

CLASSICAL SWINE FEVER (hog cholera)

SUMMARY

Classical swine fever (CSF), also known as hog cholera, is a contagious viral disease of pigs. 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 has serious consequences for trade in pigs and pig products.

The highly variable clinical picture of CSF often 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 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 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 monoclonal antibodies (MAbs) is used to determine whether the fluorescence is due to CSF or non-CSF *Pestivirus* antigens. For the detection of CSF genome, polymerase chain reaction (PCR) is commonly used. The isolation of CSFV should be attempted in the pig kidney (PK-15) cell line, or other suitable cell lines. The cultures are examined for virus growth by immunofluorescence or immunoperoxidase staining; positive isolates are further characterised by the use of MAbs and by partial genetic sequencing. Polymerase chain reaction protocols for the identification of CSFV nucleic acid have now gained international acceptance and are being used in several laboratories, both for detection of the agent and differentiation from ruminant pestiviruses. Antigen-capture enzyme-linked immunosorbent assays (ELISAs) are also useful for herd screening, but should not be used on a single animal basis.

Serological tests: Detection of virus-specific antibodies is particularly useful in herds suspected of being 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 ruminant *Pestivirus* are occasionally observed in breeding pigs, screening tests have to be followed by confirmatory tests that are CSFV-specific. Certain ELISAs are relatively CSFV-specific, but the definitive method for differentiation is the comparative neutralisation test, which compares the level of antibodies to different *Pestivirus* species.

Requirements for vaccines and diagnostic biologicals: 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 Containment Group 4 pathogens.

Effective inactivated, conventional whole virus vaccines are not available. Subunit 'marker vaccines' are now available, which, in contrast to MLV vaccines, induce antibodies that can be distinguished from antibodies induced by field virus using an accompanying diagnostic test. The presently registered 'marker vaccines' are based on the major envelope glycoprotein (E2-subunit) of CSFV, and are 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, both antigenically and structurally. Clinical signs and lesions seen at post-mortem in pigs affected with CSF are highly variable due to both viral and host factors. Furthermore, congenital infections with ruminant pestiviruses in pigs can give rise to a clinical disease that is indistinguishable from CSF (31, 33, 35).

Spread of disease in all age groups, accompanied by pyrexia, huddling, inappetance, dullness, weakness, conjunctivitis, constipation followed by diarrhoea, and an unsteady gait are the prevailing signs. Several days after the onset of clinical signs, the ears, abdomen and inner thighs may show a purple discoloration. Animals with acute disease die within 1–3 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 2–4 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, persistent infections always lead to the death of the animal. Herd mortality rates may be slightly above the expected level. 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.

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 recently been published (3), 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.

B. DIAGNOSTIC TECHNIQUES

The variability of the clinical signs and post-mortem lesions do 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 salmonellosis (especially due to *Salmonella choleraesuis*), erysipelas, pasteurellosis, actinobacillosis (due to *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 a viraemia (7), 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 a few 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. 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. Random sampling is unsuitable for CSF diagnosis. Additionally, whole blood samples for virus detection and reverse-transcription polymerase chain reaction (RT-PCR) analyses can be collected from a larger group of pigs.

CSF is subject to official control and the virus has a high risk of spread from the laboratory: consequently, a risk analysis should be carried out to determine the level of biosecurity needed for the diagnosis and characterisation of the virus. The facility should meet the requirements for the appropriate Containment Group as determined by the risk assessment and 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 Reference Laboratory.

Antibodies develop in the third week of illness and persist in the surviving animal for life. Samples for antibody detection are collected in ordinary (nonheparinised) tubes from convalescent pigs and from contact herds when \geq more than 3 weeks have elapsed since the suspected contact with a confirmed outbreak took place.

1. Identification of the agent

a) Immunological methods

o 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 (4) and transported without preservatives under cool conditions, but not frozen. Cryostat sections are stained directly with anti-CSF immunoglobulin conjugated to fluorescein isothiocyanate (FITC) or indirectly using a secondary FITC 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 (25). 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 virus isolation in cell culture (e.g. pig kidney [PK-15]) or another cell line of pig origin that is as sensitive and known to be free from *Pestivirus* contamination.

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.

o Test procedure

Include positive and negative control sections in each series of organ samples to be examined.

- 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.
- iii) Cut sections not more than 4–8 μ m thick and mount these on to 10 × 32 mm grease-free cover-slips with one corner cut-off. All sections are mounted with this corner in the same position (e.g. top right).
- iv) After drying, fix the mounted sections for 10 minutes at room temperature in acetone (analytical grade) or air-dry for 20 minutes at 37°C.
- v) 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 an incubation chamber humidified with a small volume of water placed in the bottom of the chamber.
- vi) Dispense the anti-CSF immunoglobulin at working dilution on to the entire section and incubate in the closed chamber for 30 minutes at 37°C. 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.
- vii) Wash the sections five times for 2 minutes each in PBS at room temperature.

- viii) Remove the remaining PBS 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.
- ix) Remove excess mounting fluid with tissue paper and examine the sections for fluorescence using a UV microscope. A CSF-positive section shows brilliant green fluorescing 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).

The FAT involves the use of an anti-CSF immunoglobulin prepared from a polyclonal antibody to CSFV that will not distinguish between the antigens of different pestiviruses. Conjugates used for the FAT on cryostat sections or inoculated cell cultures should be prepared from anti-CSFV gamma-globulins raised in specific pathogen free pigs. 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.

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 (22, 28). Rabbit inoculation is used to differentiate between lapinised and field strains of CSFV. In contrast to field strains, lapinised strains given intravenously cause a febrile reaction and induce an immune response in rabbits. As nucleic acid sequencing has become available and more reliable, animal inoculation is no longer necessary to differentiate between field strains and vaccine strains of CSFV.

Pigs infected with ruminant pestiviruses can give false-positive FAT reactions. Congenital infections with ruminant pestiviruses can cause clinical signs and pathological lesions indistinguishable from those in chronic CSF (31, 33, 35). Infections by CSFV or ruminant pestiviruses can be differentiated by testing sera from the dam and litter mates, or from other contacts of an FAT-positive piglet, for neutralising antibodies to each virus. If the virus was isolated, or viral nucleic acid can be detected, using RT-PCR, subsequent sequencing provides a rapid and accurate tool to distinguish ruminant pestiviruses from CSFV. Another method of differentiating these viruses is by the inoculation of seronegative piglets with a suspension of suspect material, followed at least 4 weeks by virus neutralisation (VN) tests on their sera for the respective antibodies. However, VN tests may take several days, and animal inoculation methods take several weeks.

- o **Immunoperoxidase procedure for differentiation of pestiviruses by monoclonal antibodies**

The use of a panel of three monoclonal antibodies (MAbs), either horseradish peroxidase (HRPO) or FITC-conjugated, or used in conjunction with an anti-mouse conjugate and specifically detecting all field strains of CSFV, vaccine strains of CSFV and ruminant 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 (11, 36, 38). 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 ruminant pestiviruses* (11). The use of an 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.

- o **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.
- ii) Fix the sections on to flying cover-slips for 10 minutes in acetone (analytical grade) 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 also be used, as well as unconjugated MAb provided that a secondary conjugate is used.)
- iv) After rinsing with PBS, overlay two sections with the working dilution of the respective monoclonal conjugates, and two sections with the working dilution of the polyclonal conjugate (controls).
- v) Incubate for 1 hour at 37°C in a humid chamber.
- vi) Wash the sections six times for 10 seconds each in PBS.

- vii) Stain the sections with freshly prepared chromogen–substrate solution* for 5–15 minutes at room temperature.
- viii) Rinse the sections in 0.05 M sodium acetate, pH 5.0, in distilled water and mount them on microscope slides.
- ix) Examine sections with a light microscope. Dark red staining of the cytoplasm of the epithelial cells lining the tonsillar crypts indicates recognition of the virus isolate by the respective conjugate, and is considered to be positive.
- x) 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.

o 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 (8). 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. 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.

b) 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. Isolation is best performed in rapidly dividing PK-15 cells seeded on to cover-slips 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. The cultures are examined for fluorescent foci by FAT after 24–72 hours or after 4–5 days incubation are fixed for immunoperoxidase staining.

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

A detailed procedure for virus isolation is as follows:

- 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.

* 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.

- ii) Cut 1–2 g of tissue 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 at 4°C.
- 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 up to 1 hour.
- iv) Centrifuge at 1000 **g** for 15 minutes.
- v) A PK-15 monolayer is trypsinised, the cell suspension is centrifuged at 160 **g** for 10 minutes, and resuspended to contain approximately 2×10^6 cells/ml in growth medium (Eagle's MEM with Earle's salts; 5% fetal bovine serum free from 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.

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. 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. Incubate at 37°C for a minimum of 4 hours and a maximum of 36 hours. Then drain the medium and inoculate 0.2 ml of supernatant fluid (from step iv), incubate for 1 hour at 37°C, rinse and 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, fixed with cold acetone (analytical grade) for 10 minutes, and stained with a direct anti-CSFV conjugate at its appropriate working dilution or indirectly, as described in Section B.1.a.

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 such.

- viii) 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).

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 sensitivity and simplicity whole blood is preferred (10). The procedure is as follows:

- i) Freeze a sample of whole blood at –20°C and thaw in a waterbath at 37°C.
- ii) Inoculate 300 µl haemolysed blood on to a PK-15 monolayer grown to approximately 75% confluence* in an M24-plate, and allow adsorption for 1 hour at 37°C.
- ii) Remove inoculum, wash the monolayer once with Hanks' BSS or Hanks' MEM, and add growth medium.
- iv) After a further incubation period of 3–4 days, 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.

o **Reverse-transcription polymerase chain reaction**

Many methods for RT-PCR have been described and are still being developed. (20). This internationally accepted method is rapid and more sensitive than antigen-capture ELISAs or virus isolation making it

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

particularly suited to preclinical diagnosis. Several conventional and real-time PCR protocols have been described (14, 20, 24, 26, 27) and a suitable protocol may be obtained from the literature or from the OIE Reference Laboratories for CSF (see Table given in Part 3 of this *Terrestrial Manual*). Due to its speed and sensitivity, RT-PCR is a suitable approach to screening suspect cases of disease and is now accepted by a number of countries and the European Union (EU) (1). However, it has to be kept 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. Any positive results from primary outbreaks should 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 recommended that internal controls be included. See Chapter 1.1.5 Validation and quality control of polymerase chain reaction methods used for the diagnosis of infectious diseases, for further details on PCR techniques. The test can be applied to individual or pooled blood samples as well as solid organs and has been used successfully to control outbreaks.

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 (23). 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'-noncoding region (5'NCR) of the genome (150 nucleotides) and the other lies within the E2 major glycoprotein gene (190 nucleotides). In brief, the method used is to extract virus RNA from infected PK-15 cell cultures, perform RT-PCR to amplify one or both targets within the 5'NCR or the E2 gene, and then determine the nucleotide sequence of the products and compare with stored sequence information held in the databases. A database of these sequences is available from the OIE Reference Laboratory for CSF (Hanover, Germany). Recent findings on analysing ruminant pestivirus sequences highlight the need for analysis of multiple regions in order to accurately type strains by this method (15). CSFV isolates from primary outbreaks should be sent to an OIE Reference Laboratory for investigation of molecular epidemiology. An import permit should be obtained prior to dispatch.

2. Serological tests

Detection of virus-specific antibodies is particularly useful on premises suspected of having infections with CSF strains of low virulence. Due to the immunosuppressive effect of CSFV, antibodies cannot be detected with certainty until 21 days post-infection. Serological investigations aimed at detecting residual foci of infection, especially in breeding herds, may also be useful in a terminal phase of CSF eradication.

As the incidence of infection with ruminant pestiviruses may be high in breeding stock, only tests that will discriminate between CSF and BVD/BD antibodies are useful. 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 (29) and the fluorescent antibody virus neutralisation (FAVN) test (18) 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.

a) Neutralising peroxidase-linked assay (a prescribed test for international trade)

The NPLA is carried out in flat-bottomed microtitre plates. Sera are first 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 may suffice. Appropriate controls to ensure specificity and sensitivity of reactions are incorporated into each test.

o Test procedure

- i) Dispense dilutions of serum in growth medium (Eagle's MEM, 5% fetal bovine serum and antibiotics) in 50 µl volumes into duplicate wells of a microtitre plate. The fetal bovine serum must be free from both BVDV and antibodies to it. A third well may 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 µl, and mix the contents on a microplate shaker for 20 seconds.
- iii) Incubate the plates in a CO₂ incubator for 1 hour at 37°C.
- iv) Add to all wells 50 µl of growth medium containing 2 × 10⁵ cells/ml.
- v) Allow the cells to grow at 37°C in 5% CO₂ to become confluent, usually within 3–4 days.

- vi) Discard the growth medium and rinse the plates once in 0.15 M NaCl.
- vii) Drain the plates by blotting on a towel.
- viii) The cell monolayers may be fixed in one of several ways:
 - 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
 - The plates are incubated at 70–80°C for 1–2 hours; or
 - The plates are fixed with 80% acetone and incubating at 70–80°C for 1 hour; or
 - 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.)
- ix) Add to each well 50 µl of a hyperimmune porcine CSF antiserum or monoclonal antibody, diluted in 0.5 M NaCl containing 1% Tween 80 + 0.1% sodium azide, pH 7.6. Incubate at 37°C for 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.
- x) Wash the plates five times in 0.15 M NaCl containing 1% Tween 80, pH 7.6.
- xi) 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 10 minutes at 37°C.
- xii) Wash the plates five times in 0.15 M NaCl containing 1% Tween 80, pH 7.6.
- xiii) 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.a 'Immunoperoxidase procedure for differentiation of pestiviruses by monoclonal antibodies'.
- xiv) The test is read visually. Infected cell sheets are completely or partially stained reddish brown. 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.
- xv) The following controls are included in the test: cell control, positive serum and back titration of test virus. The back-titration should confirm that virus has been used at a concentration of between 30 and 300 TCID₅₀/50 µl.

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

b) Fluorescent antibody virus neutralisation test (a prescribed test for international trade)

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 equal volumes of diluted serum and virus suspension containing 200 TCID₅₀ (50% tissue culture infective dose) per 0.1 ml for 1–2 hours at 37°C. Thus 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 below) can be followed up to step viii. 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.

Occasionally, sera from pigs infected with BVDV or BDV react in the FAVN or NPLA at low dilution as if they 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 (37). The usually high antibody levels reached after exposure to CSF infection, including strains of low virulence, allow the use of comparatively high initial dilutions in NPLA tests for CSF antibody, thus avoiding most, but not all, cross-reactions (29, 30). 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 proved 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.

c) Enzyme-linked immunosorbent assay (a prescribed test for international trade)

Competitive, blocking and indirect techniques may be used on any suitable support and a number have been described (e.g. 5, 13, 17, 21, 34). The tests used should minimise cross-reactions with BVDV and other pestiviruses. However, the test system must ensure identification of all CSF infections, and at all stages of the immune response to infection.

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.

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 (5) 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 BD antibodies can be more problematic. Positive sera are retested for confirmation by the NPLA.

Recently, a novel ELISA has been described that uses fused protein derived from viral peptides (19). The test claims to provide greater sensitivity and earlier detection of antibody than is obtained by conventional ELISAs, but, at this time, its reactivity with antibody induced by diverse strains of CSF is not known.

More information on commercial kits for diagnosis can be obtained from the OIE Reference Laboratories.

C. REQUIREMENTS FOR VACCINES AND DIAGNOSTIC BIOLOGICALS

Effective inactivated whole virus vaccines against CSF are not available.

C1. Modified live virus vaccines

MLV vaccines 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, or in non-*Suidae*, based on a seed-lot system. This must be validated with respect to identity, sterility, purity, safety, nontransmissibility, stability and immunogenicity.

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.

The vaccine production facility should operate under the appropriate biosecurity procedures and practices. If CSFV is used for vaccine production or for vaccine–challenge studies, that part of the facility where this work is done should meet the requirements for Containment Group 4 pathogens as outlined in Chapter 1.1.2.

To produce a seed lot and a final vaccine of high quality, the optimal conditions for virus yield must be determined. For vaccines produced in cell cultures, growth curve experiments must be done to study the effect of composition of the medium, regulation of pH and atmospheric CO₂ content, starting concentration of seeded cells, ratio between cell sheet surface and medium volume, phase of the cell growth at the time of viral infection, stationary or rolling conditions during viral replication, etc. For vaccines produced in animals, their age, breed, weight, the size of inoculum (number of animal ID₅₀ [50% infectious dose]), pathogenesis of the infection, and the clinical signs are factors that must be investigated to determine the peak of virus growth and the tissues to be harvested.

Regardless of the production method, the substrate should be harvested under aseptic conditions and be subjected to a freeze–thaw cycle to release cell-associated virus. Coarse cell or tissue elements are removed by filtration or low-speed centrifugation. A stabiliser is added, such as lactose at a final concentration of 5%. The vaccine is homogenised before lyophilisation to ensure a uniform batch.

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

1. Seed management

a) Characteristics of the seed

To validate a seed lot for an MLV CSF vaccine, samples of the seed lot must first pass pilot experiments. Except for tests to confirm identity, sterility, purity and stability of attenuation, pilot experiments may also be performed with representative samples of the final commercial product. These samples must originate from the same seed lot as tested above.

Except where otherwise specified, all pigs used in pilot tests are 6–8 weeks of age, healthy, free from antibodies against CSFV and BVDV, of the same breed and origin, grouped at random if necessary, and kept under the same conditions. Pregnant sows must be of equal parity.

The seed virus must be sterile and induce specific neutralising antibodies against a virulent strain of CSFV in pigs.

b) Method of culture

Production is performed in cell cultures or in a suitable host of a species not of the family *Suidae*.

c) Validation as a vaccine

i) Purity

Vaccine must be virologically pure.

Each of three seronegative pigs is inoculated intramuscularly with an amount of seed-lot virus equivalent to tenfold the amount of virus contained in one dose of vaccine. This is repeated 3 weeks later using the same dose and route of administration. Serum samples are collected 2 weeks after the last inoculation, and tested by the most sensitive method for freedom from antibodies to the viruses of African swine fever, Aujeszky's disease, BVD, foot and mouth disease (all types), transmissible gastroenteritis, swine vesicular disease, porcine reproductive and respiratory syndrome, and porcine influenza (types H1N1 and H3N2); and for porcine adenoviruses, porcine teschoviruses (types 1 and 2), porcine parvovirus and porcine circoviruses (types 1 and 2).

ii) Safety

Vaccines should be tested for any pathogenic effects on healthy pigs, and also on pigs that might be immunosuppressed due to the presence of concurrent infection or medication, as well as testing to ensure that the vaccine does not cross the placenta of pregnant sows.

For tests of safety in conventional pigs, each of ten seronegative pigs is inoculated intramuscularly with ten vaccine doses. Ten other pigs serve as controls. All pigs are observed for 3 weeks thereafter. Body temperatures are recorded and blood samples are collected daily, with an anticoagulant, for the first week. Body weights are recorded at inoculation and 2 weeks later. No animal should die or show signs of illness caused by the vaccine (seed-lot) virus. A daily group average body temperature should not reach 40.5°C or more throughout the trial period. The average weight gain should not fall significantly ($p < 0.05$) below that of the controls. Leukopenia (white blood cell [WBC] count $< 7 \times 10^6$ cells/ml) may be disregarded if it is only in one pig for 1 day.

To ensure attenuation, even in immunosuppressed pigs, each of ten pigs is immunosuppressed by daily injections, each of 2 mg prednisolone/kg body weight, for 5 consecutive days. On day 3, each animal is inoculated with the equivalent of one dose of vaccine, and kept under observation for 3 weeks thereafter. No animal should die or become ill due to the vaccine virus.

To ensure safety in pregnant animals, each of ten sows, 25–35 days pregnant, is inoculated intramuscularly with the equivalent of one dose of vaccine. A further ten animals of the same parity and gestation serve as controls. The vaccination should not interfere with normal gestation to term, and the number of live piglets born from the test group should not be significantly fewer ($p < 0.05$) than that for the control pigs.

For field trials, a minimum of 200 pigs is used, farrowed and reared by at least 20 dams, and seronegative for CSF and BVD. The litters are equally distributed over at least two farms. Half of the piglets in each litter are inoculated intramuscularly at 7–14 days of age with the equivalent of one dose of vaccine. The uninoculated littermates are controls. All piglets are weighed at inoculation and 2 weeks later, and are kept under observation for 3 weeks. A mortality rate that exceeds 5% due to causes other than vaccination invalidates the trial. No animal should die or show signs of disease due to the vaccine virus. The average weight gain of the inoculated pigs in the litters should not be more than 20% below that of the controls during the 2 weeks post-inoculation.

iii) *Nontransmissibility*

To confirm nontransmissibility, 24 seronegative pigs are divided into four groups of equal size. Five pigs in each group are inoculated intramuscularly with the equivalent of one dose of vaccine. The remaining pigs represent in-contact controls. All pigs are challenged 6 weeks later with at least 10^5 PID₅₀ (50% pig infectious dose) of a virulent strain of CSFV. All in-contact animals should be serologically negative at the time of challenge, and then die within 3 weeks. All vaccinated pigs should remain healthy and survive.

iv) *Stability of attenuation*

To confirm stability of virus attenuation, two pigs are each inoculated intramuscularly with an amount of seed-lot virus equivalent to 100 doses of vaccine, and then killed 6–7 days later. The tonsils of both pigs are pooled and made into a 10% suspension in PBS, pH 7.2. This is used to inoculate two further pigs intramuscularly with 2 ml, and these are then killed 6–7 days later. This protocol is repeated five times. During these passages, the tonsillar tissue may be stored at 4°C, if storage is to be under 24 hours, or at –70°C for longer periods. At the same time, the presence of CSF antigen is confirmed at each passage by the direct FAT in cryostat sections of the tonsils, or by virus isolation in a suitable substrate. If CSFV or antigen cannot be demonstrated after a certain passage, a second series of passages is performed to show infection, commencing with the last two pigs of the previous series.

Five pigs are inoculated intramuscularly with the sixth pig passage of the seed-lot virus, equivalent to one vaccine dose or, if this passage has not been reached, the highest passage of the two series where virus or viral antigen was detected. Five further pigs are similarly inoculated with one dose of the seed-lot virus, equivalent to one vaccine dose. All pigs are weighed at the time of inoculation and again 2 weeks later. Blood is collected daily into anticoagulant during the first week, and all pigs are kept under observation for 3 weeks. No animal should die or become ill from the vaccine virus. The average weight gain of the two groups during the first 2 weeks should not differ significantly ($p < 0.05$). Leukopenia (WBC count of $< 7 \times 10^6$ /ml) is permitted, at the most, in one pig of either group for 1 day.

v) *Immunogenicity*

To demonstrate adequate immunogenicity, ten pigs are each inoculated with an amount of virus equivalent to one dose of vaccine, and two other pigs are housed separately as uninoculated controls. All pigs are challenged 7 days later with 10^5 PID₅₀ of a virulent strain of CSFV. Only the controls should die.

In a test for duration of immunity, ten pigs are each inoculated with one dose, and two others are maintained separately as controls. Six months later, the sera of the inoculated pigs are tested for CSF antibodies; at least eight pigs should be positive. All pigs are then challenged with at least 10^5 PID₅₀ of a virulent strain of CSFV, and observed for 3 weeks. Only the controls should die.

To test protection of the development of the carrier sow syndrome, 20 pregnant sows at the same stage of gestation are randomly divided into two groups. The sows of one group are vaccinated once or twice with an amount of virus equivalent to one dose of vaccine, and intranasally challenged 4 weeks after the last vaccination with a field strain of low virulence, together with the unvaccinated control sows. All sows are killed 4 weeks after challenge and the fetuses are examined for the presence of CSFV or viral antigen. Vaccination should significantly reduce transplacental transmission of the virus.

Under the storage conditions prescribed by the manufacturer for the final product, a volume of virus equivalent to one dose of vaccine must maintain its immunogenicity at least until the end of the stated shelf life.

2. Method of manufacture

Each batch of MLV CSF vaccine must be derived from the same seed lot that has been used for the pilot tests. Also, each batch must be prepared according to the production protocol and under the conditions laid down for the registration of the final product. The properties of each batch and those of the seed lot must be verified as uniform.

3. In-process control

The protocol for production will depend on the vaccine strain, the production system (animals or cell cultures), and available facilities. The norms for cell culture vaccines may vary according to the production system, namely, primary cultures, cell lines, monolayers or suspension cultures.

4. Batch control

All pigs used in batch control tests must be 6–8 weeks of age and free from antibodies to CSFV and BVDV. They must be uniform in origin, breed, husbandry, and randomly distributed into any groups where necessary.

a) Identity

The vaccine must induce specific neutralising antibodies against a virulent strain of CSFV.

b) Sterility

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

c) Safety

Each of three pigs is inoculated intramuscularly with ten doses of the reconstituted vaccine as a single injection. The pigs are observed for 3 weeks thereafter and body temperatures are taken daily for the first week. No pig should die or show signs of disease attributable to the vaccine, the average daily body temperature must at no time reach 40.5°C or more, and the pigs should grow normally.

d) Purity

The batch must be virologically pure. To test for this, three pigs are each inoculated intramuscularly with ten vaccine doses. Serum samples are collected at the time of inoculation and again 5 weeks later. These are tested for antibodies to BVD (neutralisation for 1 hour at 37°C) and porcine parvovirus (haemagglutination inhibition using four haemagglutinating units). All three pigs must remain disease free. Tests for virological purity need not be carried out when using vaccines produced in rabbits.

e) Potency

Potency is expressed as the number of 50% protective doses (PD₅₀) contained in one vaccine dose. One vaccine dose is at least 100 PD₅₀.

Two groups of five 6–8-week-old piglets are inoculated intramuscularly with a 1/40 and a 1/160 dilution of the reconstituted vaccine, respectively, using buffered salt solution, pH 7.2. The vaccinated pigs together with two controls are challenged intramuscularly with 10^5 PID₅₀ of a virulent strain of CSFV 2 weeks later. The pigs are observed for 2 weeks thereafter, during which time the controls should die. From the pigs that survive without showing any signs of CSF, the number of PD₅₀ contained in the vaccine is calculated using the usual statistical methods.

This potency test may be replaced by an infectivity assay, provided that the manufacturer can show that there is a distinct and reproducible relationship between the virus content of the vaccine and the protection it will confer on pigs against challenge.

f) Stability

The period of validity of a batch of lyophilised CSF vaccine should not be under 1 year.

C2. Marker vaccines

Despite the existence of safe and effective MLV vaccines against CSF, their use has been discouraged in the EU and some other CSF-free or near free countries, because antibodies provoked by such vaccines cannot be distinguished from antibodies induced by the wild-type virus. A 'marker vaccine', which allows discrimination of infected from vaccinated animals (DIVA), does not have this disadvantage: it can elicit a protective immune response that can be distinguished from the immune response induced by field virus. An additional prerequisite of any DIVA strategy is the availability of a companion serological test that is highly discriminatory, for demonstrating absence of infection and in tracing residual infections.

The minimum demands for CSF marker vaccines and the companion discriminatory tests have been formulated as follows (6):

a) Vaccine

The vaccine should provide protection against any natural-contact challenge, i.e. it should prevent clinical signs and re-excretion of the virus. The efficacy of vaccination should be shown experimentally by studies in which transmission of wild-type virus in vaccinated groups of pigs is studied. The protective effect of vaccination should be achieved within the shortest possible period and ideally less than 2 weeks. A fast and reliable protection should preferably be obtained after one single application. Furthermore, it should be ensured that infection of vaccinated pregnant sows does not lead to transplacental infection and the birth of litters congenitally infected with CSFV. Duration of immunity should be at least 6 months.

Many different marker vaccines for CSF are under development, and two have been registered in the EU. Both are subunit vaccines that employ the E2 glycoprotein of CSFV as an immunogen and have been subject to independent assessment (9, 32). The E2 subunit is produced by insect cells that are infected by genetically modified baculovirus, which contains the E2 gene of CSFV. The vaccines, therefore, do not contain any CSFV, while the baculo (vector) virus is chemically inactivated. The final preparations are adjuvanted with mineral oils to form a double (water/oil/water), or a single (water in oil) emulsion. Several studies of the ability of these E2 DIVA vaccines to prevent horizontal and vertical transmission have given conflicting results (3).

b) Companion discriminatory test

The companion discriminatory serological test should be very sensitive because vaccination will reduce the prevalence of the disease. It should ideally provide discrimination within the same time-frame as for development of antibody to the immunising protein and should be used primarily as a herd test. If a high sensitivity reduces the specificity of the test, already compromised by the presence of antibodies to other pestiviruses, good and fast confirmatory assays should be available to discriminate positive from false-positive results.

The existing accompanying DIVA tests for E2 subunit vaccines are ELISAs that rely on the detection of antibody to the Erns protein (12, 16). Such tests have recently been approved by the European Commission (2), for use in determining whether herds vaccinated with an E2 subunit vaccine may also have been exposed to field virus. An assessment of their performance (12) has revealed that neither discriminatory ELISA consistently detected individual marker-vaccinated, CSF-challenged weaner pigs, hence the recommendation only to employ such a strategy at the herd level.

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