vi) After six washings, each well is filled with 100 µl of 3'3', 5'5'-tetramethylbenzidine plus 0.004% (w/v) H_2O_2 in phosphate/citrate buffer, pH 5.5.

  vii) The reaction is stopped after 15 minutes of incubation at room temperature by adding 100 µl of 0.5 M H_2SO_4. Absorbance is read at 450 nm and at 620 nm for background correction.

  viii) *Interpreting the results:* Test results are expressed as per cent positivity relative to the strong positive control [(optical density of test or control wells/optical density of strong positive control) × 100] or alternatively as a test to control (T/C) index relative to a cut-off (i.e. threshold positive) control. Profiling the NSP antibody reactivity levels in herds along with age/vaccination stratification aids interpretation of herd infection status in vaccinated populations (Bergmann *et al.,* 2003). Test cut-off values, with or without suspicious zones, need to be determined with consideration to the purpose of testing and the intended target population. Inconclusive results may be followed up using confirmatory tests, retesting with EITB or a second NSP ELISA (taking account of the conditional dependence of the two tests). The overall test system sensitivity and specificity must be taken into account when designing the serosurveillance programme. Although not a prescribed test for trade, NSP ELISAs may be a valuable adjunct in circumstances where the serotype or subtype of virus in the originating country is not known.

- **Enzyme-linked immunoelectrotransfer blot assay (EITB)**

  The EITB assay has been widely applied in South America as a confirmatory test for the above-described index screening method. Further information is available from the OIE Reference Laboratory, Panaftosa, PAHO/WHO.

  - **Preparation of test strips containing the recombinant antigens**

  i)   The five bioengineered FMDV NSPs 3A, 3B, 2C, 3D and 3ABC are expressed in *E. coli* C600 by thermo-induction. The 3D polypeptide is expressed in its complete form (McCullough *et al.,* 1992) whereas the rest of the proteins are obtained as fusions to the N-terminal part of the MS-2 polymerase gene (Strebel *et al.,* 1986).

  ii) The expressed polymerase is purified over phosphocellulose, followed by poly(U) Sepharose columns. The fused proteins 3A, 3B, 2C and 3ABC are purified by sequential extraction of the bacterial extracts with increasing concentrations of urea. The 7M fraction containing the fusion proteins is further purified on a preparative 10% SDS-PAGE (sodium dodecyl sulphate-polyacrylamide gel electrophoresis). The fusion protein band is excised from the gel and electroeluted (McCullough *et al.,* 1992).

iii) A mixture containing 20 ng/ml of each one of the purified recombinant polypeptides is separated on 12.5% SDS-PAGE and electrophoretically transferred to nitrocellulose (McCullough *et al.,* 1992).

• **Test procedure**

i) The required amount of test strips should be assessed, taking into account that for each nitrocellulose sheet, which defines one transferred gel, a positive, a weakly positive, a cut-off and a negative control serum should be assayed. In general, 24 nitrocellulose strips, each 3 mm wide, should result from a gel.

ii) A volume of 0.8 ml of saturation buffer (50 mM Tris/HCl, pH 7.5; 150 mM NaCl; 0.2% Tween 20; 5% nonfat dry milk; and 0.05% bacterial *E. coli* lysate) is added to each well. The antigen-coated strips are blocked by placing the trays on a rocker and agitating for 30 minutes at room temperature (20–22°C).

iii) A dilution of 1/200 of test sera and of each of the controls is added to the appropriate trough. The strips must be completely submerged and facing upwards, and maintained in that position during the whole process.

iv) Strips are incubated for 60 minutes on a rocker at room temperature.

v) Liquid is removed from the trays, and each test strip is washed three times with washing solution (50 mM Tris/HCl, pH 7.5; 150 mM NaCl; and 0.2% Tween 20) by agitation for 5 minutes.

vi) The alkaline-phosphatase-conjugated rabbit anti-bovine solution is added to each test well, and the strips are incubated with shaking for 60 minutes at room temperature.

vii) The liquid is removed from the trays and each test strip is washed three times with washing solution as above.

viii) Substrate solution (0.015% bromochloroindolylphosphate/0.03% nitroblue tetrazolium) is prepared in substrate buffer (100 mM NaCl; 5 mM $MgCl_2$; and 100 mM Tris/HCl, pH 9.3), and is added to each test well.

ix) Strips are incubated by placing the test tray on the orbital mixer and agitating until the cut-off control shows five distinct, discernible bands. Strips are washed with running deionised water and air-dried.

x) *Interpreting the results:* The EITB may be scanned with a densitometer but visual reading, although more subjective, is considered suitable as well. Individual control sera are tested that exhibit minimal but consistent staining for each of the five antigens. A test sample is considered positive if antigens 3ABC, 3A, 3B and 3D (±2C) demonstrate staining densities equal to or higher than that of their appropriate controls. A sample is considered negative if two or more antigens demonstrate densities below their control sera. Test samples not fitting either profile are considered indeterminate.

C. REQUIREMENTS FOR VACCINES

The control of FMD is usually a national responsibility and, in many countries, the vaccine may be used only under the control of the Competent Authority.

Guidelines for the production of veterinary vaccines are given in Chapter 1.1.8 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 in order for manufacturers to obtain an authorisation or licence for a veterinary vaccine. Where possible, manufacturers should seek to obtain such a license or authorisation for their FMD vaccines as independent verification of the quality of their product.

If virulent FMDV is used to produce FMD vaccine, the FMD vaccine production facility should operate under the appropriate biosecurity procedures and practices. The facility should meet the requirements for Containment outlined in Chapter 1.1.2 of this *Terrestrial Manual.*

Routine vaccination against FMD is used in many countries or zones recognised as free from foot and mouth disease with vaccination and in countries where the disease is endemic. In contrast, a number of disease-free countries have never vaccinated their livestock but have preferred the use of strict movement controls and culling of infected and contact animals when outbreaks have occurred. Nevertheless, many disease-free countries maintain the option to vaccinate and have their own strategic reserves of highly concentrated inactivated virus preparations. Such antigen reserves offer the potential of supplying formulated vaccine in an 'emergency' at short notice (Doel *et al.*, 1994). Chapter 1.1.10 of this *Terrestrial Manual* provides Guidelines for international standards for vaccine banks.

Traditional FMD vaccines may be defined as a fixed formulation containing defined amounts (limits) of one or more chemically inactivated cell-culture-derived preparations of a seed virus strain blended with a suitable adjuvant/s and excipients. See Chapter 1.1.8 Principles of veterinary vaccine production for biotechnology-derived vaccines such as recombinant or peptide vaccines.

Antigen banks may be defined as stockpiles of antigen components, registered or licensed according to the finished vaccine, and which can be stored under ultra-low temperatures for a very long time for subsequent formulation into vaccine as and when required.

The vaccines are formulated for their specific purpose and in the case of vaccines destined for use in cattle, both aluminium hydroxide saponin adjuvanted and oil adjuvanted vaccines may be used. For use in swine, double oil emulsions are preferred due to their efficacy.

FMD vaccines may be classified as either 'standard' or 'higher' potency vaccines. Standard potency vaccines are formulated to contain sufficient antigen and appropriate adjuvant to ensure that they meet the minimum potency level required (recommended at Section D.4.b as 3 PD_{50} [50% protective dose]) for the duration of the shelf life claimed by the manufacturer. This kind of vaccine is usually suitable for use in routine vaccination campaigns. For vaccination in naïve populations to control FMD outbreaks, higher potency vaccines (e.g.> 6 PD_{50} for the duration of the shelf life claimed by the manufacturer) are recommended for their wider spectrum of immunity as well as their rapid onset of protection.

Conventional live FMD vaccines are not acceptable due to the danger of reversion to virulence and as their use would prevent the detection of infection in vaccinated animals.

Because of the presence of multiple serotypes of the virus it is common practice to prepare vaccines from two or more different virus serotypes. In certain areas, it may be advisable to include more than one virus strain per serotype to ensure broad antigenic coverage against prevailing viruses.

1. Seed virus management

a) Characteristics of the seed virus

Selection of master seed viruses (MSVs) should ideally be based on their ease of growth in cell culture, virus yield, stability and broad antigenic spectrum (Samuel *et al.*, 1990). Isolates to prepare MSVs should be characterised and distributed, preferably by the OIE FMD Reference laboratories; they should be selected in accordance with the epidemiological importance of each variant.

The exact source of the isolate should be recorded and should include details such as the location, species and the type of material from which the virus was derived. Unique nomenclature should be used to identify the FMDV strain. The *in-vitro* passage history of the virus and details of the ingredients should be recorded in accordance with Chapter 1.1.8 of this *Terrestrial Manual.*

b) Method of culture

Methods of culture shall comply with the Chapter 1.1.8 of this *Terrestrial Manual*. Where no suitable established vaccine strain exists, new vaccine strains are derived through the establishment of MSVs from local field isolates by adapting them to growth in suspension or monolayer cells by serial passage. In order to remove the risk of contaminating lipid-enveloped viruses, it is recommended that putative MSVs undergo a validated organic solvent treatment prior to, or during, adaptation.

c) Validation as a vaccine strain

MSVs must be, well characterised and proven to be pure and free from all extraneous agents in accordance with Chapter 1.1.9 and those listed by the appropriate licensing authorities. Homology should be established with the original candidate isolates and effectiveness against the circulating strains from which they were developed should be proven. This often encompasses a number of methods, the most reliable being *in-vivo* protection assays. Alternatively, *in-vitro* tests (preferably virus neutralisation) can also be used, which require the availability of post-vaccination sera against these master seeds (see Section D of this chapter).

Seed viruses may be stored at low temperature (e.g. –70°C) or freeze-dried. Working seed viruses may be expanded in one or a few more passages from the master seed stock and used to infect the final cell culture.

Consideration should also be given to minimising the risk of transmission of transmissible spongiform encephalopathy agents (TSEs) by ensuring that TSE risk materials are not used as the source of the virus or in any of the media used in virus propagation.

d) **Emergency procedures for provisional acceptance of new MSV, and subsequent release of formulated vaccines**

In the case of incursion in a region of a new strain that is antigenically distinct from existing vaccine strains, it may be necessary to develop a new vaccine strain from a representative field isolate. Before the new MSV can be accepted, full compliance should be demonstrated with the relevant guidelines to demonstrate freedom from all extraneous agents listed by the appropriate licensing authorities using both general and specific tests, and to establish homology to the original candidate isolates. The time taken to raise the specific antisera necessary to neutralise the new strain for use in the general tests for detection of extraneous agents and to conduct other specific tests that require specialised techniques may be lengthy. Therefore, in emergency situations where there is insufficient time to complete full testing of the MSV, provisional acceptance of the new strain should be based on a risk analysis of the possibility of contamination of the antigen produced from the new MSV with extraneous agents. This risk assessment should take into account that a validated procedure to inactivate enveloped viruses must be used when establishing the MSV and that the virus is inactivated using a chemical inactivant with first order kinetics. Further assurance is provided by the requirement for the kinetics of inactivation to be monitored and recorded for each production batch.

2. Method of manufacture

The recommended method of virus propagation for antigen production is the growth of FMDV in large-scale suspension cultures or monolayers using cell lines under sterile conditions.

Cattle tongue epithelium in surviving conditions in medium with salts but without products of biological origin, may be acceptable for vaccine production but only if the method of production is entirely compliant with the standard requirements referred to in Chapter 1.1.8 of the *Terrestrial Manual*. In addition, in order to remove the risk of contaminating lipid-enveloped viruses, the harvested virus suspension must undergo a validated organic solvent treatment prior to BEI/EI inactivation. A validated procedure is applied to ensure inactivation of all possible extraneous agents and each batch is independently tested in an official laboratory for absence of extraneous agents. Adequate in-process and final products tests are in place to ensure consistency and safety of the final product. Consideration should also be given to minimising the risk of transmission of transmissible spongiform encephalopathy agents (i.e. BSE) by ensuring safe sourcing of the epithelium.

A suitable strain of the virus is used to infect a suspension or monolayers of an established cell line, such as BHK. Such cell cultures should be proven to be free from contaminating microorganisms.

It is common practice to keep stocks of BHK cells over liquid nitrogen and revive as necessary. On revival, they are expanded in nutrient medium to a volume and cell density appropriate to seeding the main culture.

When the virus is expected to have reached its maximum yield, the culture is clarified, often by chloroform treatment followed by centrifugation and/or filtration. The virus is subsequently inactivated by addition of an inactivant of first order, usually ethyleneimine (EI) in the form of binary ethyleneimine (BEI) (Bahnemann, 1975; 1990). It is important that the necessary safety precautions for working with BEI/EI are fully observed.

The BEI is added to a virus suspension, to give a predetermined final concentration. Inactivation must be duly validated and documented to show the inactivation kinetic and the results of the inactivation controls. The time period for BEI treatment and temperature used for inactivation must be validated for the actual conditions and equipment used.

To decrease the likelihood of live virus failing to contact the inactivant, e.g. EI/BEI, it is essential to transfer the vessel contents immediately to a second sterile vessel where inactivation is allowed to go to completion according to the validated inactivation kinetic and taking into account possible regulatory requirements for additional waiting times.

During inactivation, the virus titre is monitored by a sensitive and reproducible technique. The inactivation procedure is not satisfactory unless the decrease in virus titre, plotted logarithmically, is linear and extrapolation indicates that there is less than 1 infectious virus unit per 10^4 litres of liquid preparation at the end of inactivation.

After inactivation any residual EI/BEI in the harvest can be removed, or neutralised, for example by adding excess sodium thiosulphate solution to a final concentration of 2%.

The inactivated virus may be concentrated/purified by procedures such as ultrafiltration, polyethylene glycol precipitation or polyethylene oxide adsorption (Adamowicz *et al.,* 1974; Wagner *et al.,* 1970). Concentrated inactivated virus may be purified further by procedures such as chromatography. These concentrated and purified

antigens can be formulated into vaccines or stored at low temperatures for many years, and made into vaccine when required by dilution in a suitable buffer and addition of adjuvants (Doel & Pullen, 1990).

Conventional FMD vaccines are usually formulated as oil adjuvanted or aqueous. Oil-adjuvanted vaccines are usually formulated as water-in-oil emulsion using mineral oils, such as Marcol and Drakeol. The mineral oil is usually premixed with an emulsifying agent before the addition of a proportion, or all, of the aqueous phase of the vaccine, and emulsified by use of a colloid mill or continuous mechanical or flow ultrasonic emulsifier.

More complex double emulsions (water/oil/water) may be produced by emulsifying once more in an aqueous phase containing a small amount of detergent such as Tween 80 (Barnett *et al.,* 1996, Doel *et al.,* 1994; Herbert, 1965).

The aqueous vaccine is prepared by adsorbing the virus on to aluminium hydroxide gel, one of the adjuvant constituents of the final vaccine blend.

The final blend of the vaccine may include other components, such as antifoam, phenol red dye, lactalbumin hydrolysate, tryptose phosphate broth, amino acids, vitamins, buffer salts and other substances. An adjuvant, such as saponins, may also be incorporated, as well as preservatives.

Preservatives may be used as long as their usefulness as a preservative and absence of interference with FMDV antigen has been properly.

When using novel components, including adjuvants or preservatives, in any vaccine it is important to take into account that its status with regard to residues in products derived from food-producing species must be assessed to ensure that adequate assurance can be giving to licensing authorities in relation to safety for consumers. This requirement limits considerably the choice of adjuvants and preservatives for use in food-producing species.

3. In-process control

In general, virus titres reach optimum levels within about 24 hours of the cell culture being infected. The time chosen to harvest the culture may be based on a number of assays; for instance cell death. Virus concentration may be assessed by an infectivity test, sucrose density gradient (Bartelling & Meloen, 1974; Fayet *et al.,* 1971) or serological techniques. It is preferable to use a method for measuring antigenic mass, such as sucrose density gradient analysis, as well as one that measures infectivity, as the two properties do not necessarily coincide and the different methods may complement one another.

a) Inactivation kinetics

During inactivation of the virus, timed samples should be taken at regular intervals for the purpose of monitoring the rate and linearity of the inactivation process. Virus titres in the samples are determined by inoculation of cell cultures proven to be highly susceptible to FMDV, e.g. BHK. Such cultures permit the testing of statistically meaningful samples under reproducible conditions. The $\log_{10}$ infectivities of the timed samples are plotted against time, and the inactivation procedure is not considered to be satisfactory unless at least the latter part of the slope of the line is linear and extrapolation indicates that there would be less than one infectious particle per 10^4 litres of liquid preparation at the end of the inactivation period.

b) Inactivation control

The test for innocuity is an in-process test that should be carried out for every batch of antigen. Cells used to test for absence of residual live virus are not suitable if use of an amount of virus corresponding to 1 µg of 146S antigen gives a titre of less than 10^6 $TCID_{50}$ (European Pharmacopoeia, 2008). Following inactivation, a sample of each batch of inactivated antigen representing at least 200 doses should be tested for freedom from infectious virus by inoculation of sensitive monolayer cell cultures, preferably of the same origin as those used for the production of antigen. It may be preferable to concentrate the antigen to do this, in which case it must be shown that the concentrated material does not interfere with the sensitivity or reading of the assay. The cell sheets are examined daily over a period of 2–3 days, after which the spent medium is transferred to fresh monolayers and the original monolayers are replenished with fresh medium. Using this method, traces of live virus can be amplified by the passage procedure and detected on the basis of CPE observed. Two to three passages of the original virus preparation are commonly used. A variant on this method is to freeze–thaw the old monolayers to release intracellular virus, which can be detected by further passage.

4. Final product batch tests

a) Sterility

The bulk inactivated antigen, concentrated antigen and the final formulated product should undergo sterility testing. The preferred method is to collect any contaminating microorganisms by membrane filtration of the material to be examined and to detect any organisms present by incubation of the membranes with culture media. This procedure allows the removal of preservatives, etc., which may inhibit the detection of microorganisms. Guidelines on techniques and culture media, which allow the detection of a wide range of organisms, are described in the European Pharmacopoeia (2008) (see also Chapter 1.1.9 Tests for sterility and freedom from contamination of biological materials).

b) Identity testing

The bulk inactivated antigen, concentrated antigen and the final formulated product should undergo identity testing to demonstrate that the relevant strains are present. No other FMD virus serotype registered on the manufacturing site should be present in the vaccine, to be assured by adequate tests.

c) Viral nonstructural protein testing

Nonstructural proteins refer to proteins not present in the FMD viral capsid. Only products claiming to be purified from NSPs have to demonstrate their level of purification. Unless consistency of purification 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 of the *Terrestrial Manual,* NSP lack of reactivity has to be demonstrated in the final product (see Section C.5. Requirements for registration of vaccine).

Confirmation of vaccine purity may be shown by testing sera from animals vaccinated at least twice with the batch for absence of antibodies to nonstructural proteins.

d) Safety

Unless consistent safety of the product is demonstrated and approved in the registration dossier and the production process is approved for consistency in accordance with the standard requirements referred to in Chapter 1.1.8 of the *Terrestrial Manual*, batch safety testing is to be performed.

This final product batch safety test is conducted to detect any abnormal local or systemic adverse reactions,

For the purposes of batch release, each of at least two healthy sero-negative target animals is 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 undue 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.

e) Potency

Potency is examined on the final formulated product, or alternatively for antigen banks on a representative batch of vaccine prepared from the same bulk inactivated antigen.

The potency testing standard is the vaccination challenge test. However, for batch release indirect tests can also be used for practicability and animal welfare considerations, as long as correlation has been validated to percentage of protection in the target animal. Frequently indirect potency tests include antibody titration after vaccination of target species. Alternative methods could be used if suitably validated.

Ideally, indirect tests are carried out for each strain for one species and each formulation of vaccine to establish correlation between the indirect test results and the vaccine efficacy.

i) *Expected percentage of protection* (*EPP*) (*Maradei et al.*, *2008; Periolo et al*., *1993*)

The EPP estimates the likelihood that cattle would be protected against a challenge of 10,000 bovine infective doses after a single vaccination.

- Individual sera collected 30–60 days post-vaccination using a full dose of the vaccine are required from a group of either 16 or 30 18–24 month-old cattle.

- This panel of sera and sera of two control cattle are tested for antibody titres to the homologous FMD vaccine strain in a strongly correlated LPB-ELISA (see Sections B.2.a and B.2.c).

- The antigens used in the ELISA may be inactivated using BEI.

- The EPP is determined by reference to predetermined tables of correlation between serological titres and clinical protection (Maradei *et al.,* 2008; Periolo *et al.,* 1993).

- Batches with at least 75% EPP (with 16 vaccinated cattle) or at least 70% EPP (with 30 vaccinated cattle) are satisfactory for potency.

The presence of more than one serotype in a vaccine does not diminish the induction of antibodies against another serotype or the correlation of antibody titre with protection.

ii) *Other methods for evaluating protection*

Other tests were published using different ELISA methods and VNT methods to indirectly evaluate the protection given by vaccines. Their results could be accepted only if a strong correlation with protection in relation to the vaccine strain being tested and the serological method being used has been scientifically demonstrated and published in a peer-reviewed journal (Ahl *et al.,* 1990).

5. Requirements for registration of vaccine

a) Manufacturing process

For registration of vaccine, all relevant details concerning manufacture of the vaccine and quality control testing (see Sections C.1–4) should be submitted to the 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.

b) Safety

For the purposes of gaining regulatory approval, a trial batch of vaccine should be tested for local and systemic toxicity by each recommended route of administration in an *in-vivo* test in eight animals of each target species (European Pharmacopoeia, 2008). Single dose and repeat dose tests using vaccines formulated to contain the maximum permitted payload and number of antigens 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.

c) Efficacy

Vaccine efficacy is estimated in vaccinated animals directly, by evaluating their resistance to live virus challenge. The uncertainty of measurement in this test should be taken into account when interpreting its significance (Goris *et al.,* 2007; 2008). Vaccine efficacy should be established for every strain to be authorised for use in the vaccine.

Live reference FMD viruses corresponding to the main vaccine virus strains used in the region are available under certain conditions from OIE Reference Laboratories for FMD in the region. These reference viruses are stored at ultralow temperatures; they are already titrated for immediate use in challenge tests in cattle and are sent, in strict accordance with shipping regulations, with instructions for use at a predetermined dilution in PD_{50} and PGP challenge tests, described hereafter.

Each challenge virus is prepared at an OIE Reference Laboratory for FMD as follows: tongue tissue infected with FMDV should be obtained from the original FMD field case, which has been sent to the Reference Laboratory in glycerol buffer as described in Section B of this chapter.

The preparation of cattle challenge virus follows the process described in Section B.1.a Virus isolation, with the aim of obtaining a sterile 10% suspension in Eagle's minimal essential medium with 10% sterile fetal bovine serum.

The stock of challenge virus to be aliquoted is prepared from lesions collected from two cattle above 6 months of age that have been recognised as free from FMDV antibodies. These animals are tranquillised, for example using Xylazine 100 mg/ml (following the instructions for use), then inoculated intradermally in the tongue with the suspension in about 20 sites of 0.1 ml each. The vesiculated tongue tissue is harvested at the peak of the lesions, approximately 2 days later.

A 2% suspension is prepared as above, filtered through a 0.2 μm filter, aliquoted and frozen in the gas phase of liquid nitrogen; this constitutes the challenge virus stock. The infective titres of this stock are determined both in cell culture ($TCID_{50}$) and in two cattle (BID_{50}). Two tranquilised cattle are injected

intradermally in the tongue with tenfold dilutions (1/10 through 1/10,000), using four sites per dilution (Henderson, 1949). The cattle titrations are read 2 days later. Titres are usually above 10^6 $TCID_{50}$ for 0.1 ml and above 10^5 BID_{50} for 0.1 ml, calculated using the Spearman–Kärber method. The dilution for use in the cattle challenge test is 10 000 DIB_{50} in a total volume of 2 × 0.1 ml by intradermo-lingual injection both for the PD_{50} test and PGP test.

i) *PD_{50} test:*

The number of protective doses in a vaccine is estimated from the resistance to live virus challenge of animal groups receiving different amounts of vaccine. Cattle of at least 6 months of age, obtained from areas free from FMD that have not previously been vaccinated against FMD and are free from antibodies to FMDV should be used. Three groups of no fewer than five cattle per group should be vaccinated by the route recommended by the manufacturer. The vaccine should be administered at different doses per group by injecting different volumes of the vaccine. For example, if the label states that the injection of 2 ml corresponds to the administration of 1 dose of vaccine, a 1/4 dose of vaccine would be obtained by injecting 0.5 ml, and a 1/10 dose would be obtained by injecting 0.2 ml. These animals and a control group of two nonvaccinated animals are challenged either 3 weeks (aqueous) or up to 4 weeks (oil) after vaccination with a suspension of bovine virus that is fully virulent and appropriate to the virus types in the vaccine under test by inoculating the equivalent of a total of 10,000 BID_{50} (50% bovine infectious dose) intradermally into two sites on the upper surface of the tongue (0.1 ml per site). Animals are observed for at least 8 days. Unprotected animals show lesions at sites other than the tongue. Control animals must develop lesions on at least three feet. From the number of animals protected in each group, the PD_{50} content of the vaccine is calculated. There are a variety of methods for calculating PD_{50} (FAO, 1997), but procedures based on the Kärber (1931) method are generally preferred when interpreting PD_{50} estimates calculated in this way. The vaccine should contain at least 3 PD_{50} per dose for cattle.

ii) *PGP test* (*protection against generalised foot infection*)

For this method, a group of 16 FMD-seronegative cattle of at least 6 months of age, with the same characteristics described for the PD_{50} test, are vaccinated with a bovine dose by the route and in the volume recommended by the manufacturer. These animals and a control group of two nonvaccinated animals are challenged 4 weeks or more after vaccination with the challenge strain, which is a suspension of bovine virus that is fully virulent and appropriate to the virus types in the vaccine under test by inoculating a total of 10,000 BID_{50}, intradermally into at least two sites on the upper surface of the tongue. Unprotected animals show lesions on the feet within 7 days after inoculation. Control animals must develop lesions on at least three feet; for routine prophylactic use, the vaccine should protect at least 12 animals out of 16 vaccinated. Animals are observed at 7–8 days after challenge (Vianna Filho *et al.*, 1993). This test does not provide an estimate of how many protective doses are in a single vaccine dose but gives a certain measure of the protection following the injection of single commercial bovine doses of vaccine in a limited cattle population (Maradei *et al.*, 2008; Periolo *et al.*, 1993).

iii) *Efficacy in other species*

Efficacy tests in other target species, such as sheep, goats, pigs or buffalo are either different or not yet standardised. In general, a successful test in cattle 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 cattle, it may be more appropriate to potency test the vaccine in that same species. With respect to sheep, goats and African (*Syncerus caffer*) or Asiatic buffalo (*Bubalus bubalis*), due to the often inapparent nature of the disease in these species, potency results from a cattle test may be a more reliable indicator of vaccine quality than attempting a potency test reliant on the detection of clinical signs in these other species.

d) Purity: testing for antibody against nonstructural proteins

The OIE *Terrestrial Animal Health Code* stipulates that a criterion for regaining FMD free status following an outbreak, if vaccine is used, is to test the vaccinated animals for antibody against NSP. Likewise, countries wishing to be recognised as FMD free with vaccination must demonstrate the absence of virus circulation by showing that vaccinated animals are free from antibody to NSPs arising as a result of infection. Consequently, FMD antigens used to formulate vaccines that may be used in these circumstances should be purified to reduce the NSP content. With current manufacturing techniques it is possible to exclude the majority of NSPs so that they induce little, if any, NSP-specific antibody. Under these circumstances, detection of NSP antibodies can provide evidence that vaccinated animals have been exposed to FMDV. Vaccine manufacturers may wish to exploit this potential by including a claim that their vaccines do not induce antibody to one or more NSPs and can be used in conjunction with an appropriate diagnostic test. In addition to providing supporting documentation on the processes involved in such purification, manufacturers should demonstrate lack of immunogenicity against NSPs as part of the licensing procedure in order to make such a claim on their product literature. A recommended test method that can be used is to vaccinate not less than 8 naïve cattle with a trial blend of the vaccine containing the maximum number of

strains and amounts of antigen permitted on the authorisation. Cattle should be vaccinated at least three times at 21- to 30-day intervals and then tested before each revaccination and 30–60 days after the last vaccination for the presence of antibody to NSPs using the tests described in Section B.2.d of this chapter. Negative results in NSP assays may support claims that the vaccine does not induce antibody to NSPs for the number of injections tested. These cattle may be the same as those used for the safety test described in Section C.5.b of this chapter.

e)   Duration of immunity

The duration of immunity (D.O.I) of an FMD vaccine will depend on the efficacy (formulation and antigen payload). As part of the authorisation procedure the manufacturer should be required to demonstrate the D.O.I. 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, in compliance with Section 5.c. D.O.I. studies should be conducted in each species for which the vaccine is indicated or the manufacturer should indicate that the D.O.I. for that species is not known. Likewise, the manufacturer should demonstrate the effectiveness of the recommended booster regime in line with these guidelines, usually by measuring the magnitude and kinetics of the serological response observed.

In endemic or outbreak situations, vaccine is usually given as a primary course consisting of one or two doses of vaccine 3–4 weeks apart (based on animal population immunological status, vaccine potency, virus-vaccine matching, virus challenge levels, and other factors), followed by revaccination every 6–12 months. The frequency of revaccination will depend on the epidemiological situation and the type and quality of vaccine used.

For target animals born to vaccinated dams, vaccination should be delayed to allow decline of maternally derived antibodies. Primary vaccination of offspring to nonvaccinated dams can occur as early as 1 week of age (Auge De Mello *et al.,* 1989).

Information should be provided by manufacturers to indicate the appropriate vaccination programme(s) to minimise interference with maternally derived antibodies in target species.

f)   Stability

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

The shelf life of conventional FMD vaccines is usually 1–2 years at 2–8°C. Vaccines should never be frozen or stored above the target temperature.

g)   Precautions (hazards)

Current FMD vaccines are innocuous and present no toxic hazard to the vaccinators. Manufacturers should provide adequate warnings that medical advice should be sought in case of self-injection of an oil-emulsion vaccine.

6.   Storage and monitoring of concentrated antigens

Chapter 1.1.10 of the *Terrestrial Manual* provides guidelines for international standards for vaccine/antigen banks.

The process of storing concentrated antigens at ultra-low temperature for later formulation into FMD vaccine as described in Section C 2, is a well-established procedure for building stocks of immunogenic material ready to be formulated into vaccines in case of need. It not only forms the basis for the storage of antigens in a strategic reserve for emergency purposes, but allows the manufacturer immediate access to many different antigen strains that can be rapidly formulated and dispatched to the customer (Lombard & Fussel, 2007). Such stockpiling minimises delays subsequent to an order, particularly where a multivalent vaccine is requested. Another advantage of this procedure is that much of the quality testing can be completed well in advance of shipment. It is necessary to state that the concentrated antigens have to be controlled using standards indicated in Sections C.1–4.

a)   Storage conditions

- **Facilities**

It is important that all aspects of the storage of concentrated antigens conform fully to internationally accepted requirements such as those referred to in Chapter 1.1.8 of the *Terrestrial Manual.* Housing,

facilities and procedures should ensure the security of the stored antigen and prevent tampering, contamination or damage.

- **Containment of stored antigens**

The dose numbers or volumes stored are an important consideration, particularly where a reserve is shared between OIE Members and there is variation in number of doses perceived to be needed by each Member in an emergency. Where the requirement is for a large stockpile of a particular vaccine strain that can only be produced from several separate production runs, vaccine bank managers must consider the need to either formulate each lot into a representative final blend for testing purposes or mixing the individual batches, at some convenient point, for ease of formulating and/or testing.

The type of container used to hold antigen concentrate is important. Under ultra-low temperature conditions it is important to use containers made from materials that do not become brittle or fragile at a temperature range allowing for heat sterilisation and cold storage.

- **Labelling of stored antigens**

The concentrated antigens do not need to be labelled according to final or finished vaccine requirements and may be labelled as "in process" materials. Under ultra-low temperature conditions, the method of labelling must be of a durable nature. From experience, wire tagging bottles is the most preferred option using a metal/plastic tag sizeable enough to allow the necessary detail. Such detail should include the antigen/vaccine strain, batch number, date received and should also include an individual container or stock number. This information should be clear to read and marked on the tag using an indelible marker pen. Storage records and positions of containers should be carefully maintained.

b) **Monitoring of stored concentrated antigens**

It is vitally important that antigen concentrates are optimally maintained and routinely monitored in order to have some assurance that they will be efficacious when needed. Therefore arrangements should be made to monitor these antigen concentrates on a routine basis and to include where necessary, and at appropriate time intervals, a testing regime to ensure integrity of the antigen component or acceptable potency of the final product.

146S quantification, vaccination serology or vaccination challenge studies can be used for monitoring FMD antigen banks (Barnett & Statham, 1998; Bartelling & Meloen, 1974; Doel & Pullen, 1990; Fayet *et al.,* 1971). It is recommended to carry out these tests on receipt (year 0) and every 5 years thereafter.

To support these testing requirements for depositories of antigen, concentrates should include a number of small samples that are representative of the larger stock. Small aliquots/stocks of FMD antigen have usually consisted of a volume representing approximately one milligram of antigen. These aliquots should be stored side by side with the bulk antigen,

7. Emergency release of vaccines prepared from concentrated antigens

In situations of extreme urgency and subject to agreement by the competent authority, a batch of vaccine may be released before completion of the tests and the determination of potency if a test for sterility has been carried out on the bulk inactivated antigen and all other components of the vaccine and if the test for safety and the determination of potency have been carried out on a representative batch of vaccine prepared from the same bulk inactivated antigen. In this context, a batch is not considered to be representative unless it has been prepared with not more than the amount of antigen or antigens and with the same formulation as the batch to be released (European Pharmacopoeia, 2008).

D. VACCINE MATCHING TESTS

1. Introduction

Appropriate vaccine strain selection is an important element in the control of FMD and is necessary for the application of vaccination programmes in FMD-affected regions as well as for the establishment and maintenance of vaccine antigen reserves to be used in the event of new FMD incursions.

Vaccination against one serotype of FMDV does not cross-protect against other serotypes and may also fail to protect fully or at all against other strains of the same serotype. The most direct and reliable method to measure

cross-protection is to vaccinate relevant target species and then to challenge them by exposure to the virus isolate against which protection is required. This will take account of both potency and cross-reactivity.

However, such an approach requires the use of live FMDV and appropriate biosecurity procedures and practices must be used. The facility should meet the requirements for Containment Group 4 pathogens as outlined in Chapter 1.1.2 of this *Terrestrial Manual.* In addition to the safety concerns, this procedure is slow and expensive and requires specific expertise that is best available in OIE Reference laboratories. The use of animals for such studies should be avoided where possible by the use of *in vitro* alternatives.

A variety of *in vitro* serological methods can be used to quantify antigenic differences between FMDV strains and thereby estimate the likely cross-protection between a vaccine strain and a field isolate. Genetic characterisation and antigenic profiling can also reveal the emergence of new strains for which vaccine matching may be required and, conversely, may indicate that an isolate is similar to one for which vaccine matching information is already available. Such tests should be carried out in laboratories that work according to the standard specified in Chapters 1.1.2 and 1.1.3 of this *Terrestrial Manual*, preferably OIE Reference Laboratories for FMD.

Shipping of samples should be in accordance with Chapter 1.1.2 Sections H and I and Chapter 1.1.1 of this *Terrestrial Manual*.

Vaccine potency also contributes to the range of antigenic cover provided by a vaccine. A highly potent vaccine that stimulates a strong immune response may give greater protection against a heterologous virus than an equally cross-reactive vaccine that stimulates a weaker immune response (Brehm *et al.,* 2008). Furthermore, booster doses of vaccine can increase efficacy and the subsequent breadth of antigenic cover provided by a given vaccine, although the onset of full protection may be delayed (Pay, 1984).

2.   Selection of field viruses for vaccine matching

For vaccine matching, preferably, more than one representative isolate should be evaluated from any outbreak.

Viruses should be selected based on epidemiological information, for instance isolation at different stages of an outbreak, from different geographical locations, or from different hosts (Alonso *et al.,* 1987). Field evidence for a suspected lack of vaccine efficacy, as shown by reduced apparent protection, is an important criterion for vaccine matching.

The serotype of the field isolate is usually determined by ELISA or CFT using type-specific serological reagents, although methods based on MAbs or genetic typing may also be used. If the number of viruses exceeds the capacity of the laboratory to carry out methods described in Section D.4-Vaccine matching tests, a pre-selection of isolates should be done.

In order to minimise the risk of missing a relevant sample, the pre-selection should be carried out using all the isolates received by the laboratory. The recommended approach is to engage in serological validated antigenic profiling methods using ELISA. The performance of VP1 sequencing could be used to verify the homogeneity of the virus isolate population.

Only isolates showing important differences with vaccine strains are selected for vaccine matching tests.

3.   Selection of vaccine strains to be matched

The serotype of the virus, the region of origin and any information on the characteristics of the field isolate and, as appropriate, the vaccine strain used in the region of origin, may give indications as to the vaccine strains to be selected for vaccine matching tests. The availability of reagents for matching to particular vaccine strains may limit the extent of testing that is possible. Antigenic characterisation has two purposes; first, to choose the most effective vaccine strain for use in a particular circumstance and, second, to monitor, on an ongoing basis, the suitability of vaccine strains maintained in strategic antigen reserves.

4.   Vaccine matching test

The serological relationship between a field isolate and a vaccine virus ('r' value) can be determined by VNT or ELISA (Kitching *et al.,* 1988; Mattion *et al.,* 2009; Pereira, 1977). One way testing is recommended (r_1) with a vaccine antiserum, rather than two way testing (r_2), which also requires an antiserum against the field isolate to be matched. VNT using chequer-board titration method will give more accuracy to the results obtained. *In-vitro* neutralisation may be more relevant to predict *in-vivo* protection by the vaccine than other measures of virus-antibody interaction.

VNT is the method of choice (Mattion *et al.,* 2009) compared with the ELISA, which can be used only as a screening method for vaccine matching.

For either VNT or ELISA, post-vaccination sera should be derived from at least five cattle 21–30 days after inoculation. The titre of antibody to the vaccine strain is established for each serum. Sera are used individually or pooled, after excluding low responders.

a) Relationship between the field isolate and the vaccine strain

The recommended standard test is the VNT. The ELISA can be used as a screening method.

i) *Vaccine matching by two-dimensional* (*chequerboard*) *neutralisation test*

This test uses antiserum raised against a vaccine strain. The titres of this serum against 100 $TCID_{50}$ of the homologous vaccine strain and the same dose of a field isolate are compared to determine how antigenically 'similar' the field virus is to the vaccine strain.

• **Test procedure**

The procedure is similar to that of the VNT (see Section B.2.a).

Additional biological reagents are: monovalent 21–30 day post-vaccination bovine sera (inactivated at 56°C for 45–60 minutes), the homologous vaccine strain; and the test virus, a field isolate of the same serotype as the vaccine strain.

a) Field isolates are passaged on cell cultures until adapted to give 100% CPE in 24 hours. Passages should be kept to a minimum. When adapted, determine the virus titre (log_{10} $TCID_{50}$/ml) by end-point titration.

b) For each test and vaccine virus a chequerboard titration is performed of virus against vaccine serum along with a back-titration of virus. Cells are added and incubated at 37°C for 48–72 hours after which time CPE is assessed.

c) Antibody titres of the vaccine serum against the vaccine strain and field isolate for each virus dose used are calculated using the Spearman–Kärber method. The titre of the vaccine serum against 100 $TCID_{50}$ of each virus can then be estimated by regression. The relationship between the field isolate and the vaccine strain is then expressed as an 'r' value as:

$$r_1 = \frac{\text{reciprocal arithmetic titre of reference serum against field virus}}{\text{reciprocal arithmetic titre of reference serum against vaccine virus}}$$

• **Interpretation**

Interpretation of the results: It is generally accepted that in the case of neutralisation, r_1 values greater than 0.3 indicate that the field isolate is sufficiently similar to the vaccine strain that use of a vaccine based on this strain is likely to confer protection against challenge with the field isolate (Rweyemamu, 1984).

Conversely, values less than 0.3 suggest that the field isolate is sufficiently different from the vaccine strains tested that a vaccine based on these strains is less likely to protect. In this case, either the field isolate should be examined against other vaccine strains or the field isolate could be tested against existing vaccines in a heterologous cross protection challenge test. Alternatively, a suitable field isolate could be adapted to become a new vaccine strain.

Tests should always be repeated more than once. The confidence with which 'r' values can be taken to indicate differences between strains is related to the number of times that the examination is repeated (Rweyemamu & Hingley, 1984). In practice, a minimum of at least three repetitions is advised.

ii) *Vaccine matching by ELISA*

The use of the Liquid-phase blocking ELISA for vaccine matching has been reported (Ferris & Donaldson, 1992).

b) Testing the fitness for purpose of a vaccine

Only when the r-value suggests an insufficient match of a certain vaccine strain, the suitability of a vaccine based on such a vaccine strain could be demonstrated by a heterologous cross-protection challenge test carried out as described in Section C.4.b. in animals vaccinated with a known vaccine and challenged with the (heterologous) field virus. If the r value is under 0.3, the following differences in the previously described test are recommended respecting the instructions for vaccination. Vaccinate at least seven cattle without FMD antibodies, with a commercial dose of the current vaccine to be used in the region. Between 28 to

30 days later, boost all these animals with a second commercial dose in the same conditions and vaccinate a second group of at least seven animals with the same vaccine dosage and same route., Challenge the two groups plus two control animals (not vaccinated) 30 days later with the equivalent of a total of 10.000 BID 50% (50% bovine infective dose) of the new field strain duly titrated. The results are valid if each of the two control animal shows podal lesions on at least three feet. Final results are reported either as the number of protected animals (without podal lesion) over the total number of animal per group, or by percentage of protection where 100% is the total number of animals used per group. If results in the group of once vaccinated cattle indicate a protection level under 75%, and in the group of twice vaccinated cattle, protection under 100%, the change for a more appropriate vaccine strain is recommended (Henderson, 1949).

The use of the Expected Percentage of Protection (EPP) method (Alonso *et al*., 1987) is not recommended under heterologous conditions. This method measures the reactivity of a panel of post-vaccination antisera using either VNT or ELISA and relates the serological titres to the probability of protection, established through correlation tables associating antibody titres with protection against the homologous vaccine strain. Consequently the correlation from the panels of antisera and accompanying challenge tests cannot be extrapolated to any other strain (Robiolo *et al*., 2010).

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NB: There are OIE Reference Laboratories for foot and mouth disease
(see Table in Part 3 of this *Terrestrial Manual* or consult the OIE Web site for the most up-to-date list:
http://www.oie.int/en/our-scientific-expertise/reference-laboratories/list-of-laboratories/).
Please contact the OIE Reference Laboratories for any further information on
FMD diagnostic tests, reagents and vaccines.