

CHAPTER 2.8.3.

**CLASSICAL SWINE FEVER
(hog cholera)
(INFECTION WITH
CLASSICAL SWINE FEVER VIRUS)**

SUMMARY

Classical swine fever (CSF), also known as hog cholera, is a contagious viral disease of pigs, including wild boar. The causative virus is a member of the genus Pestivirus of the family Flaviviridae, and is closely related to the viruses of bovine viral diarrhoea and border disease. There is only one serotype of CSF virus (CSFV).

The disease may run an acute, subacute, chronic, late onset, or inapparent course, depending on a variety of viral and host factors of which the age of the animals, the virulence of the virus and the time of infection (pre- or post-natal) are of greatest importance. Adult pigs usually display less severe signs of disease than young animals and stand a better chance of survival. In pregnant sows, the virus may cross the placental barrier and reach the fetuses. In-utero infection with strains of the virus of moderate or low virulence can result in what is referred to as the 'carrier sow' syndrome followed by prenatal or early post-natal death, the birth of diseased piglets or an apparently healthy but persistently infected litter. An outbreak of CSF in domestic pigs has serious consequences for trade in pigs and pig products.

The highly variable clinical picture of CSF precludes a diagnosis on clinical and pathological grounds alone. Laboratory methods are therefore essential for an unambiguous diagnosis. Detection of virus or viral nucleic acid in anticoagulated whole blood and of antibodies in serum are the methods of choice for diagnosing CSF in live pigs, whereas detection of virus, viral nucleic acid or antigen in organ samples is most suitable when the pig is dead.

Identification of the agent: *The isolation of CSFV should be attempted in pig kidney (PK-15, SK-6) cell lines, or other CSFV permissive cell lines. The cultures, which are generated from stocks that are Pestivirus-free (and preferably free of other contaminants, e.g. mycoplasmas, porcine circovirus), are examined for virus growth by immunofluorescence or immunoperoxidase staining; positive isolates are further characterised by partial genetic sequencing or, if that method is not available, by the use of monoclonal antibodies (MAbs). Reverse-transcription polymerase chain reaction protocols for the identification of CSFV nucleic acid have now gained international acceptance and are being used in many laboratories, both for detection of the agent and differentiation from other pestiviruses. The direct fluorescent antibody test (FAT) on cryostat sections of organs from affected pigs can be used for the detection of CSF antigen. A panel of MAbs is used to determine whether the fluorescence is caused by CSF or non-CSF Pestivirus antigens. Antigen-capture enzyme-linked immunosorbent assays (ELISAs) are also useful for herd screening, but must not be used on a single animal basis.*

Serological tests: *Detection of virus-specific antibodies is particularly useful in herds suspected of having been infected at least 21 days previously with CSFV. Serological methods are also valuable for monitoring and for prevalence studies, and are essential if a country wishes to be internationally recognised as being free from the disease in the absence of vaccination.*

As CSFV cross-reactive antibodies against other pestiviruses are occasionally observed in pigs, screening tests have to be followed by confirmatory tests that are CSFV-specific. Certain ELISAs are relatively CSFV-specific, but the definitive method of choice for differentiation is the

comparative neutralisation test, which compares the neutralising titre of antibodies to different Pestivirus isolates.

Requirements for vaccines: *Vaccines against CSF are based on live virus that has been attenuated by passage through cell cultures or through a suitable host species that is not of the family Suidae. The production of these modified live virus (MLV) vaccines is based on a seed-lot system that has been validated with respect to virus identity, sterility, purity, safety, nontransmissibility, stability and immunogenicity. If CSFV is used in the production of vaccine or in challenge studies, the facility should meet the OIE requirements for the appropriate Containment Group as determined by risk assessment.*

Effective inactivated, conventional whole virus vaccines are not available. In contrast to conventional MLV vaccines, new generation MLV 'marker vaccines' capable of inducing antibodies that can be distinguished from antibodies induced by field virus when an appropriate companion discriminatory diagnostic test is used, may become available. The presently registered subunit 'marker vaccine' is based on the major envelope glycoprotein (E2-subunit) of CSFV, and is produced in insect cells using recombinant DNA technology.

A. INTRODUCTION

The viruses that cause classical swine fever (CSF), bovine viral diarrhoea (BVD) and border disease (BD) are members of the family *Flaviviridae*, genus *Pestivirus*, and are closely related to each other, both antigenically and structurally. Clinical signs and lesions seen at post-mortem examination in pigs affected with CSF are highly variable due to both viral and host factors. Furthermore, (congenital) infections with ruminant pestiviruses in pigs occasionally give rise to a clinical disease that is indistinguishable from CSF (Terpstra & Wensvoort, 1988; Vannier & Carnero, 1985; Wensvoort & Terpstra, 1988). A recent review of the disease is provided by Moennig *et al.* (2013).

CSF affects the immune system, a main characteristic being generalised leukopenia, which can often be detected before the onset of fever. Immunosuppression may lead to concurrent infections that can mask the clinical picture.

Pyrexia, huddling, inappetance, dullness, weakness, conjunctivitis and constipation followed by diarrhoea are the prevailing signs of disease in all age groups. In addition, animals may display a staggering gait, ataxia or convulsions. Several days after the onset of clinical signs, the ears, abdomen and inner thighs may especially show petechial haemorrhages or a purple discoloration. Animals with acute disease die within 1–4 weeks. Sudden death in the absence of clinical illness is not symptomatic of CSF.

Under certain circumstances related to the animals' age and condition, as well as to the virus strain involved, subacute or chronic clinical illness may develop, which can be protracted for several weeks or even months. Chronic illness leads to a stunting of growth, anorexia, intermittent pyrexia and diarrhoea.

Congenital persistent infections may go undetected for months and may be confined to only a few piglets in the herd or may affect larger numbers. The clinical signs are nonspecific: wasting in the absence of pyrexia. Chronic and persistent infections always lead to the death of the animal. Herd mortality rates may be slightly above the expected level.

In acute cases, gross pathological lesions might be inconspicuous or absent. In typical cases, the lymph nodes are swollen and marbled red, and haemorrhages occur on serosal and mucosal membranes of the intestinal organs. Splenic infarctions may occur. In subacute and chronic cases, necrotic or 'button' ulcers may be observed in the mucosa of the gastrointestinal tract, epiglottis and larynx, in addition to the above lesions.

Histopathological findings are not pathognomonic. Lesions may include parenchymatous degeneration of lymphatic tissue, cellular proliferation of vascular interstitial tissue, and a nonsuppurative meningo-encephalomyelitis, with or without vascular cuffing.

A useful critique of diagnostics and vaccination for CSF, from an authoritative source, has been published (Blome *et al.*, 2006), which, as well as general guidance, also provides sources of information on validation and scientific opinion on the applicability of certain commercial products in these areas.

There is no known risk of human infection with CSF virus. The virus has a high risk of spread from the laboratory, and biocontainment measures should be determined by risk analysis as described in Chapter 1.1.4 *Biosafety and*

biosecurity: Standard for managing biological risk in the veterinary laboratory and animal facilities. Countries lacking access to an appropriately equipped laboratory should send specimens to an OIE Reference Laboratory.

B. DIAGNOSTIC TECHNIQUES

Table 1. Test methods available for the diagnosis of classical swine fever and their purpose

Method	Purpose					
	Population freedom from infection	Individual animal freedom from infection prior to movement	Contribute to eradication policies	Confirmation of clinical cases	Sero-prevalence of infection – surveillance	Immune status in individual animals or populations post-vaccination
Agent identification¹						
Virus isolation	–	+	–	+++	–	–
PCR	+	+	++	+++	++	–
ELISA (antigen)	++	+	+	+	–	–
FAT	–	–	+	+	–	–
Detection of immune response						
ELISA (antibody)	+++	+++	+++	–	+++	+++
VN (FAVN or NPLA)	+	+++	++	++	+++	+++

Key: +++ = recommended method; ++ = suitable method; + = may be used in some situations, but cost, reliability, or other factors severely limits its application; – = not appropriate for this purpose.

Although not all of the tests listed as category +++ or ++ have undergone formal validation, their routine nature and the fact that they have been used widely without dubious results, makes them acceptable.

PCR = polymerase chain reaction; ELISA = enzyme-linked immunosorbent assay; VN = virus neutralisation; FAT = fluorescent antibody test.

The variability of the clinical signs and post-mortem lesions does not provide firm evidence for unequivocal diagnosis. Other viral diseases, such as African swine fever, porcine dermatitis and nephropathy syndrome (PDNS), and post-weaning multisystemic wasting syndrome (PMWS), thrombocytopenic purpura and various septicaemic conditions including, amongst others, salmonellosis (especially caused by *Salmonella choleraesuis*), erysipelas, pasteurellosis, actinobacillosis (caused by *Actinobacillus suis*) and *Haemophilus parasuis* infections may be confused with acute CSF. In fact, these bacteria often cause concurrent infections, and isolating these pathogens may obscure the real cause of disease, the CSF virus (CSFV). Similarly concurrent PDNS can lead to oversight of an underlying CSF infection.

A tentative diagnosis based on clinical signs and post-mortem lesions must therefore be confirmed by laboratory investigations. As pyrexia is one of the first signs of CSF and is accompanied by viraemia (Depner *et al.*, 1994), detection of virus or viral nucleic acid in whole blood, collected in heparin or ethylene diamine tetra-acetic acid (EDTA), or in tissues, collected from febrile animals, is the method of choice for detecting infected herds at an early stage. This is all the more necessary in view of the serious consequences of an outbreak of CSF for trade in pigs and pig products.

Laboratory methods for diagnosis of CSF are aimed at detection of the virus, viral nucleic acid or viral antigens, or detection of specific antibodies. Targeted and risk-based sampling should be performed, random sampling only being applied in cases where no clinical signs of disease are present. To increase the sensitivity of detection of virus, viral antigen or nucleic acid, clinically diseased animals and febrile animals should primarily be sampled.

¹ A combination of agent identification methods applied on the same clinical sample is recommended.

For the detection of antibodies, animals that have recovered from disease or animals that have been in contact with infected or diseased animals should be primarily targeted.

For a correct interpretation of the test results, the inspecting veterinarian should pay particular attention to the simultaneous and clustered occurrence of two or more of the prevailing signs of disease listed above. In suspected primary cases an initial positive test result needs to be confirmed using a second test method.

Antibodies develop in the third week of illness and persist in the surviving animal for years or even life (except for chronic cases). Samples for antibody detection are collected in ordinary (non-heparinised) tubes from convalescent pigs and from contact herds. All methods and protocols need to be validated in the respective laboratory and the laboratory has to prove that it is capable of performing the tests it uses for diagnostic purposes with satisfactory results. Validation should be done in accordance with the OIE validation standard (see Chapter 1.1.6 *Principles and methods of validation of diagnostic assays for infectious diseases*).

1. Identification of the agent

1.1. Isolation of virus

Isolation of virus in cell cultures is a more sensitive but slower method for diagnosis of CSF than immunofluorescence on frozen sections. Organ preparations, leukocyte preparations, or whole blood samples can be used. Isolation is best performed in rapidly dividing PK-15 cells seeded on to coverslips simultaneously with a 2% suspension of the tonsil in growth medium. Other pig cell lines may be used, but should be demonstrably at least as sensitive as PK-15 cells for isolation of CSFV and must be free of pestiviruses and pestivirus antibodies. It is generally advantageous to use more than one porcine cell line for inoculation, to enhance the chances of a positive result. As growth of the virus does not cause a cytopathic effect, its presence must be demonstrated by an immunostaining method, which may be carried out after one or two virus passages. This can be done by examining the cultures for fluorescent foci by FAT after 24–72 hours or by immunoperoxidase staining after 3–4 days' incubation. NB: Positive and negative controls always need to be included.

The tonsil is the most suitable organ for virus isolation from pigs that died or were killed for diagnostic purposes. Alternatively or in addition, spleen, kidney, ileum or lymph nodes can also be used.

Fetal bovine serum (FBS) used in any diagnostic assay always needs to be free of pestiviruses and pestivirus antibodies. It might not be sufficient to rely on manufacturers' declarations and for this reason it is recommended that each lot of FBS be tested for the presence of pestiviruses and pestivirus antibodies prior to its use in diagnostic assays.

1.1.1. Virus isolation – Test procedure 1

- i) Prepare a 100-fold strength glutamine–antibiotic stock solution: dissolve glutamine (2.92 g) in 50 ml distilled water (solution A) and sterilise by filtration. Dissolve each of the following antibiotics in 5–10 ml sterile distilled water: penicillin (10^6 International Units [IU]); streptomycin (1 g); mycostatin (5×10^5 U); polymixin B (15×10^4 U); and kanamycin (1 g). Pool these solutions (solution B). Mix aseptically solutions A and B, make up to 100 ml with sterile distilled water, and store in 5 ml aliquots at -20°C . Exact antibiotic constitution is not critical, provided sterility is achieved and cells are not affected.
- ii) Cut 1–2 g of tissue (organ sample of approx. 1 cm^3) into small pieces and, using a mortar and pestle or other device, grind in a small amount of cell culture medium with sterile sand into a homogeneous paste. Alternatively, use an appropriate crushing machine or automatic homogenisator at 4°C . (Attention: high speeds can heat the sample and affect the virus!)
- iii) Make a 20% (w/v) suspension by adding Hanks' balanced salts solution (BSS) or Hanks' minimal essential medium (MEM); 1 ml of the glutamine–antibiotic stock is added for each 10 ml of suspension. This mixture is held at room temperature for 1 hour.
- iv) Centrifuge at 1000 or 2500 **g** for 15 minutes. The supernatant is used for inoculation of cell cultures. A 1/100 dilution can be processed in parallel in case of cytotoxic effects. Sterile filtration can be performed, if considered necessary using syringe filters ($0.45 \mu\text{m}$ followed by $0.22 \mu\text{m}$).
- v) A PK-15 monolayer is trypsinised, the cell suspension is centrifuged at 160 **g** for 10 minutes. The supernatant is discarded and the pellet is resuspended to contain approximately 2×10^6 cells/ml in growth medium (Eagle's MEM with Earle's salts; 5% fetal bovine serum free of ruminant pestiviruses and pestivirus antibodies; and 0.2 ml of the

glutamine–antibiotic stock solution per 10 ml cell suspension). As a guide, one 75 cm² flask will give approximately 50 ml of cell suspension at the appropriate concentration. It usually contains about 8.5×10^6 cells.

Alternatively a protocol without centrifugation can be performed:

Growth medium is removed from a PK-15 monolayer and cells are washed once or twice with 5 ml of adjusted trypsin/versen (ATV) solution (5 ml ATV for a 250 ml flask). ATV is removed and replaced with fresh ATV (2 ml ATV for a 250 ml flask). The flask is incubated at 37°C for 15 minutes or until cells are detached. It is then filled with cell culture medium containing 5% FBS (8 ml medium for a 250 ml flask) and the cells are resuspended.

vi) Either:

Suspension inoculation: mix nine parts of cell suspension (from step v) and one part of supernatant fluid (from step iv) and inoculate 1.0–1.5 ml into 6–8 Leighton tubes with cover-slips or other appropriate cell culture flasks or plates. Three tubes are inoculated with 1.0–1.5 ml of cell suspension alone as controls. After completion of the sample inoculations, three tubes are inoculated with CSFV as positive controls. Careful precautions must be taken to avoid cross-contamination with this known positive virus suspension. Negative cultures should also be prepared. Incubate at 37°C.

Or:

Pre-formed monolayer inoculation: for each tissue, inoculate 1.0–1.5 ml of cell suspension (prepared as in step v) into 6–8 Leighton tubes with cover-slips or other appropriate cell culture flasks or plates. Incubate at 37°C for a minimum of 4 hours and a maximum of 36 hours (until 50–80% confluency is reached). Then drain the medium and inoculate 0.2 ml of supernatant fluid (from step iv), incubate for 1–2 hours at 37°C, rinse once with PBSM (PBS without Ca/Mg), overlay with 1 ml of growth medium and incubate at 37°C.

vii) At 1, 2 and 3 days after inoculation, two cultures, together with a positive and negative control culture are washed twice for 5 minutes each in Hanks' BSS, Hanks' MEM or PBS and fixed. Cell fixation is performed by 100% acetone (analytical grade) for 5 minutes for cell cultures grown on glass surfaces.

viii) After fixation, staining with a direct or indirect anti-CSFV conjugate at its appropriate working dilution is performed as described in Section B.1.2. After washing in PBS three times for 5 minutes each, the cover-slip cultures are mounted in 90% carbonate/bicarbonate buffered glycerol, pH>8.0, and examined for fluorescent foci.

Instead of Leighton tubes, 6-well plates with cover-slips can be used. Alternatively, cultures growing on flat-bottomed microtitre plates or M24-plates can also be used for virus isolation. In such case, plates are fixed and stained as described later for the neutralising peroxidase-linked assay (NPLA; Section B.2.1).

ix) If the 2% tonsil suspension proves to be toxic for the cells, then the test should be repeated using a higher dilution or another organ. Use of the method employing pre-formed monolayers (above) will help to avoid this problem.

1.1.2. Virus isolation – Test procedure 2

Whole blood (heparin or EDTA-treated) from clinically diseased pigs is a suitable sample for early CSF diagnosis. The leukocyte fraction or other components may be used, but for reasons of simplicity the use of whole blood is more practical and therefore preferred (De Smit *et al.*, 1994). The procedure is as follows:

- i) Freeze a sample of whole blood at –20°C and thaw in a waterbath at 37°C to lyse the cells.
- ii) Inoculate 300 µl haemolysed blood on to a PK-15 monolayer grown to approximately 50–80% confluence* in an M24-plate or Leighton tubes with cover slips, and allow adsorption for 1–2 hours at 37°C. Duplicate cultures of each sample should always be prepared.
- iii) Remove inoculum, wash the monolayer once with Hanks' BSS or Hanks' MEM or PBSM, and add fresh growth medium.

* Simultaneous inoculation, though slightly more sensitive, is less suitable as the anticoagulant may interfere with the adhesion of cells on to the surface.

- iv) After a further incubation period of 3–4 days at 37°C in a CO₂ incubator, the plates are washed, fixed and stained, as described later for the NPLA, using in each step a volume of 300 µl to compensate for the larger cell surface.

NOTE: This method is less sensitive than conventional virus isolation for the detection of acute CSF.

1.1.3. Virus isolation – Test procedure 3

To improve the sensitivity, virus isolation can be performed over two passages:

- i) Inoculate a cell culture tube with 200–300 µl of organ preparation or blood lysate (see above). Duplicate cultures should always be prepared.
- ii) Incubate the cell cultures for 37°C for 1–2 hours, and wash twice with PBSM.
- iii) Incubate the cultures for 72 hours at 37°C in a CO₂ incubator. Eagle's MEM with 10% FBS is the ideal medium for virus growth. Simultaneous inoculation is possible if the sample is fresh and a cytotoxic effect is unlikely.
- iv) Freeze the cell culture tubes or plates at –80°C for at least 1 hour and then thaw at room temperature.
- v) When using cell culture tubes, the tubes are centrifuged for 10 minutes at 778 *g*.
- vi) Incubate 200–300 µl of the supernatant for 1–2 hours on a well of a multi-dish plate or Leighton tube as described above.
- vii) Wash the cell culture tubes or plates with PBSM, refill with cell culture medium and incubate for 72–96 hours in a CO₂ incubator.
- viii) Cells are fixed and stained as described in Section B.2.1.

If a slow-growing isolate is suspected, a second passage in a culture tube can be done, leading to a third passage in a culture dish.

Positive and negative controls must always be included and processed in the same way.

1.1.4. Reverse-transcription polymerase chain reaction

Many methods for RT-PCR have been described or are being developed (McGoldrick *et al.*, 1998). By using RT-PCR techniques, infected animals can be detected early during the incubation period and for a longer period of time in cases where the pigs recover. RT-PCR detects viral nucleic acid only and positive results may be obtained in cases where virus isolation or other techniques yield negative results. RT-PCR is therefore more sensitive than other techniques (such as antigen-capture ELISA, and FAT).

Owing to its speed and sensitivity, RT-PCR is a suitable approach for screening and confirmation of suspected cases of disease and is now accepted by a number of countries and the European Union (EU) (European Commission, 2002). It is however, important to bear in mind that false positive results due to laboratory contamination can occur as well as false negative results due to inhibitors contained in the sample. Therefore, any positive results from primary outbreaks must always be confirmed by other tests. It is mandatory to include an adequate number of positive and negative controls in each run; it is also strongly recommended that internal controls be included. See Chapter 1.1.6 *Principles and methods of validation of diagnostic assays for infectious diseases* for further details on PCR techniques.

The test can be applied to blood and serum samples as well as solid organs and cell culture supernatants and has been used successfully in case of outbreaks.

Isolation of RNA is a critical step in RT-PCR analysis. RNA integrity is at the highest risk prior to and after extraction. Thus, treatment of samples prior to RNA extraction and storage of isolated RNA have to be carefully considered as they will influence the quality of the yielded RNA and the test result. Different methods for RNA isolation have been described and a wide variety of extraction kits is commercially available. RNA isolation must also be validated in the laboratory.

Several conventional and real-time PCR protocols have been described (Hoffmann *et al.*, 2005; McGoldrick *et al.*, 1998; Paton *et al.*, 2000b; Risatti *et al.*, 2003; 2005) and a suitable protocol may be obtained from the literature or from the OIE Reference Laboratories for CSF (see Table

given in Part 4 of this *Terrestrial Manual*). Evaluation of RT-PCR results can either be performed by agarose gel electrophoresis (standard RT-PCR) or by real-time techniques (RT-qPCR). Any RT-PCR protocol to be used must be thoroughly validated in each individual laboratory to show that the method is fit for purpose, before it can be used for diagnosis in that laboratory. Any RT-PCR protocol used must prove to be at least as sensitive as virus isolation. The RT-qPCR protocol described by Hoffmann *et al.* (2005) is widely used and the method yielded consistent results in international interlaboratory comparison testing.

In principle, pooling of samples is possible, but sensitivity must be shown to be at least as high as the sensitivity of virus isolation performed on single samples. Pooling must be properly validated prior to its use in each individual laboratory.

Quality control is an essential issue in PCR diagnosis and prevention of laboratory contamination is crucial.

1.1.5. Molecular epidemiology and genetic typing

The molecular epidemiology of CSF is based on the comparison of genetic differences between virus isolates. RT-PCR amplification of CSFV RNA followed by nucleotide sequencing is the simplest way of obtaining the sequence data to make these comparisons. A number of different regions of the CSFV genome may be targeted for molecular epidemiological studies (Paton *et al.*, 2000a). Two regions have been extensively studied and provide large sets of sequence data with which new isolates can be compared. One of these regions lies within the 5'-nontranslated region (5'NTR) of the genome (150 nucleotides) and the other lies within the E2 major glycoprotein gene (190 nucleotides). In brief, the method used involves extracting virus RNA from clinical samples or cell cultures infected with low passage CSFV, performing RT-PCR to amplify one or both targets within the 5'NTR or the E2 gene, and then determining the nucleotide sequence of the products and comparing with stored sequence information held in the databases (Greiser-Wilke *et al.*, 1998; Lowings *et al.*, 1996). A database of these sequences is available from the OIE Reference Laboratory for CSF in Germany. Recent findings on analysing other pestivirus sequences highlight the need for analysis of multiple regions in order to accurately type strains by this method (Becher *et al.*, 2003; Hurtado *et al.*, 2003; Liu *et al.*, 2009; Vilcek *et al.*, 2010). CSFV isolates from primary outbreaks should be sent to an OIE Reference Laboratory for investigation of molecular epidemiology. The receiving laboratory should be contacted first and an import permit should be obtained prior to dispatch.

1.2. Immunological methods

1.2.1. Fluorescent antibody test

The fluorescent antibody test (FAT) is a rapid test that can be used to detect CSFV antigen in cryostat sections of tonsils, spleen, kidney, lymph nodes or distal portions of the ileum. Tissues should be collected from several (febrile and/or diseased) animals (Bouma *et al.*, 2001) and transported without preservatives under cool conditions, but not frozen. Cryostat sections are stained directly with anti-CSF immunoglobulin conjugated to a fluorescence marker such as fluorescein isothiocyanate (FITC) or indirectly using a secondary fluorescent conjugate and examined by fluorescence microscopy. During the first stage of the infection, tonsillar tissue is the most suitable, as this is the first to become affected by the virus irrespective of the route of infection (Ressang, 1973). In subacute and chronic cases, the ileum is frequently positive and occasionally may be the only tissue to display fluorescence.

A negative FAT result does not completely rule out CSF infection. When suspicion of CSF continues, further samples should be obtained or attempts made at reverse-transcription polymerase chain reaction (RT-PCR) or virus isolation in cell culture. In some cases during the terminal stage of disease, neutralising antibodies can mask a positive reaction.

There is a relatively high risk of false (positive and negative) results when FAT is used by laboratories not thoroughly acquainted with the method. Thus FAT should only be used by laboratories that have experience of using the technique, perform the technique on a routine basis and have had training in interpreting the fluorescence.

a) Test procedure

Include positive and negative control sections in each series of organ samples to be examined. In indirect labelling, an infected control section should also be included, which is treated without incubation of the first antibody. The control sections can be prepared in advance and stored after acetone fixation for 2–3 years at –70°C until use.

- i) Cut out a piece of tonsil, spleen, kidney and ileum of approximately 1 × 1 × 0.5 cm, and mount it with a cryo-embedding compound or distilled water on a cryostat table.
- ii) Freeze the piece of organ on to the cryostat table. The freezing temperature should be –15 to –20°C. Shock-freezing of the tissue in n-Heptan cooled with liquid N₂ is ideal.
- iii) Cut sections not more than 4–8 µm thick and mount these on to grease-free cover-slips. It is helpful to mark these cover-slips by one cut-off corner and to mount them with this corner in the same position (e.g. top right).
- iv) Prepare several cover slips for each tissue sample.
- v) Dry for 20 minutes at room temperature.
- vi) After drying, fix the mounted sections for 10 minutes in acetone (analytical grade) at –20°C or air-dry for 20 minutes at 37°C.
- vii) Immerse the sections briefly in phosphate-buffered saline (PBS), remove excess fluid with tissue paper and place them (cut off corner top right) on a frame in a humid incubation chamber.
- viii) Dispense the anti-CSF immunoglobulin at working dilution (dilution in PBS) on to the entire section and incubate in a dark, closed chamber for 30 minutes at 37°C. Check afterwards that the conjugate solutions have not evaporated and that the tissues have not dried out.

If a secondary FITC conjugate is required, wash the section five times for 2 minutes each in PBS at room temperature, then add the FITC conjugate at working dilution and incubate as previously described.
- ix) Wash the sections five times for 2 minutes (or three times for 5 minutes) each in PBS at room temperature.
- x) Immerse the section briefly in double-distilled water (solvent).
- xi) If necessary, counterstain in Evans Blue for 30 seconds.
- xii) Remove the remaining fluid by touching the cover-slip against tissue paper and mount the cover-slip (with the section between cover-slip and slide) with mounting buffer on to a microscope slide.
- xiii) Remove excess mounting fluid with tissue paper and examine the sections for fluorescence using a UV microscope.

A CSF-positive section shows brilliant fluorescence in the cytoplasm of infected cells. In the tonsils, fluorescence in the epithelial lining of the crypts is particularly evident. In kidney sections, fluorescence is most abundant in the proximal and distal tubules of the renal cortex and the collecting ducts in the medulla. In the ileum, fluorescence is most prominent in the epithelial cells of the Lieberkühn glands, whereas in the spleen reactivity is more diffuse, with concentrations of lymphoid cells in the periarterial lymphoid sheath (PALS).

It is recommended to use anti-CSFV gamma-globulins prepared from polyclonal antibodies against CSFV raised in specific pathogen free pigs. This ensures that no minor variant viruses will be missed, but has the disadvantage that the test will not distinguish between the antigens of different pestiviruses. Thus, pigs infected with other pestiviruses can yield a positive result. To differentiate CSFV from other pestiviruses, especially in CSFV-free areas, duplicate samples from FAT-positive samples should be examined using monoclonal antibodies (MAbs) that can distinguish between CSFV and other pestiviruses (especially BVDV and BDV). Alternatively, confirmatory diagnosis should await results of RT-PCR (followed by genetic typing) or virus isolation in cell culture with subsequent typing by MAbs.

Strains of modified live virus (MLV) vaccine multiply mainly in the regional lymph nodes and in the crypt epithelium of the tonsils. Pigs vaccinated with MLV strains may yield a positive FAT for 2 weeks after vaccination (Ogawa *et al.*, 1973; Terpstra, 1978). RT-PCR followed by nucleic acid sequencing of the RT-PCR amplicon allows differentiation between field isolates and vaccine strains of CSFV.

The working dilution of the conjugates (at least 1/30) should combine a maximum brilliance with a minimum of background. The test should only be performed on samples from freshly dead animals, as autolysis and bacterial contamination can often result in high background staining.

1.2.2. Immunoperoxidase procedure for differentiation of pestiviruses by monoclonal antibodies

The use of a panel of three MAbs that are conjugated to either horseradish peroxidase (HRPO) or a fluorescence marker, or used in conjunction with an anti-mouse conjugate and capable of specifically detecting all field strains of CSFV, vaccine strains of CSFV and other pestiviruses, respectively, would allow an unambiguous differentiation between field and vaccine strains of CSFV on the one hand, and between CSFV and other pestiviruses on the other (Edwards *et al.*, 1991; Wensvoort *et al.*, 1986; 1989b). A prerequisite is that the MAb against CSFV recognises all field strains and that the anti-vaccine MAb recognises all vaccine strains used in the country. *No single MAb selectively reacts with all other pestiviruses* (Edwards *et al.*, 1991). The use of a MAb to differentiate a CSF vaccine strain can be omitted in nonvaccination areas. A polyclonal anti-CSF immunoglobulin conjugated to HRPO serves as a positive control. Caution should be exercised when using evidence of a single MAb as sole confirmation of an isolate as CSF. Advice on suitable MAbs and their suppliers should be sought from the OIE Reference Laboratories for CSF.

Positive and negative control sections need to be included in each series of organ samples to be examined. In the case of indirect labelling, an infected control section, which is treated without incubation of the first antibody, should also be included. The control sections can be prepared in advance and stored after acetone fixation for 2–3 years at –70°C until use.

a) Test procedure

- i) Cut eight or more cryostat sections (4–8 µm) of the FAT-positive tonsil, or another positive organ if the tonsil is not available (as described above for the FAT method).
- ii) Place the sections on to cover-slips, allow to dry for 20 minutes at room temperature and fix for 10 minutes in acetone (analytical grade) at –20°C and allow to air dry.
- iii) Prepare working dilutions of the respective MAb-peroxidase conjugates in PBS + 0.01% Tween 80 + 5% horse serum, pH 7.6. (FITC–MAb can be used as well as unconjugated MAb provided that a secondary conjugate is used.)
- iv) Immerse the sections briefly in PBS, remove excess fluid with tissue paper and place them (cut off corner top right) on a frame in a humid incubation chamber.
- v) Overlay two sections each with the working dilution of the respective monoclonal conjugates, and two sections with the working dilution of the polyclonal conjugate (controls).
- vi) Incubate in a dark, closed chamber for 30 minutes at 37°C. Check afterwards that the solutions have not evaporated and that the tissues have not dried out.
- vii) Wash the sections six times for 10 seconds each in PBS at room temperature.
- viii) Stain the sections with freshly prepared chromogen–substrate solution* for 5–15 minutes at room temperature.
- ix) Rinse the sections in 0.05 M sodium acetate, pH 5.0, in distilled water and mount them on microscope slides.
- x) Examine sections with a light microscope. Dark red staining of the cytoplasm indicates recognition of the virus isolate by the respective conjugate, and is considered to be positive.

* Chromogen–substrate solution

- A. Stock solution of chromogen: 0.4% 3-amino-9-ethyl carbazole; N,N-dimethyl-formamide (1 ml).
Caution **TOXIC compound**. Both chemicals are carcinogens and irritants to eyes, skin and respiratory tract.
- B. 0.05 M sodium acetate, pH 5.0; 19 ml (sterile filtered through a membrane).
- C. Stock solution of substrate (30% hydrogen peroxide).

Keep stock solutions A and C at 4°C in the dark and solution B at room temperature. Stock solution A can be kept at 4°C for at least 6 months and solution C for 1 year. Immediately before use, dilute 1 ml of solution A in 19 ml of solution B. Then add 10 µl of stock solution C. Mix well and stain the sections.

xi) Interpretation of the test:

Polyclonal antibody	Monoclonal antibody specific for			Interpretation
	CSF strain	CSF vaccine strain	BVD/BD strain	
+	+	–	–	CSF field strain
+	+	+	–	CSF vaccine strain
+	–	–	+	BVD/BD strain
+	–	–	–	Other non-CSF <i>Pestivirus</i> [†]

[†]The existence of novel strains of CSF should always be considered and any isolate from cases where CSF is still suspected should be sent to an OIE Reference Laboratory.

1.2.3. Antigen-capture assay

For rapid diagnosis of CSF in live pigs, antigen-capture enzyme-linked immunosorbent assays (ELISAs) have been developed for screening herds suspected of having been recently infected. The ELISAs are of the double-antibody sandwich type, using monoclonal and/or polyclonal antibodies against a variety of viral proteins in either serum, the blood leukocyte fraction or anticoagulated whole blood; in addition, some test kits can be used to test clarified tissue homogenates (Depner *et al.*, 1995) or serum. The technique is relatively simple to perform, does not require tissue culture facilities, is suitable for automation and can provide results within half a day. The disadvantage of being less sensitive than virus isolation, especially in adult pigs and mild or subclinical cases, may be compensated by testing all pigs of the suspect herd showing pyrexia or clinical signs of disease. However, the lowered specificity of these tests should also be taken into consideration.

The test is not suitable for the diagnosis of CSF in a single animal, but should only be used at the herd level.

In any primary case, positive results must be confirmed using another test (i.e. virus isolation, RT-PCR or FAT).

2. Serological tests

Due to the immunosuppressive effect of CSFV, antibodies cannot be detected with certainty until at least 21 days post-infection. Serological investigations aimed at detecting residual foci of infection, especially in breeding herds, may be useful in a terminal phase of CSF eradication. Antibody titres provide valuable epidemiological information and may be of help in determining the entry route of the virus.

As the incidence of infection with ruminant pestiviruses may be high, particularly in breeding stock, only tests that will discriminate between CSF and BVD/BD antibodies are useful. Virus neutralisation (VN) and the ELISA using MAbs satisfy the requirements for sensitivity, but positive results should be confirmed by comparative VN testing.

Neutralisation tests are performed in cell cultures using a constant-virus/varying-serum method. As CSFV is noncytopathic, any non-neutralised virus must be detected, after multiplication, by an indicator system. The NPLA (Terpstra *et al.*, 1984) and the fluorescent antibody virus neutralisation (FAVN) test (Liess & Prager, 1976) are the most commonly used techniques. Both tests can be carried out in microtitre plates. The NPLA system is now favoured, being easier to read and having the advantage that the results can be determined by use of an inverted light microscope, though a crude assessment of titre can be made with the naked eye.

2.1. Neutralising peroxidase-linked assay

The NPLA is carried out in flat-bottomed microtitre plates. Sera can first be inactivated for 30 minutes at 56°C. For international trade purposes, it is best to test with an initial serum dilution of 1/5 (1/10 final dilution). For surveillance schemes within a country, a screening dilution of 1/10 (1/20 final dilution) may suffice. Appropriate controls to ensure specificity and sensitivity of reactions are incorporated into each test.

2.1.1. Test procedure

- i) Dispense dilutions of serum in growth medium (Eagle's MEM, 5% FBS and antibiotics) in 50 µl volumes into duplicate wells of a microtitre plate. The FBS must be free from both BVDV and antibodies to it. A third well should be included for each sample. This well

contains serum and not virus and is used as a serum control (for cytotoxicity and/or nonspecific staining).

- ii) Add 50 µl of virus suspension to the wells, diluted in growth medium to contain approximately 100 TCID₅₀ (50% tissue culture infective dose)/50 µl, and mix the contents on a microplate shaker for 20 seconds. A commonly used virus is CSF Alfort 187 (genotype 1.1). Although there is only one CSFV serotype, it is recommended that recent genotypes or field virus isolates circulating in the country or relevant other countries should also be used as antibody titres can vary depending on the virus genotype used in the assay.
- iii) Incubate the plates in a CO₂ incubator in a moist chamber for 1 hour at 37°C.
- iv) Add to all wells 50 µl of growth medium containing 2 × 10⁵ PK-15 cells/ml.
- v) Back titrate the virus and incubate together with the neutralisation plate.
- vi) Allow the cells to grow at 37°C in 5% CO₂ to become confluent, usually within 3–4 days.
- vii) Discard the growth medium and rinse the plates once in 0.15 M NaCl or PBS.
- viii) Drain the plates by blotting on a towel.
- ix) The cell monolayers may be fixed, and the virus inactivated, in one of several ways:
 - a) The plates are incubated for 45 minutes at 37°C, and then for at least a further 45 minutes at –20°C. The plates are removed from the freezer, the wells are filled with 100 µl 4% paraformaldehyde in PBS and reincubated for 5–10 minutes at room temperature. The paraformaldehyde is discarded and the plates are rinsed with 0.15 M NaCl;
or
 - b) The plates are incubated at 70–80°C for 2–3 hours;
or
 - c) The plates are fixed with 80% acetone and incubating at 70–80°C for 1 hour;
or
 - d) The plates are fixed in 20% acetone in PBS for 10 minutes followed by thorough drying at 25–30°C for 4 hours. (This can be done quickly with the aid of a hair-dryer: after 3–5 minutes complete dryness is obtained as observed by the whitish colour of the cell monolayer.)
or
 - e) The plates are washed with ice-cold 99.9% ethanol and fixed with 99.9% ethanol for 45 minutes at 4°C. (Staining should be done immediately.)
- x) Add to each well (of a 96-well plate) 50 µl of a hyperimmune porcine CSF antiserum or MAb, diluted in 0.5 M NaCl containing 1% Tween 80 + 0.1% sodium azide, pH 7.6. Incubate at 37°C for at least 15 minutes. The working dilution of the antiserum should be determined by prior titration: i.e. a serum with an NPLA titre of 1/30,000 could be used at 1/100.
- xi) Wash the plates five times in 0.15 M NaCl containing 1% Tween 80, pH 7.6 or PBS containing Tween and once in distilled water.
- xii) Add to each well 50 µl of an anti-porcine or anti-murine (as appropriate) IgG-HRPO conjugate, diluted to its working concentration in 0.5 M NaCl with 1% Tween 80, pH 7.6, and then incubate for at least 15 minutes at 37°C.
- xiii) Wash the plates five times in 0.15 M NaCl containing 1% Tween 80, pH 7.6.
- xiv) Add 50 µl of chromogen–substrate solution to each well and stain for 15–30 minutes at room temperature. This solution is described in Section B.1.2.2 Immunoperoxidase procedure for differentiation of pestiviruses by MAbs.
- xv) Discard the supernatant and wash once with 1/3 PBS/distilled water.
- xvi) The test is read visually. Infected cell sheets are completely or partially stained reddish brown in the cytoplasm. The monolayer should be examined by low-power microscopy to determine the end-point of the titration. The cytoplasm of infected cells is stained dark red. Neutralisation titres are expressed as the reciprocal of the highest serum dilution that

prevents virus growth in 50% of two replicate wells. The titre can be calculated according to the equation of Karber (1931)

- xvii) The following controls are included in the test: cell control, positive serum and back titration of test virus. The virus dilution added to the neutralisation plate undergoes a back-titration, which should cover a range of 4 log dilutions. The back-titration, which acts as an internal quality control, should confirm that virus has been used at a concentration of between 30 and 300 TCID₅₀/50 µl. A CSF antibody positive reference serum with known titre needs to be included. If the reference serum does not give the expected result and the back-titration is out of the limit, the test has to be repeated. Reference sera should be monitored over time using internal laboratory tracking charts.
- xviii) The back-titration titre is calculated using the method described by Reed & Muench.

NOTE: The incubation times given above are for guidance only. Longer incubation times, with reagent dilutions optimised to such times, may be used, to conserve reagents.

2.2. Fluorescent antibody virus neutralisation test

2.2.1. Leighton tube method:

- i) Seed a suspension of PK-15 cells at a concentration of 2×10^5 cells/ml into Leighton tubes with a cover-slip.
- ii) Incubate the cultures for 1–2 days at 37°C until they reach 70–80% confluency.
- iii) Inactivate the sera for 30 minutes at 56°C. For international trade purposes, it is best to test with an initial serum dilution of 1/5 (1/10 final dilution).
- iv) Incubate the diluted serum with an equal volume of a virus suspension that contains 200 TCID₅₀ of CSFV for 1–2 hours at 37°C; in this way, a constant amount of CSFV of 100 TCID₅₀ is used for each reaction well.
- v) Remove the cover-slips from the Leighton tubes, wash briefly in serum-free medium, overlay the cell sheet with the serum/virus mixture (from step iv) and incubate for 1 hour at 37°C in a humid atmosphere.
- vi) Place the cover-slip in a clean Leighton tube and incubate the cultures in maintenance medium for 2 more days.
- vii) Remove the cover-slips from the Leighton tubes, wash the monolayers twice for 5 minutes each in PBS, pH 7.2, fix in pure acetone for 10 minutes and stain with the working dilution of the conjugate for 30 minutes at 37°C before washing.
- viii) Mount the cover-slips on grease-free microscope slides with 90% carbonate/bicarbonate buffered glycerol, pH>8.0, and examine for fluorescence.

When the FAVN test is performed in microtitre plates, the procedure for the NPLA (see Section B.2.1.1) can be followed up to step ix. The plates are then stained with the working dilution of the conjugate for 30 minutes at 37°C and examined for fluorescence. NOTE: When detecting fluorescence, microplates are best examined from above, using a long focal-length objective and an inverted microscope.

Sera from pigs infected with BVDV or BDV may show cross-neutralising antibody titres that react in the FAVN or NPLA as if the pigs were infected with CSFV. The extent of cross-reactivity depends on the strain of ruminant pestivirus involved and the interval between infection and time of sampling (Wensvoort *et al.*, 1989a). In case of continued doubt, comparative tests using a strain of CSFV, a strain of BVDV and a strain of BDV, that are representative for the country or region, have proven useful. Comparative neutralisation tests are end-point titrations in which the same series of twofold dilutions of the suspected serum sample is tested in duplicate against 100 TCID₅₀ of each selected virus strain. The comparative tests are performed according to the protocols described for the FAVN or NPLA; the cell lines used must be suitable for BVDV and BDV. Neutralisation titres are expressed as the reciprocal of the highest serum dilution that prevents virus growth in 50% of two replicate wells. A three-fold difference or more between end-points of two titrations should be considered decisive for an infection by the virus species yielding the highest titre. It may be necessary to use different strains of the same genotype, and/or to test several pigs from an infected herd to obtain a definitive result.

2.3. Enzyme-linked immunosorbent assay

Competitive, blocking and indirect techniques may be used on any suitable support and a number have been described (e.g. Colijn *et al.*, 1997; Have, 1987; Leforban *et al.*, 1990; Moser *et al.*, 1996; Wensvoort *et al.*, 1988). The tests used should minimise cross-reactions with BVDV, BDV and other pestiviruses. However, the test system must ensure identification of all CSF infections, and at all stages of the immune response to infection. Most commercially available test systems are based on the immunodominant glycoprotein E2.

2.3.1. Antigen

The antigen should be derived from or correspond to viral proteins of one of the recommended CSFV strains. Cells used to prepare antigen must be free from any other *Pestivirus* infection.

2.3.2. Antisera

Polyclonal antisera for competitive or blocking assays should be raised in pigs or rabbits by infection with one of the recommended CSFV strains or with the lapinised C strain. MABs should be directed against or correspond to an immunodominant viral protein of CSFV. Indirect assays should use an anti-porcine immunoglobulin reagent that detects both IgG and IgM.

The sensitivity of the ELISA should be high enough to score positive any serum from convalescent animals, i.e. at least 21 days post-inoculation that reacts in the neutralisation test. The ELISA may only be used with serum or plasma samples derived from individual pigs. If the ELISA procedure used is not CSF-specific, then positive samples should be further examined by differential tests to distinguish between CSF and other pestiviruses.

The complex-trapping blocking ELISA (Colijn *et al.*, 1997) is a one-step method and is suitable for use in automated ELISA systems, e.g. robots. The sera are tested undiluted. The test is fast and easy to perform, and detects antibodies against low virulence strains of CSFV at an early stage after infection. As the MABs are specific for CSFV, the complex-trapping blocking ELISA will only rarely detect antibodies against BVDV, although BDV antibodies can be more problematic. Positive sera are retested for confirmation by the NPLA or FAVN.

The use of marker vaccines depends on a discriminatory test able to distinguish between vaccinated and naturally infected animals. In combination with the E2 subunit vaccine, ELISAs detecting antibodies directed against the E^{ms} protein can be used as discriminatory tests. However, commercially available E^{ms}-specific ELISAs are less sensitive and specific than conventional CSF E2 antibody ELISAs. It is recommended to use the discriminatory tests on a herd basis and not for diagnostic analysis on samples of single animals (European Commission, 2003; Floegel-Niesmann, 2001; Schroeder *et al.*, 2012).

More information on commercial kits for diagnosis can be obtained from the OIE Reference Laboratories. Even though commercial test kits may have been thoroughly validated before licensing, each lab must perform batch control with selected (positive and negative) reference sera prior to use.

C. REQUIREMENTS FOR VACCINES

1. Background

CSF has severe clinical and socio-economic consequences for pig production worldwide. The control of the disease is usually a national responsibility, and in many countries vaccination is carried out as part of a national control programme under the auspices of the veterinary 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 additional requirements relating to quality, safety and efficacy will apply in particular countries or regions for manufacturers to obtain an authorisation or licence for a veterinary vaccine.

Wherever CSFV is handled, the appropriate biosecurity procedures and practices should be used. The CSF vaccine production facility should meet the requirements for containment outlined in Chapter 1.1.4 *Biosafety and biosecurity: Standard for managing biological risk in the veterinary laboratory and animal facilities*.

Modified live vaccines (MLVs) based on several attenuated virus strains (e.g. C-strain, Thiverval, PAV-250, GPE-, K-strain) are most widely used, and many of them have proven to be both safe and efficacious. In addition, E2 subunit vaccines produced in baculovirus systems are available. Inactivated whole virus vaccines are presently not available.

Information regarding these vaccines can be found in review publications (Blome *et al.*, 2006; Ganges *et al.*, 2008; Geiser-Wilke & Moennig, 2004; Uttenthal *et al.*, 2010; Van Oirschot, 2003; Vannier *et al.*, 2007).

New generations of marker vaccines are also being developed and one is undergoing the licensing process (Reimann *et al.*, 2004).

Different strategies are available to differentiate infected from vaccinated animals (DIVA) by serological methods (e.g. ELISA) or genome detection methods (e.g. RT-PCR). A opinion published by the European Food Safety Authority (EFSA, 2008) demonstrated that the combination of a vaccine that uses the C-strain with RT-PCR to detect viral genome in slaughtered animals can be successfully used in a vaccination-to-live strategy (Li *et al.*, 2007; Zhao *et al.*, 2008).

CSF vaccines are used in different epidemiological settings and situations. Most countries free of the disease have adopted a control strategy without prophylactic vaccination but established legal provisions for emergency vaccination scenarios. In endemic situations, vaccination is mainly used to lower the impact of the disease or as a first step in an eradication programme. During epidemic incidents in otherwise free areas, emergency vaccination can be an additional tool to control and eradicate the disease.

Moreover, oral vaccination of affected wild boar populations may be considered. These different scenarios and the different systems of pig production may require different vaccine characteristics or may influence the focus of requirements.

Limited antigen and vaccine banks exist and can be used for emergency situations.

The optimal CSF vaccine should have the following general characteristics: short- and long-term safety for target and non-target species (especially for oral vaccines), stability, rapid induction of a stable, preferably life-long immunity, efficacy against all strains and types of field viruses, full clinical protection and protection against carrier states, prevention of horizontal and vertical transmission. Furthermore, marker vaccines will have to be accompanied by reliable discriminatory tests.

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

2.1. Characteristics of the seed

CSF vaccines prepared in live animals do not follow OIE animal welfare principles. Their production and use should be discontinued.

2.1.1. Biological characteristics of the master seed

MLVs are produced from CSFV strains that have been attenuated by passage either in cell cultures or in a suitable host species not belonging to the family *Suidae*. Production is carried out in cell cultures, based on a seed-lot system.

Master seed viruses (MSVs) for MLVs should be selected and produced, based on their ease of growth in cell culture, virus yield and stability.

The exact source of the underlying CSFV isolate, its sequence, and the passage history must be recorded.

2.1.2. Quality criteria

Only MSVs that have been established as sterile, pure (free of extraneous agents as described in Chapter 1.1.9 *Tests of biological materials for sterility and freedom from contamination* and those listed by the appropriate licensing authorities) and immunogenic, should be used for vaccine virus (working seed viruses and vaccine batches) production. Live vaccines must be shown not to cause disease or other adverse effects in target animals injected in accordance with chapter 1.1.8 (section on *Safety tests* [for live attenuated MSVs]).

Identity of the MSV has to be confirmed using appropriate methods (e.g. through the use of specific MABs or vaccine strain-specific genome detection methods).

2.1.3. Validation as vaccine strain

The vaccine derived from the MSV must be shown to be satisfactory with respect to safety and efficacy.

Even if pigs are not known for susceptibility to transmissible spongiform encephalopathy (TSE) agents, consideration should also be given to minimising the risk of transmission 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.

The vaccine virus in the final product should generally not differ by more than five passages from the master seed lot. The commercial vaccine should be produced in batches in lyophilised form as a homogeneous product.

2.2. Method of manufacture

2.2.1. Procedure

The virus is used to infect an established cell line. Such cell culture should be proven to be free from contaminating microorganisms and shall comply with the requirements in chapter 1.1.8.

Regardless of the production method, the substrate should be harvested under aseptic conditions and may be subjected to appropriate methods to release cell-associated virus (e.g. freeze–thaw cycles). The harvest can be further processed by filtration and other methods. A stabiliser may be added as appropriate. The vaccine is homogenised before lyophilisation to ensure a uniform batch/serial.

2.2.2. Requirements for ingredients

All ingredients used for vaccine production should be in line with requirements in chapter 1.1.8.

2.2.3. In-process controls

In-process controls will depend on the protocol of production: they include virus titration of bulk antigen and sterility tests.

2.2.4. Final product batch/serial test

i) Sterility

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

ii) Identity

Appropriate methods (specific antibodies or specific genome detection methods) should be used for confirmation of the identity of the vaccine virus.

iii) Residual moisture

The level of moisture contained in desiccated products should be measured as described in chapter 1.1.8.

iv) Safety

Batch safety testing is to be performed 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.

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

For batch/serial safety testing, use two healthy piglets, 6–10 weeks old, that do not have antibodies against pestiviruses. Administer to each piglet by a recommended route a tenfold dose of the vaccine. Observe the piglets daily for at least 14 days. The vaccine complies with the test if no piglet shows notable signs of disease or dies from causes attributable to the vaccine.

v) Batch/ serial potency

Virus titration is a reliable indicator of vaccine potency once a relationship has been established between the level of protection conferred by the vaccine in pigs and titre of the modified live vaccine *in vitro*.

In the absence of a demonstrated correlation between the virus titre and protection, an efficacy test will be necessary (see Section C.2.3.3).

2.3. Requirements for authorisation/registration/licensing

2.3.1. Manufacturing process

For registration of a vaccine, all relevant details concerning preparation of MSV, manufacture of the vaccine and quality control testing (see Sections C.2.1 and C.2.2) should be submitted to the authorities. This information shall be provided from three consecutive vaccine batches originating from the same MSV, with a volume not less than 1/3 of the typical industrial batch volume.

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

2.3.2. Safety requirements

For the purpose of gaining regulatory approval, the following safety tests should be performed satisfactorily.

Vaccines should be tested for any pathogenic effects on healthy pigs, and in sows to evaluate the safety in pregnant animals and their offspring.

i) Safety in young animals

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

Use no fewer than eight piglets of 6–8 weeks of age that do not have antibodies against pestiviruses. Administer to each piglet a quantity of the vaccine virus equivalent to not less than ten times the maximum virus titre likely to be contained in 1 dose of the vaccine. Observe the piglets daily for at least 14 days. The body temperature of each vaccinated piglet is measured on at least the 3 days preceding administration of the vaccine, at the time of administration, 4 hours after and then daily for at least 14 days. The vaccine complies with the test if the average body temperature increase for all piglets does not exceed 1.50°C, no piglet shows a temperature rise greater than 1.50°C for a period exceeding 3 consecutive days, and no piglet shows notable signs of disease or dies from causes attributable to the vaccine.

Blood samples are taken at 7 days after vaccination and tested for leukopenia. The average white blood cell (WBC) count should exceed 7×10^6 cells/ml.

In addition, the vaccines in their commercial presentation should be tested for safety in the field (see chapter 1.1.8, section on *Field tests [safety and efficacy]*).

ii) Safety test in pregnant sows and test for transplacental transmission

Carry out the test with vaccination by a recommended route using no fewer than eight healthy sows or gilts of the same age and origin, between the 55th and 70th days of gestation, that do not have antibodies against pestiviruses. Use vaccine virus at the least attenuated passage level that will be present in a batch of the vaccine.

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

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

Vaccine virus or antibodies against CSFV must not be present in blood samples from newborn piglets.

iii) Non-transmissibility

Keep together for the test no fewer than 12 healthy piglets, 6–10 weeks old and of the same origin, that do not have antibodies against pestiviruses. Use vaccine virus at the least attenuated passage level that will be present between the master seed lot and a batch of the vaccine. Administer by a recommended route to no fewer than six piglets a quantity of the vaccine virus equivalent to not less than the maximum virus titre likely to be contained in 1 dose of the vaccine.

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

After 45 days, kill all piglets humanely. Carry out appropriate tests on the piglets to detect antibodies against CSFV and on the control piglets to detect CSFV in the tonsils. The vaccine complies with the test if antibodies are found in all vaccinated piglets and if no antibodies and no virus are found in the control piglets.

iv) Reversion-to-virulence

The test for increase in virulence consists of the administration of the vaccine virus from the master seed lot or one or two passages above to piglets that do not have antibodies against pestiviruses.

This protocol is repeated five times. Administer to each of two healthy piglets free of antibodies to pestiviruses, 6–10 weeks old, by a recommended route, a quantity of the vaccine virus equivalent to not less than the maximum virus titre likely to be contained in 1 dose of the vaccine. Collect an appropriate quantity of blood from each piglet daily between day 2 and day 7 after administration of the vaccine virus, and pool the samples taken on the same day. Then kill the piglets and take the tonsils of both of them, pool the tonsils and prepare a 10% suspension in PBS, pH 7.2 kept at 4°C or at –70°C for longer storage. At the same time, the presence of CSF antigens is confirmed at each passage. Blood and pooled tonsillar tissue are used to inoculate two further pigs of the same age and origin by the same route as before.

Administer 2 ml of the pooled material (blood and tonsillar tissue) with the highest virus titre by a recommended route to each of two other piglets of the same age and origin. If no virus is found, repeat the administration once again with the same material and another two piglets. If no virus is found at this point, end the process here. If, however, virus is found, carry out a second series of passages by administering 2 ml of positive material by a recommended route to each of two other piglets of the same age and origin.

Carry out this passage operation no fewer than four times (in total five groups from the start of the test should be vaccinated), verifying the presence of the virus at each passage in blood and tonsils. Care must be taken to avoid contamination by virus from previous passages.

The vaccine virus complies with the test if no indication of increasing virulence (monitored by clinical observations) of the maximally passaged virus compared with the unpassaged virus is observed.

If virus is not recovered at any passage level in the first and second series of passages, the vaccine virus also complies with the test.

2.3.3. Efficacy requirements

i) Protective dose

Vaccine efficacy is estimated in vaccinated animals directly, by evaluating their resistance to live virus challenge and is expressed by the number of 50% protective doses (PD₅₀) for pigs contained in the vaccine dose.

The test consists of a vaccination/challenge trial in piglets aged 6–10 weeks using different dilutions of the vaccine in question and five piglets per dilution. An additional group of two piglets of the same age and origin are used as controls. All animals have to be free from

antibodies against pestiviruses prior to the trial. Each group of piglets, except the control group, is vaccinated with an appropriate dilution of the reconstituted vaccine (e.g. 1/40 and 1/160 using a suitable buffer solution).

Fourteen days after the single injection of vaccine, challenge the piglets by a suitable route with a dose of a virulent strain of CSFV that kills at least 50% of the non-vaccinated piglets in less than 21 days. Observe the piglets for 21 days and record the body temperature 3 days before challenge and daily after challenge for 21 days. The PD_{50} content of the vaccine is calculated from the number of animals protected in each group using the Spearman-Kärber method.

The test is invalid if less than 50% of the control piglets display typical signs of serious infection with CSFV, and die, and if less than 100% of the control piglets show clinical signs of disease within the 21 days following challenge.

The vaccine complies with the test if the minimum dose corresponds to not less than 100 PD_{50} .

ii) Protection against transplacental infection

Use eight sows that do not have antibodies against pestiviruses, randomly allocated to either the vaccine group ($n = 6$) or the control group ($n = 2$).

Between the 34th and 49th day of gestation, all sows allocated to the vaccine group are vaccinated once with 1 dose of vaccine containing not more than the minimum titre stated on the label. Three weeks after vaccination, all eight sows are challenged by a suitable route with a dose of virulent strain of CSFV that would be sufficient to kill at least 50% of non-vaccinated piglets in less than 21 days.

Just before farrowing, the sows are killed humanely and their fetuses are examined for CSFV. Serum samples from sows and fetuses are tested for the presence of antibodies against CSFV. Isolation of CSFV is carried out from blood of the sows (collected 7 and 9 days after challenge and at euthanasia), and from homogenised organ material (tonsils, spleen, kidneys, lymph nodes) of the fetuses.

The test is valid if virus is found in at least 50% of the fetuses from the control sows (excluding mummified fetuses).

The vaccine complies with the test if no virus is found in the blood of vaccinated sows and in fetuses from the vaccinated sows, and antibodies against CSFV should not be found in the serum of the fetuses from the vaccinated sows

In addition, where appropriate, the vaccines should be tested for efficacy in the field (see chapter 1.1.8, section on *Field tests [safety and efficacy]*).

2.3.4. Duration of immunity

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

At least ten vaccinated pigs are each inoculated with an amount of virus corresponding to 10^5 PID_{50} (median pig infectious dose) of a virulent strain of CSFV and observed for 3 weeks. The vaccinated animals have to remain healthy, only the controls should die.

The duration of immunity after vaccination against CSF shall not be less than 6 months.

2.3.5. Stability

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

The period of validity of lyophilised CSF vaccine should be shown to be at least 1 year.

3. Requirements for other vaccines

3.1. Oral vaccine

3.1.1. Background

The most widely applied concept of oral bait vaccination of wild boar against CSF, including bait design and immunisation scheme was developed, evaluated, and optimised by Kaden *et al.* (2010). The respective vaccines are conventional MLVs. Immunisation occurs by uptake of the oral vaccine through the lymphoid tissues of the oral mucosa and tonsils, where expression of virus stimulates the immune system (Kaden *et al.*, 2000; 2002; 2003; 2004; Kaden & Lange, 2004; Rossi *et al.*, 2010).

Safety is of paramount consideration for oral vaccine use, not only for the target animals, but for the environment (see chapter 1.1.8) and other species that may come in contact with the vaccine.

3.1.2. Outline of production and minimum requirements for vaccines

In addition to the outline of production described for injectable vaccines above, the following specific requirements must be met:

i) Method of manufacture

a) Final product batch/serial test

After combining all of the ingredients, the final blend contains the definitive formulation that is usually used in liquid form. The last step in production of a batch/serial is filling the final blend into blisters/capsules to be included in baits or filling directly into the bait. This final batch/serial is tested as described for the injectable vaccines, with the following differences:

- Residual moisture test

The residual moisture test does not apply if the oral vaccine is presented in liquid form.

- Safety

Administer orally by syringe to each piglet a volume corresponding to ten oral doses as indicated by the manufacturer.

ii) Requirements for authorisation/registration/licensing

In addition to the requirements described for injectable vaccines, the following specific requirements must be met.

a) The bait

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

- Designed for and attractive to the target species and adapted to the mode of distribution;
- Keep its form and shape under a wide range of temperature and weather conditions;
- Ingredients are non-harmful, comply with animal feed standards and should not interfere with vaccine activity;
- Feature a labelling system with a public warning and identification of the product.

b) Safety requirements

For all the tests the liquid vaccine is administered orally with a syringe (not in the final bait formulation) to ensure that each animal receives the full dose.

- Precaution hazards

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

- c) Efficacy requirements

Efficacy should be proven using the liquid vaccine administered by syringe to ensure that each animal receives the full dose. Proof-of-concept studies for the final formulation (vaccine integrated into bait) should be provided.

3.2. Biotechnology-based vaccines

3.2.1. Background

As described in Guideline 3.3 Section E *Subunit vaccines*, conventional, live attenuated CSF vaccines have a rapid onset of immunity and are effective at preventing transmission of infection (Van Oirschot, 2003), but have the disadvantage that it is not possible using serological methods (e.g. ELISA) to differentiate infected pigs from those that have merely been vaccinated. Commercial E2 subunit vaccines (Marker vaccine) have a slower onset of immunity and reduce, but may not completely prevent, viral shedding and transplacental infection. However, these vaccines enable a DIVA strategy to be followed thereby facilitating a 'vaccination to live' strategy.

The vaccine only elicits antibodies against the E2 glycoprotein and therefore antibodies against other CSFV antigens, such as the E^{RNS} antigen, can be used as markers of infection.

3.2.2. Outline of production

- i) Characteristics of the seed

E2 subunit marker vaccine is prepared by the use of *Baculovirus* expressing the E2 antigen of CSFV. The vaccine therefore does not contain any CSFV while the baculo (vector) virus is chemically inactivated.

- a) Biological characteristic of the master seed

Production is carried out in insect cell cultures, based on a seed-lot system.

Selection of MSVs should ideally be based on their ease of growth in cell culture, virus yield and stability.

The exact source of the isolate including its sequence and passage history should be recorded.

- b) Quality criteria

Only MSVs that have been established as sterile and pure (free of extraneous agents as described in chapter 1.1.9 and those listed by the appropriate licensing authorities), and immunogenic, shall be used for preparing the vaccine virus production.

Appropriate methods (specific antibodies or specific genome detection methods) should be used for confirmation of the identity of the MSV.

- c) Validation as vaccine strain

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

In accordance with chapter 1.1.8, consideration should also be given to minimising the risk of transmission of TSE agents 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.

The vaccine virus used to produce the final product should not differ by more than five passages from the material used for validating the seed lot. The commercial vaccine is inactivated for residual baculovirus and adjuvanted.

ii) Method of manufacture

a) Procedure

The baculovirus is used to infect an established insect cell line. Such cell culture should be proven to be free from contaminating microorganisms and shall comply with requirements in chapter 1.1.8.

Regardless of the production method, the substrate should be harvested under aseptic conditions and may be subjected to appropriate methods to release cell-associated virus. The harvest can be further processed by filtration and other methods. Inactivation of residual baculovirus is performed, preferably using a first order inactivant. The antigen is homogenised before formulation with adjuvant.

b) Requirements for ingredients

All ingredients used for vaccine production should be in line with requirements in chapter 1.1.8.

c) In-process controls

Infectivity, sterility and antigenic mass are monitored. After inactivation a test for innocuity is carried out on every batch of antigen. The cells used to test for absence of residual live baculovirus are the same cell line used for production or potentially equally or more sensitive cells.

d) Final product batch/serial test

- Sterility

Must comply with chapter 1.1.8.

- Identity

The identity test is performed by a specific MAb-based virus neutralisation against CSFV or an appropriate molecular identification. Sera prepared to be used for identity testing should not be prepared using the homologous vaccine virus or baculovirus expressed subunit antigen but from another source. This test may be combined with the potency test (see below).

- Safety and prove of marker concept

Batch safety testing is to be performed 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.

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

For batch/serial safety testing, use two healthy piglets, 6–10 weeks old, that do not have antibodies against pestiviruses. Administer to each piglet by a recommended route a double dose of the formulated vaccine. Observe the piglets daily for at least 14 days for local and systems reactions to vaccination. After 14 days they are each injected with a second single dose of vaccine.

Any adverse reaction attributable to the vaccine should be assessed and may prevent acceptance of the batch. The vaccine should elicit antibodies against CSFV E2 but not against CSFV-E^{RNS} antigen.

- Batch/serial potency

Induction of specific anti-E2 antibodies in vaccinated pigs can be used to confirm the potency of each batch once the titre has been correlated with the results of the efficacy test.

iii) Requirements for authorisation /registration/ licensing

a) Manufacturing process

See Section C.2.3.1.

b) Identity

The identity test is performed by virus neutralisation using immune sera against CSFV. Sera prepared to be used for identity testing should not be prepared using the homologous vaccine virus or baculovirus expressed subunit antigen but from another source.

c) Safety requirements

- Safety in young animals

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 eight piglets of 6–8 weeks of age. Single-dose and repeat-dose tests using vaccines formulated to contain the maximum permitted payload should be conducted. The repeat dose test should correspond to the primary vaccination schedule (e.g. two injections) plus the first revaccination (i.e. a total of three injections). The animals are observed for local and systemic reaction 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. It has to be proven that the vaccine does not elicit antibodies against CSFV-ERNS antigen.

- Safety in pregnant sows

For the purpose of gaining regulatory approval a trial batch of vaccine should be tested for local and systemic toxicity by each recommended route of administration corresponding to the primary vaccination schedule (e.g. two injections) in eight pregnant sows. The sows are observed for local and systemic reactions to vaccination. The observation period must last until parturition to examine any harmful effects during gestation or on progeny. Any undue reaction attributable to the vaccine should be assessed and may prevent acceptance of the vaccine. It has to be proven that the vaccine does not elicit antibodies against CSFV-ERNS antigen.

d) Efficacy requirements

- Protective dose

Vaccine efficacy is estimated in animals vaccinated according to the manufacturer's recommendation, following the methods described in Section C.2.3.3.

- Protection against transplacental infection

The trial vaccine should comply with the test described in Section C.2.3.3.

e) Duration of immunity

As part of the authorisation procedure the manufacturer should demonstrate the duration of immunity (see Section C.2.3.4).

f) Stability

The stability of all vaccines should be demonstrated as part of the shelf-life determination studies for authorisation. The period of validity of a batch of biotechnology-based CSF vaccine should be shown to be at least 1 year (see Section C.2.3.5).

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