

SECTION 2.8.

SUIDAE

CHAPTER 2.8.1.

AFRICAN SWINE FEVER

SUMMARY

African swine fever (ASF) is an infectious disease of domestic and wild pigs of all breeds and ages, caused by a virus that produces a range of syndromes. Acute disease is characterised by high fever, haemorrhages in the reticuloendothelial system, and a high mortality rate. Soft ticks of the Ornithodoros genus, especially O. moubata and O. erraticus, have been shown to be both reservoirs and transmission vectors of ASF virus (ASFV).

ASFV is the only member of the Asfarviridae family, genus Asfivirus.

Laboratory diagnostic procedures for ASF fall into two groups: the first contains the tests for virus isolation and the detection of virus antigens and genomic DNA, while the second contains the tests for antibody detection. The selection of the tests to be carried out depends on the disease situation and laboratory diagnostic capacity in the area or country.

Identification of the agent: *Laboratory diagnosis must be directed towards isolation of the virus by simultaneously carrying out the inoculation of pig leukocyte or bone marrow cultures, the detection of antigen in smears or cryostat sections of tissues by fluorescent antibody test (FAT) and the detection of genomic DNA by the polymerase chain reaction (PCR). The PCR is an excellent, highly sensitive, and rapid technique for ASFV detection and it is very useful under a wide range of circumstances. It is especially useful if the tissues are unsuitable for virus isolation and antigen detection.*

In doubtful cases, the material is passaged in leukocytes cell cultures and the procedures described above are repeated.

Serological tests: *Pigs that survive natural infection usually develop antibodies against ASFV from 7–10 days post-infection and these antibodies persist for long periods of time. Where the disease is endemic, or where a primary outbreak is caused by a strain of low virulence, the investigation of new outbreaks should include the detection of specific antibodies in serum or extracts of the tissues submitted. A variety of methods such as indirect fluorescent antibody (IFA), the enzyme-linked immunosorbent assay (ELISA) and the immunoblotting test is available for antibody detection.*

Requirements for vaccines and diagnostic biologicals: At present, there is no vaccine for ASF.

A. INTRODUCTION

African swine fever virus (ASFV) is a complex large icosahedral enveloped DNA virus that exhibits many features common to both iridovirus and poxvirus families (Arias & Sánchez-Vizcaíno, 2002a; Vinuela, 1985). This virus is currently classified as the only member of a family called *Asfarviridae* (Dixon *et al.*, 2005). At least 28 structural proteins have been identified in intracellular virus particles (200 nm) (Sánchez-Vizcaíno, 2006). More than a hundred infectious proteins have been identified in infected porcine macrophages, and at least 50 of them react with sera from infected or recovered pigs. The virus genome comprises between 170 and 192 kilobases (kb), with a conserved central region of about 125 kb and variable ends. These variable regions encode five multigene families that are directly involved with the variability of the virus genome (Blasco *et al.*, 1989). The complete

analysis of the sequence of several ASFV strains has been accomplished (Chapman *et al.*, 2008; De Villier *et al.*, 2010). Different strains of ASFV vary in their ability to cause disease, but at present there is only one recognised serotype of the virus detectable by antibody tests.

The molecular epidemiology of the disease is investigated by sequencing of the C- terminal end of VP72 gene, which differentiates up to 22 distinct genotypes (Boshoff *et al.*, 2007; Lubisi *et al.*, 2005). Full genome sequence of the *p54*-gene has been confirmed as a valuable additional genotyping method for molecular epidemiological studies (Gallardo *et al.*, 2009). Enhanced discrimination is obtained by analysis of the central variable region (CVR) within the B602L-gene, described as the most variable locus to distinguish between closely related isolates and identify virus subgroups within several of the 22 genotypes (Gallardo *et al.*, 2009).

ASF viruses produce a range of syndromes varying from peracute, acute to chronic disease and apparently healthy virus carriers. Pigs are the only domestic animal species that is naturally infected by ASFV. European wild boars and feral pigs are also susceptible to the disease, exhibiting clinical signs and mortality rates similar to those observed in domestic pigs. In contrast African wild pigs such as warthogs (*Phacochoerus aethiopicus*), bush pigs (*Potamochoerus porcus*) and giant forest hogs (*Hylochoerus meinertzhageni*) are resistant to the disease and show few or no clinical signs. These species of wild pig act as reservoir hosts of ASFV in Africa (Sánchez-Vizcaíno, 2006).

The incubation period in nature is usually 4–19 days. The more virulent strains produce peracute or acute haemorrhagic disease characterised by high fever, loss of appetite, haemorrhages in the skin and internal organs, and death in 4–10 days, sometimes even before the first clinical signs are observed. Mortality rates may be as high as 100%. Less virulent strains produce mild clinical signs – slight fever, reduced appetite and depression – which can be readily confused with many other conditions in pigs and may not lead to suspicion of ASF. Low virulent, non-haemadsorbing strains occasionally produce mainly subclinical non-haemorrhagic infection and seroconversion, but some animals may develop discrete lesions in the lungs or on the skin in areas over bony protrusions and other areas subject to trauma. Animals which have recovered from either acute or chronic infections may become persistently infected, acting as virus carriers. The biological base for the persistence of ASFV is still not well understood (Carrillo *et al.*, 1994). Recovered ASFV carrier pigs and persistently infected wild pigs constitute the biggest problems in controlling the disease. The serological recognition of carrier pigs has been vital for the success of eradication programmes (Arias & Sánchez-Vizcaíno, 2002b).

ASF cannot be differentiated from classical swine fever (hog cholera; CSF) by either clinical or post-mortem examination, and both diseases should be considered in the differential diagnosis of any acute febrile haemorrhagic syndrome of pigs. Bacterial septicaemias may also be confused with ASF and CSF. Laboratory tests are essential to distinguish between these diseases.

In countries free from ASF but suspecting its presence, the laboratory diagnosis must be directed towards isolation of the virus by simultaneously carrying out the inoculation of pig leukocyte or bone marrow cultures, the detection of antigen in smears or cryostat sections of tissues by the fluorescent antibody test (FAT) and, by the detection of genomic DNA by the polymerase chain reaction (PCR), which is the most sensitive technique for detecting the presence of the agent in persistently infected animals and is particularly useful if samples submitted are unsuitable for virus isolation and antigen detection because they have undergone putrefaction. ASFV can be detected by PCR from a very early stage of infection in tissues, ethylene diamine tetra-acetic acid (EDTA)-blood and serum samples. Pigs recovered from acute or chronic infections usually exhibit a viraemia for several weeks making the PCR test a very useful tool for the detection of ASFV DNA in pigs infected with low or moderately virulent strains.

As no vaccine is available, the presence of ASFV antibodies is indicative of previous infection and, as antibodies are produced from the first week of infection and persist for long periods, they are a good marker for the diagnosis of the disease. The early appearance (from 7 to 10 days post-infection) and subsequent long-term persistence of antibodies make antibody detection techniques, such as ELISA, immunoblotting or IFA test, very useful in diagnosing the subacute and chronic forms of disease.

ASF epidemiology is complex with different epidemiological patterns of infection occurring in Africa and Europe. ASF occurs through transmission cycles involving domestic pigs, wild boars, wild African suids, and soft ticks (Sánchez-Vizcaíno *et al.*, 2009). In regions where *Ornithodoros* soft-bodied ticks are present, the detection of ASFV in these reservoirs of infection contributes to a better understanding of the epidemiology of the disease. This is of major importance in establishing effective control and eradication programmes (Basto *et al.*, 2006).

ASF is not a zoonotic disease and does not affect public health (Sánchez-Vizcaíno *et al.*, 2009).

ASFV should be handled in laboratories that have been approved by their Competent Authorities following OIE guidelines for the biocontainment of Group 3 and 4 pathogens.

B. DIAGNOSTIC TECHNIQUES

1. Identification of the agent

Where ASF is suspected, the following samples should be sent to the laboratory: blood in anticoagulant (EDTA), spleen, lymph nodes, tonsil and kidney. These should be kept as cold as possible, without freezing, during transit. After the samples arrive at the laboratory, they should be stored at -70°C if processing is going to be delayed. As maintaining a cold chain is not always possible, samples can be submitted in glycerosaline; this may slightly decrease the likelihood of virus isolation, but it may facilitate the submission of samples to the laboratory so that an outbreak can be confirmed.

1.1. Sample preparation for haemadsorption test

- i) Prepare suspensions of tissues by grinding 0.5–1.0 g pieces with a pestle and mortar containing sterile sand, and then add 5–10 ml of a buffered salt solution or tissue culture medium containing antibiotics.
- ii) Clarify the suspensions by centrifugation at 1000 *g* for 5 minutes. Use the supernatant for cell culture/haemadsorption (Section B.1.2 below).

1.2. Haemadsorption test

The haemadsorption (HAD) test (Malmquist & Hay, 1960) is based on the fact that pig erythrocytes will adhere to the surface of pig monocyte or macrophage cells infected with ASFV and that most virus isolates produce this phenomenon of haemadsorption. A positive result in the HAD test is definitive for ASF diagnosis. A very small number of 'non-haemadsorbing' viruses have been isolated, most of which are a virulent, but some do produce typical acute ASF. The test is carried out by inoculating blood or tissue suspensions from suspect pigs into primary leukocyte cultures, (Procedure 1 below) or into alveolar macrophages cell cultures, and also by preparing leukocyte cultures from the blood of pigs inoculated at the laboratory or from the blood of suspect pigs collected in the field (Procedure 2 below). Up to 300 cultures in tubes can be prepared from each 100 ml of defibrinated blood collected. It is essential to carry out all procedures in such a way as to prevent contamination of the cultures.

1.2.1. Procedure 1: Haemadsorption test in primary leukocyte cultures

- i) Collect the required volume of fresh defibrinated pig blood.
- ii) Centrifuge at 700 *g* for 30 minutes and collect the buffy coat cells. Add three volumes of 0.83% ammonium chloride to the leukocytes obtained. Mix and incubate at room temperature for 15 minutes. Centrifuge at 650 *g* for 15 minutes and carefully remove the supernatant. Wash pellet in medium or phosphate buffered saline (PBS).
- iii) Resuspend the cells at a concentration of 10^6 – 10^7 cells/ml in tissue culture medium containing 10–30% pig serum and antibiotics. In order to prevent nonspecific haemadsorption, the medium should contain serum or plasma from the same pig from which the leukocytes were obtained. If a large volume of samples is to be tested, the homologous serum can be replaced by serum that has been identified by pre-screening as capable of preventing the nonspecific auto-rosette formation.
- iv) Dispense the cell suspension in 96-well plates with 200 μl per well (300,000 cells/well) and incubate at 37°C in a humidified 5% CO_2 incubator. This procedure can also be performed in aliquots of 1.5 ml in 160 \times 16 mm tubes and incubate in a sloping position (5 – 10° from the horizontal) at 37°C .

Note: For routine diagnosis, only 2–4-day-old cultures are sufficiently sensitive.

- v) After 3 days inoculate three tubes or well plates by adding 0.2 ml/tube or 0.02 ml (1/10 final dilution)/well of prepared samples. It is advisable to inoculate ten-fold and hundred-fold dilutions into cultures, and this is especially important when the field material submitted is in poor condition.
- vi) Inoculate positive control cultures with haemadsorbing virus. Uninoculated negative controls are essential to monitor the possibility of nonspecific haemadsorption.
- vii) Add 0.2 ml of a fresh preparation of 1% pig erythrocytes in buffered saline to each tube. In the case of the 96-well plates, add 0.02 ml of 1% pig erythrocytes per well.
- viii) Examine the cultures daily for 7–10 days under a microscope for cytopathic effect (CPE) and haemadsorption.

ix) *Reading the results*

Haemadsorption consists of the attachment of large numbers of pig erythrocytes to the surface of infected cells. A CPE consisting of a reduction in the number of adherent cells in the absence of haemadsorption may be due to the cytotoxicity of the inoculum, Aujeszky's disease virus or non-haemadsorbing ASFV, which can be detected by the FAT on the cell sediment or by use of PCR (see below). If no change is observed, or if the results of the immunofluorescence and PCR tests are negative, subinoculate the supernatant up to three times into fresh leukocyte cultures. All isolations should be confirmed by PCR and sequencing.

1.2.2. Procedure 2: Haemadsorption 'autorosette' test with peripheral blood leukocytes from infected pigs

This procedure is quicker than the preparation and inoculation of primary pig leukocyte cultures (described in Procedure 1 above) and will give more rapid results in positive cases. It can be performed in laboratories that are not equipped for routine virological examinations; the minimum requirements are slides and cover-slips, a microscope and sterile medium, tubes or bottles and pipettes. Blood from suspect pigs in the field, or those inoculated in the laboratory, is collected in heparin and leukocyte cultures are prepared for direct examination for haemadsorption. However, the results of the test are difficult to evaluate and it is now being replaced by the PCR.

- i) Collect 20 ml of whole blood in a syringe containing 2000 IU heparin in 2 ml of saline, mix and transfer to a glass tube or narrow bottle.
- ii) Place the tube/bottle vertically in an incubator or water bath at 37°C, and allow the cells to settle. Sedimentation is improved by the addition of 2 ml of a plasma volume expander, such as 'Dextravan150' which is a solution of Dextran 150 in 0.9% NaCl for injection (Fisons, United Kingdom).
- iii) Incubate the cultures for 6–8 hours at 37°C, and then examine the cultures at 2–3-hour intervals by transferring small aliquots of the white-cell-rich supernatant, together with some erythrocytes, on to a glass slide.
- iv) *Reading the results*

The presence of haemadsorbing cells identified under a microscope indicates the presence of ASFV. Haemadsorption consists of the attachment of large numbers of pig erythrocytes to the surface of infected cells. Any evidence of haemadsorption would be enough to consider repeating the assay or confirming the presence of ASFV by another test such as PCR.

1.3. Antigen detection by fluorescent antibody test

The FAT (Bool *et al.*, 1969) can be used as an additional method to detect antigen in tissues of suspect pigs in the field or those inoculated at the laboratory. Positive FAT plus clinical signs and appropriate lesions can provide a presumptive diagnosis of ASF. It can also be used to detect ASFV antigen in leukocyte cultures in which no HAD is observed and can thus identify non-haemadsorbing strains of virus. It also distinguishes between the CPE produced by ASFV and that produced by other viruses, such as Aujeszky's disease virus or a cytotoxic inoculum. However, it is important to note that in subacute and chronic disease, FAT has a significantly decreased sensitivity. This reduction in sensitivity may be related to the formation of antigen-antibody complexes in the tissues of infected pigs which block the interaction between the ASFV antigen and ASF conjugate (Sánchez-Vizcaíno, 2006).

1.3.1. Test procedure

- i) Prepare cryostat sections or impression smears of test tissues, or spreads of cell sediment from inoculated leukocyte cultures on slides, air dry and fix with acetone for 10 minutes at room temperature.
- ii) Stain with fluorescein isothiocyanate (FITC)-conjugated anti-ASFV immunoglobulin at the recommended or pretitrated dilution for 1 hour at 37°C in a humid chamber.
- iii) Fix and stain positive and negative control preparations similarly.
- iv) Wash by immersing four times in fresh clean PBS, mount stained tissues in PBS/glycerol, and examine under an ultraviolet light microscope with suitable barrier and exciter filters.

v) *Reading the results*

Tissues are positive if specific granular cytoplasmic fluorescence is observed in paracortical tissue of lymphoid organs or in fixed macrophages in other organs, or in inoculated leukocyte cultures.

1.4. Detection of virus genome by the polymerase chain reaction

PCR techniques have been developed, using primers from a highly conserved region of the genome, to detect and identify a wide range of isolates belonging to all the known virus genotypes, including both non-haemadsorbing viruses and isolates of low virulence. The PCR techniques are particularly useful for identifying virus DNA in pig tissues that are unsuitable for virus isolation or antigen detection because they have undergone putrefaction, or when there is good reason to believe that virus may have been inactivated before samples are received in the laboratory.

Two validated PCR procedures are described and consist of a sample preparation followed by the test procedure. These procedures serve as a general guideline and a starting point for the PCR protocol. Optimal reaction conditions (incubation times and temperatures, models and suppliers of equipment, concentrations of assay reagents such as the primers and dNTPs) may vary so the described conditions should be evaluated first.

1.4.1. Sample preparation procedure (Agüero *et al.*, 2003; 2004)

A sensitive extraction procedure using the commercial High Pure PCR Template Preparation Kit¹ is described below. A number of other DNA extraction kits are commercially available for the preparation of template suitable for PCR depending on the sample submitted for analysis and may be appropriate for use. Different samples can be used in this procedure such as cell culture supernatants, EDTA-blood, serum and tissue homogenates, even if the latter have been kept in warm conditions and undergone a degree of putrefaction. This procedure has the advantage in that it can be used for both the extraction of ASFV DNA and CSFV RNA which enables the simultaneous detection of both viruses in a multiplex PCR assay (Agüero *et al.*, 2004).

The High Pure PCR Template Preparation Kit¹ includes the following reagents: Binding Buffer, Proteinase K, Inhibitor Removal Buffer, Wash Buffer, and High Pure Filter Tubes and collection tubes.

For organ and tissue samples, first prepare a 1/10 homogenate of the material in PBS, then centrifuge to clarify at 12,000 *g* for 5 minutes. Extract DNA/RNA from the resultant supernatant fluid. Sometimes it is recommended to process a 1/10 dilution of the supernatant in parallel.

Extraction for control samples: at least one positive and one negative control should be included in each nucleic acid extraction run. Positive control samples should be 200 µl of ASFV-positive serum, EDTA-blood, or 1/10 tissue homogenates (same kind of tissue as samples to be analysed): the negative control should be 200 µl of water or ASFV-negative EDTA-blood, or tissue homogenate. Controls should be processed with the test samples.

1.4.2. Preparation of working solutions

i) Lyophilised proteinase K

Dissolve proteinase K in 4.5 ml sterile distilled water, and aliquot the solution in 500 µl vials. Store at –20°C until use.

ii) Inhibitor removal buffer

Add 20 ml absolute ethanol to the original vial. Label and date bottle accordingly.

iii) Wash buffer

Add 80 ml absolute ethanol to the original vial. Label and date bottle accordingly.

1 Roche Diagnostics.

1.4.2.1. Preparation method

- i) Pipette 200 µl of sample into a 1.5 ml microcentrifuge tube.
- ii) Add 200 µl of binding buffer and 40 µl of proteinase K. Mix immediately. Incubate for 10 minutes at 72°C.
- iii) Briefly centrifuge the 1.5 ml microcentrifuge tube to remove drops from the inside of the lid.
- iv) Add 100 µl of isopropanol to the sample tube.
- v) Place the High Pure filter tube in a collection tube and pipette the sample in the upper reservoir. Centrifuge for 1 minute at 8000 rpm. (With blood samples, repeat the centrifugation step if sample remains in the filter tube.)
- v) Discard the collection tube and place the filter tube into a clean collection tube.
- vii) Add 500 µl of Inhibitor Removal Buffer to the upper reservoir and centrifuge for 1 minute at 8000 rpm.
- viii) Discard the collection tube and place the filter tube into a clean collection tube.
- ix) Add 450 µl of wash buffer to the upper reservoir and centrifuge for 1 minute at 8000 rpm.
- x) Discard the collection tube and repeat the washing step.
- xi) Discard the collection tube and place the filter tube into a clean collection tube. Centrifuge for 10 seconds at 13000 rpm to remove residual wash buffer.
- xii) Discard the collection tube and place the filter tube in a clean 1.5 ml microcentrifuge tube.
- xiii) For the elution of nucleic acids, add 50 µl of prewarmed (70°C) sterile water to the upper reservoir (be careful not to use the Elution Buffer included in the kit for CSFV RNA). Centrifuge for 1 minute at 8000 rpm.
- xiv) Use immediately or store at –20°C for future use.

1.4.3. PCR amplification by conventional PCR (Agüero *et al.*, 2003)

The ASFV primer set described in this procedure can be combined with a specific primer set for CSFV in a multiplex RT-PCR method that allows the simultaneous and differential detection of both virus genomes in a single reaction (Agüero *et al.*, 2004).

1.4.4. Stock solutions

- i) Nuclease-free sterile water.
- ii) Hot Start Taq Gold DNA polymerase, 10× PCR Buffer II, and magnesium chloride are commercially available.
- iii) PCR nucleotide mix containing 10 mM of each dNTP is commercially available.
- iv) Primers at a concentration of 20 pmol/µl: Primer PPA-1 sequence 5'-AGT-TAT-GGG-AAA-CCC-GAC-CC-3' (forward primer); primer PPA-2 sequence 5'-CCC-TGA-ATC-GGA-GCA-TCC-T-3' (reverse primer).
- v) 10× Loading buffer
0.2% xylene cyanol, 0.2% bromophenol blue, 30% glycerol.
- vi) TAE buffer (50×) for agarose gel
Tris base (242 g); glacial acetic acid (57.1 ml); 0.5 M EDTA, pH 8.0 (100 ml); distilled water (to 1 litre).
- vii) Marker DNA
100 base-pair ladder is commercially available.

1.4.4.1. Preparation method

- i) In a sterile 1.5 ml microcentrifuge tube prepare the PCR reaction mixture described below for each sample. Prepare the reaction mixture in bulk for the number of samples to be assayed allowing for at least one extra sample.

- ii) Nuclease-free or sterile distilled water (17.375 µl), 10 ×PCR Buffer II (2.5 µl), magnesium chloride 25 mM (2 µl), dNTP mix 10mM (0.5 µl), primer PPA-1, 20 pmol/µl (0.25 µl), primer PPA-2, 20 pmol/µl (0.25 µl), Taq Gold DNA polymerase 5 U/µl (0.125 µl).
- iii) Add 23 µl of the PCR reaction mix to the required number of 0.2 ml PCR tubes.
- iv) Add 2µl of extracted sample template to each PCR tube. Include a positive reaction control (2 µl of ASFV DNA) and a negative reaction control (2 µl of distilled water) for each PCR run.
- v) Place all the tubes in an automated thermal cycler and run the following programme:
One cycle at 95°C for 10 minutes.
40 cycles at 95°C for 15 seconds, 62°C for 30 seconds and 72°C for 30 seconds.
One cycle at 72°C for 7 minutes.
Hold at 4°C.
- vi) At the end of the programme, remove PCR tubes and add 2.5 µl of 10x loading buffer to each tube.
- vii) Load all the samples in a 2% agarose gel in TAE buffer containing ethidium bromide at a final concentration of 0.5 µg/ml.
- viii) Add marker DNA to one lane on each side of the gel.
- ix) Run the gel at a constant voltage of 150-200 volts for about 30 minutes.
- x) *Reading the results*
Examine the gel over a UV light source. In a positive sample, a discrete band will be present that should co-migrate with the PCR product of the positive control. Calculate the size of the PCR products in the test samples and the positive control by reference to the standard markers. The PCR product of the positive control has a size of 257 base pairs. No bands should be seen in the negative control.
- xi) *Optional*
An additional confirmatory assay could be performed by BsmA I restriction endonuclease digestion of the amplified products. For this assay, incubate for 2.5 hours at 55°C a total of 5 µl of amplified DNA product in a final volume of 20 µl digestion mix: 2 µl of 10x buffer, 1 µl of BsmA I (5 U/µl) and 12 µl of sterile distilled water. Then, run the samples in a 3% agarose gel as described above. The restriction pattern should include two fragments of 173–177 and 84–80 base pairs in the positive samples.

1.4.5. PCR Procedure: TaqMan® PCR protocol (King *et al.*, 2003)

1.4.5.1. Sample preparation

A number of DNA extraction kits are commercially available for the preparation of template suitable for PCR depending on the sample submitted for analysis and may be appropriate for use in Reference Laboratories.

The QIAamp® Viral RNA Mini Kit (QIAGEN) procedure (spin protocol) is described below. This kit can be used for blood from suspected swine fever animals. Blood from infected swine should be taken in EDTA. The detection of ASFV can be performed in parallel to that for CSF virus (see Chapter 2.8.3 *Classical swine fever* for CSFV molecular detection methods).

- i) Pipette 560 µl of the supplied buffer AVL into a 1.5 ml microcentrifuge tube.
- ii) Add 140 µl of test or control sample and mix by pulse-vortexing for about 15 seconds. Negative ASF control samples consisting of spleen homogenates from uninfected pigs and uninfected porcine bone marrow (PBM) and peripheral blood mononuclear (PBL) cells should be processed alongside the test samples. Additional extraction negative controls can also be prepared for each test sample and uninfected negative control by running parallel extractions of nuclease-free water (all controls should subsequently be assayed by the PCR procedure along with the test samples).
- iii) Incubate at room temperature for at least 10 minutes.
- iv) Briefly centrifuge the 1.5 ml microcentrifuge tube to remove drops from the inside of the lid.

- v) Add 560 µl ethanol to the sample, pulse-vortex for approximately 15 seconds and briefly centrifuge to remove drops from the inside of the lid.
- vi) Add 630 µl of the solution from v) to a QIAamp spin column (in a 2-ml collection tube) without wetting the rim. Close the cap and centrifuge for 1 minute at 6000 *g*. Place the spin column into a clean 2 ml collection tube and discard the tube containing the filtrate.
- vii) Carefully open the QIAamp spin column and repeat step vi.
- viii) Carefully open the QIAamp spin column and add 500 µl of Buffer AW1. Close the cap and centrifuge for 1 minute at 6000 *g*. Place the spin column into a clean 2-ml collection tube and discard the tube containing the filtrate.
- ix) Carefully open the QIAamp spin column and add 500 µl of Buffer AW2. Close the cap and centrifuge for 3 minutes at 20,000 *g*.
- x) Place the QIAamp spin column in a new 1.5 ml microcentrifuge tube. Discard the old collection tube containing the filtrate. Carefully open the QIAamp spin column and add 60 µl of Buffer AVE. Close the cap and incubate at room temperature for 1 minute. Centrifuge for 1 minute at 6000 *g*.
- xi) Discard the QIAamp spin column. Store the extracted DNA (60 µl) at –20°C until required for PCR amplification procedure.

1.4.5.2. Stock solutions

- i) Nuclease-free or another appropriate sterile water and TaqMan® PCR reaction master mix (2×).
- ii) Primers at a concentration of 50 pmol/µl: Primer 1 sequence 5'-CTGCT-CATGG-TATCA-ATCTT-ATCGA-3' (positive strand); Primer 2 sequence 5'-GATAC-CACAA-GATC(AG)-GCCGT-3' (negative strand).
- iii) TaqMan® probe at a concentration of 5 pmol/µl: (5'-[6-carboxy-fluorescein (FAM)]-CCACG-GGAGG-AATAC-CAACC-CAGTG-3'-[6-carboxy-tetramethyl-rhodamine (TAMRA)]).

1.4.5.3. PCR amplification by TaqMan® assay (Fernández-Pinero *et al.*, 2010)

- i) In a sterile 1.5 ml microcentrifuge tube prepare the PCR reaction mixture described below for each sample. Prepare the reaction mixture in bulk for the number of samples to be assayed but allowing for one extra sample.

Nuclease-free or sterile water (7.5 µl); (2× conc.) TaqMan® PCR reaction master mix (12.5 µl); primer 1, 50 pmol (1.0 µl); primer 2, 50 pmol (1.0 µl); TaqMan® probe, 5 pmol (1 µl).

- ii) Add 22 µl PCR reaction mix to a well of a MicroAmp® optical reaction plate for each sample to be assayed.
- iii) Add 3 µl of extracted sample template or blank extraction control and securely cover each well with a cap.
- iv) Spin the plate for 1 minute in a suitable centrifuge to mix the contents of each well.
- v) Place the plate in a TaqMan® Sequence Detection System for PCR amplification and run the following programme:

One cycle at 50°C for 2 minutes,

One cycle at 95°C for 10 minutes,

Forty cycles at 95°C for 15 seconds, 58°C for 1 minute.

Note: If a TaqMan® thermal cycler is not available, an ordinary thermal cycler can be used and the PCR products analysed by end-point fluorescence readers or alternatively by electrophoresis on a 1.5% agarose gel. A product of 250 bp is expected.

- vi) *Reading the results*

Assign a threshold cycle (C_T) value to each PCR reaction from a scan of all amplification plots (a plot of the fluorescence signal versus cycle number). Negative test samples, uninfected negative or extraction blank controls should have a C_T value >40.0. Positive

test samples and controls should have a C_T value < 40.0 (strongly positive samples have a C_T value <30.0).

Modifications of this protocol using a range of commercial amplification kits can provide even higher PCR yields, however these amplification kits should be fully validated prior to use.

2. Serological tests

Antibodies persist in recovered pigs for long periods after infection, sometimes for life, and a number of tests are available for detecting these antibodies, although only a few of them have been developed for routine use in diagnostic laboratories (Escribano *et al.*, 1990; Pastor *et al.*, 1990; Sánchez-Vizcaíno, 1987). The most commonly used is the ELISA (Sánchez-Vizcaíno *et al.*, 1983; Wardley *et al.*, 1979), which is suitable for examining either serum or fluid from the tissues. Confirmatory testing of ELISA-positive samples should be carried out in critical cases using an alternative test, such as the IFA test, immunoperoxidase staining or immunoblotting (Escribano *et al.*, 1990; Pastor *et al.*, 1989). Antibody is usually not detected in pigs infected with virulent ASFV as they die before it is produced. Antibodies are produced in pigs infected with low or moderately virulent ASF viruses, but these are not fully neutralising antibodies.

Recently, an extensive study carried out to assess the specificity and sensitivity of ASF serological tests in the different epidemiological scenarios of Africa and Europe, with currently circulating ASFV isolates (including the Caucasian genotype II and the eastern African isolates exhibiting more variability), has shown that the OIE prescribed tests are able to detect with accuracy and high sensitivity the presence of ASF antibodies in all the epidemiological situations evaluated (Gallardo *et al.*, 2010).

Where ASF is endemic, confirmation of suspected cases of disease is best done using a standard serological test (ELISA), combined with an alternative serological test (IFA) or an antigen-detection test. In some countries, over 95% of positive cases have been identified using a combination of IFA tests and FAT (Sánchez-Vizcaíno, 2006).

It should be noted that when pigs have been infected with avirulent isolates or those of low virulence, serological tests may be the only way of detecting infected animals.

2.1. Enzyme-linked immunosorbent assay (the prescribed test for international trade)

The ELISA (Pastor *et al.*, 1990) is a direct test that can detect antibodies to ASFV in pigs that have been infected by viruses of low or moderate virulence. A highly sensitive and specific commercial ELISA kit based on a competition format that has been validated for use under different epidemiological situations is available. A cheaper alternative is to prepare a soluble antigen for use in an indirect ELISA, and the procedure using this soluble antigen is described below.

ELISAs show a decreased sensitivity when the serum samples to be tested are poorly preserved. To solve this problem, several new ELISAs based on the use of new ASFV recombinant proteins are now being validated (Gallardo *et al.*, 2006).

Carrying out a second confirmatory test such as the immunoblotting test, IFA test or Immunoperoxidase test described below is recommended in the case of a doubtful result or a positive result when sera are suspected to be poorly preserved.

2.1.1. Antigen preparation for ELISA

The ELISA antigen is prepared from infected cells grown in the presence of pig serum (Escribano *et al.*, 1989).

- i) Infect MS (monkey stable) cells at multiplicity of infection of 10 with adapted virus, and incubate in medium containing 2% pig serum.
- ii) Harvest the cells at 36–48 hours post-infection, when the CPE is extensive. Wash in PBS, sediment at 650 *g* for 5 minutes, wash the cell pellet in 0.34 M sucrose in 5 mM Tris/HCl, pH 8.0, and centrifuge to pellet cells.

Carry out steps (iii) to (v) on ice:

- iii) Resuspend the cell pellet in 67 mM sucrose in 5 mM Tris/HCl, pH 8.0 (1.8 ml per 175 cm² flask), and leave for 10 minutes with agitation after 5 minutes.
- iv) Add nonionic detergent Nonidet P-40 to a final concentration of 1% (w/v), and leave for 10 minutes (with agitation after 5 minutes) to lyse the cells.

- v) Add sucrose to a final concentration of 64% (w/w) in 0.4 M Tris/HCl, pH 8.0, and centrifuge at 1000 **g** for 10 minutes to pellet nuclei.
- vi) Collect the supernatant and add EDTA (2 mM final concentration), beta-mercaptoethanol (50 mM final concentration) and NaCl (0.5 M final concentration) in 0.25 mM Tris/HCl, pH 8.0, and incubate for 15 minutes at 25°C.
- vii) Centrifuge at 100,000 **g** for 1 hour at 4°C over a layer of 20% (w/w) sucrose in 50 mM Tris/HCl, pH 8.0.

Remove the band immediately above the sucrose layer and use as the ELISA antigen. Store at –20°C.

2.1.2. Indirect ELISA procedure (Pastor *et al.*, 1990)

- i) Coat ELISA polysorp microtitre plate(s) with antigen by adding 100 µl of the recommended or pretitrated dilution of antigen in 0.05 M carbonate/bicarbonate buffer, pH 9.6, to each well.
- ii) Incubate at 4°C for 16 hours (overnight) and then wash five times with 0.05% Tween 20 in PBS, pH 7.2.
- iii) Dilute the test sera and positive and negative control sera 1/30 in 0.05% Tween 20 in PBS, pH 7.2, and add 100 µl of each diluted serum to duplicate wells of the antigen-coated plate(s).

If four pairs of each positive and negative control serum are added to wells in different parts of the plate, 40 sera can be tested in duplicate on one plate, as shown on the plate plan below.

	1	2	3	4	5	6	7	8	9	10	11	12
A	+											–
B	+											–
C				+					–			
D				+					–			
E				–					+			
F				–					+			
G	–											+
H	–											+

- iv) Incubate plates at 37°C for 1 hour (optionally on a plate shaker), and then wash five times with 0.05% Tween 20 in PBS.
- v) To each well add 100 µl of protein-A/horseradish-peroxidase conjugate (Pierce) at the recommended or pretitrated dilution in 0.05% Tween 20 in PBS.
- vi) Incubate the plates at 37°C for 1 hour, and then wash five times with 0.05% Tween 20 in PBS.
- vii) Substrate: Add hydrogen peroxide to the substrate solution (0.04% orthophenylene-diamine (OPD) in phosphate/citrate buffer, pH 5.0) at the rate of 10 µl/25 ml, and add 100 µl of substrate to each well.

Alternatively, DMAB/MBTH substrate solution can be used instead of OPD: Add 200 µl of substrate to each well (10 ml DMAB 80.6 mM Solution + 10 ml of MBTH 1.56mM solution + 5 µl H₂O₂).

- a) DMAB/MBTH substrate preparation

DMAB – 3-Dimethylaminobenzoic acid (SIGMAD-07871643); MBTH – 3-Methyl-2-benzothiazolinone hydrazone hydrochloride monohydrate (SIGMA M-8006).

b) DAMB 80.6 mM solution

Dissolve 13.315 g of DAMB acid in 1000 ml of 0.1 M phosphate buffer, pH 7 (5.3 g KH_2PO_4 , 8.65 g Na_2HPO_4 made up to 1000 ml in distilled water) by continuous agitation for 1 hour at room temperature, adjusting the pH to 7 with NaOH (5 M). Filter through a funnel.

c) MBTH 1,56 mM solution

Dissolve 0.3646 g of MBTH in 1000 ml of 0.1 M phosphate buffer, pH 7 (5.3 g KH_2PO_4 , 8.65 g Na_2HPO_4 made up to 1000 ml in distilled water) by continuous agitation for 1 hour, adjusting the pH to 6,25 with concentrated Hydrochloric acid. Filter through a funnel.

The volume required per plate is: 10 ml DMAB + 10 ml MBTH + 5 μl H_2O_2 30%

Substrate can be prepared as stock solutions, aliquoted and kept at -20°C . Mix the DAMB and the MBTH solutions (1:1) just before use and add the required quantity of 30% H_2O_2 .

viii) Incubate at room temperature for approximately 6–10 minutes (before the negative control begins to be coloured). The time necessary for the colour to develop will depend on both the temperature of the substrate when added to the wells, and the room temperature.

ix) Stop the reaction by adding 100 μl of 3 N sulphuric acid to each well.

x) Reading the results: Positive sera have a clear colour (yellow in case of OPD substrate, blue in case of DMAB/MBTH substrate) and can be read by eye, but to ensure that all positive sera are identified, it is necessary to read the absorbance in each well spectrophotometrically, at 492 nm (OPD substrate) or at 600–620 nm (in case of DMAB/MBTH), in an ELISA reader. Using OPD substrate any serum is considered to be positive if it has an absorbance value of more than twice the mean absorbance value of the control negative sera on that plate. Using DMAB/MBTH substrate, the test is validated when the mean absorbance value of the positive control is more than four times the mean of absorbance of the negative control.

To correctly interpret the results it is necessary to calculate a cut-off point which enables the differentiation of negative, inconclusive and positive results. The cut-off point is established by the following equation:

The cut-off point = optical density negative serum \times 1 + optical density positive serum \times 0.2.

- a) Sera with an optical density below the cut-off point – 0.1 can be considered negative.
- b) Sera with an optical density higher than cut-off point + 0.1 can be considered positive.
- c) Sera with an optical density between cut-off point \pm 0.1 can be considered inconclusive and the result needs to be confirmed by the IB technique.

2.2. Indirect fluorescent antibody test

This test (Pan *et al.*, 1974) should be used as a confirmatory test for sera from areas that are free from ASF and are positive in the ELISA, and for sera from endemic areas that give an inconclusive result in the ELISA.

2.2.1. Test procedure

- i) Prepare a suspension of ASFV-infected pig kidney or monkey cells at a concentration of 5×10^5 cells/ml, spread small drops on glass slides, air dry and fix with acetone at room temperature for 10 minutes. Note that slides can be stored at -20°C until ready for use.
- ii) Heat inactivate test sera at 56°C for 30 minutes.
- iii) Add appropriate dilutions of test sera and positive and negative control sera in buffered saline to slides of both infected and uninfected control cells, and incubate for 1 hour at 37°C in a humid chamber.
- iv) Wash the slides by immersing four times in fresh clean PBS and then distilled water.

- v) Add predetermined or recommended dilutions of anti-pig immunoglobulin/FITC or protein-A/FITC conjugate to all slides, and incubate for 1 hour at 37°C in a humid chamber.
- vi) Wash the slides by immersing four times in fresh clean PBS and then distilled water, mount in PBS/glycerol, and examine under an ultraviolet light microscope with suitable barrier and exciter filters.
- vii) *Reading the results:* The control positive serum on infected cells must be positive and all other controls must be negative before the test can be read. Sera are positive if infected cultures show specific fluorescence.

2.3. Immunoblotting test (Escribano *et al.*, 1990; Pastor *et al.*, 1989)

This test should be used as an alternative to the IFA test to confirm equivocal results with individual sera. The immunoblotting test is very specific and enables easier and more objective interpretation of the results and a better recognition of weak-positive samples.

Viral proteins that induce specific antibodies in pigs have been determined. These polypeptides have been placed on antigen strips and have been shown in the immunoblotting test to react with specific antibodies from 9 days post-infection.

2.3.1. Preparation of antigen strips

- i) Prepare cytoplasmic soluble virus proteins as described for the preparation of ELISA antigen in Section B.2.1.
- ii) Electrophorese through 17% acryl-amide/N,N'-diallyltartardiamide (DATD) gels with appropriate molecular weight standards.
- iii) Transfer the proteins on to a 14 × 14 cm² nitrocellulose membrane by electrophoresis at a constant current of 5 mA/cm in transfer buffer (20% methanol in 196 mM glycine, 25 mMTris/HCl, pH 8.3).
- iv) Dry the membrane and label the side on to which the proteins were electrophoresed.
- v) Cut one strip from the edge of the filter and carry out the immunoblotting procedure described below. Identify the region containing proteins of 23–35 kDa by comparison with the molecular weight standards run in parallel, and cut this region into 0.5 cm wide strips. Label each strip on the side on to which the proteins were electrophoresed.

These strips (approximately 4 cm long) constitute the antigen strips used for immunoblotting and contain proteins with which antibodies in both acute and convalescent pig sera will react. These antibodies persist for life in some pigs.

2.3.2. Preparation of chloranaphthol substrate solution

This solution must be prepared immediately before use.

- i) Dissolve 6 mg of 4-chloro-1-naphthol in 2 ml of methanol and add this solution slowly to 10 ml of PBS while it is being stirred.
- ii) Remove the white precipitate that is formed by filtration through Whatman No.1 filter paper (optional).
- iii) Add 4 µl of 30% hydrogen peroxide.

2.3.3. Test procedure

The antigen strips must be kept with the labelled side uppermost during the immunoreaction procedure.

- i) Incubate the antigen strips in blocking buffer (2% non-fat dried milk in PBS) at 37°C for 30 minutes with continuous agitation.
- ii) Prepare 1/40 dilutions of test sera and positive and negative control sera in blocking buffer.
- iii) Incubate the antigen strips in the appropriate serum at 37°C for 45 minutes with continuous agitation. Incubate one antigen strip in positive control serum and one in

negative control serum. These two strips are controls. Wash four times in blocking buffer; the final wash should be for 5 minutes with continuous agitation.

- iv) Add protein-A/horseradish-peroxidase conjugate at the recommended or pretitrated dilution (usually at 1/1000 dilution) in blocking buffer to all antigen strips. Incubate at 37°C for 45 minutes with continuous agitation. Wash four times in blocking buffer; the final wash should be for 5 minutes with continuous agitation.
- v) Prepare the substrate solution, add to the antigen strips, and incubate at room temperature for 5–15 minutes with continuous agitation.
- vi) Stop the reaction with distilled water when the protein bands are suitably dark.
- vii) *Reading the results:* Positive sera react with more than one virus protein in the antigen strip; they must give a similar protein pattern and have the same intensity of colour as the antigen strips stained with positive control serum.

C. REQUIREMENTS FOR VACCINES

At present there is no vaccine for ASF.

REFERENCES

- AGÜERO M., FERNÁNDEZ J., ROMERO L., SANCHEZ C., ARIAS M. & SÁNCHEZ-VIZCAÍNO J.M. (2003). Highly sensitive PCR assay for the routine diagnosis of African swine fever virus in clinical samples, *J. Clin. Microbiol.*, **41** (9), 4431–4434.
- AGÜERO M., FERNÁNDEZ J., ROMERO L., ZAMORA M.J., SANCHEZ C., BELÁK S., ARIAS M. & SÁNCHEZ-VIZCAÍNO J.M. (2004). A highly sensitive and specific gel-based multiplex RT-PCR assay for the simultaneous and differential diagnosis of African swine fever and Classical swine fever. *Vet. Res.*, **35**, 1–13.
- ARIAS M. & SÁNCHEZ-VIZCAÍNO J.M. (2002a). African swine fever. *In: Trends in Emerging Viral Infections of Swine*, Iowa State University press, pp 119–124. ISBN 0813803837.
- ARIAS M. & SÁNCHEZ-VIZCAÍNO J.M. (2002b). African swine fever eradication: the Spanish model. *In: Trends in Emerging Viral Infections of Swine*, Iowa State University Press, pp 133–139. ISBN 0813803837.
- BASTO A.P., PORTUGAL R.S., NIX R.J., CARTAXEIRO C., BOINAS F., DIXON L.K., LEITAO A. & MARTINS C. (2006). Development of a nested PCR and its internal control for the detection of African swine fever virus (ASFV) in *Ornithodoros erraticus*. *Arch. Virol.*, **151** (4), 819–826.
- BLASCO R., AGÜERO M., ALMENDRAL J.M. & VIÑUELA E. (1989). Variable and constant region in African swine fever virus DNA. *Virology*, **168**, 330–338.
- BOOL P.H., ORDAS A. & SANCHEZ BOTIJA C. (1969). El diagnostico de la peste porcina africana por inmunofluorescencia. (The diagnosis of African swine fever by immunofluorescence). *Bull. OIE*, **72**, 819–839.
- BOSHOF C.I., BASTOS A.D., GERBER L.J. & VOSLOO W. (2007). Genetic characterisation of African swine fever viruses from outbreaks in southern Africa (1973–1999). *Vet. Microbiol.*, **121** (1–2), 45–55.
- CARRILLO C., BORCA M.V., AFONSO C.L., ONISK D.V. & ROCK D.L. (1994). Long-term persistent infection of swine monocytes/macrophages with African swine fever virus. *J. Virol.*, **68**, 580–583.
- CHAPMAN D.A., TCHEREPANOV V., UPTON C. & DIXON L.K. (2008). Comparison of the genome sequences of non-pathogenic and pathogenic African swine fever virus isolates. *J. Gen. Virol.*, **89**, 397–408.
- DEVILLIER E.P., GALLARDO C., ARIAS M., DA SILVA M., UPTON C., MARTIN R. & BISHOP R.P. (2010). Phylogenomic analysis of 11 complete African swine fever virus genome sequences. *Virol.*, **400** (1), 128–136.
- DIXON L.K., ESCRIBANO J.M., MARTINS C., ROCK D.L., SALAS M.L. & WILKINSON P.J. (2005). *In: Virus Taxonomy*, VIIIth Report of the ICTV, Fauquet C.M., Mayo M.A., Maniloff J, Desselberger U. & Ball L.A., eds. Elsevier/Academic Press, London, UK. 135–143.

ESCRIBANO J.M., PASTOR M.J., ARIAS M. & SANCHEZ VIZCAINO J.M. (1990). Confirmación de sueros positivos a ELISA- peste porcina africana, mediante la técnica de 'Immunoblotting'. Utilización de las proteínas inducidas por el virus con pesos moleculares comprendidos entre 23 y 35 kilodaltons, en el desarrollo de un 'kit' de diagnóstico. (Confirmation of sera positive by ASF ELISA with the immunoblotting technique. Use of virus-induced proteins of 23–25 kDa in the development of a diagnostic kit.) *Med. Vet.*, **7**, 135–141.

ESCRIBANO J.M., PASTOR M.J. & SANCHEZ VIZCAINO J.M. (1989). Antibodies to bovine serum albumin in swine sera: implications for false positive reactions in the sero diagnosis of African swine fever. *Am. J. Vet. Res.*, **50**, 1118–1122.

FERNÁNDEZ-PINERO J., GALLARDO C., ELIZALDE M., RASMUSSEN T.B., STAHL K., LOEFFEN W., BLOME S., BATTEN C., CROOKE H., LE POTIER M.F., UTTENTHAL Å., LEBLANC N., ALBINA E., KOWALCZYK A., MARKOWSKA-DANIEL I., TIGNON M., DE MIA G.M., GIAMMARIOLI M., ARIAS M. & HOFFMANN B. (2010). EPIZONE ring trial on ASFV real-time PCR. Annual Meeting of National African Swine Fever Laboratories, 18 May 2010, Pulawy, Poland.

GALLARDO C., BLANCO E., RODRÍGUEZ M.J., CARRASCOSA A. & SANCHEZ-VIZCAINO J.M. (2006). Antigenic properties and diagnostic potential of African swine fever virus protein pp62 expressed in insect cells. *J. Clin. Microbiol.*, **44** (3), 1489–1495.

GALLARDO C., MWAENGO D.M., MACHARIA J.M., ARIAS M., TARACHA E.A., SOLER A., OKOTH E., MARTÍN E., KASITI J. & BISHOP R.P. (2009). Enhanced discrimination of African swine fever virus isolates through nucleotide sequencing of the p54, p72, and pB602L (CVR) genes. *Virus Genes*, **38** (1), 85–95.

GALLARDO C., SOLER A., SIMÓN A., MARTÍN E., MARTÍN R., PELAYO V., OKOTH E., BISHOP R., SÁNCHEZ M.A., DE MIA G., FASINA F.O., SÁNCHEZ-VIZCAÍNO, J.M. & ARIAS M. (2010). African Swine Fever Threat: Evaluating diagnostic tools with ASFV circulating strains. Annual Meeting of National African Swine Fever Laboratories, 18 May 2010, Pulawy, Poland.

KING D.P., REID S.M., HUTCHINGS G.H., GRIERSON S.S., WILKINSON P.J., DIXON L.K., BASTOS A.D.S. & DREW T.W. (2003). Development of a TaqMan® PCR assay with internal amplification control for the detection of African swine fever virus. *J. Virol. Methods*, **107**, 53–61.

LUBISI B.A., BASTOS A.D., DWARKA R.M. & VOSLOO W. (2005). Molecular epidemiology of African swine fever in East Africa. *Arch Virol.*, **150** (12), 2439–2452.

MALMQUIST W.A. & HAY D. (1960). Haemadsorption and cytopathic effect produced by African swine fever virus in swine bone marrow and buffy coat cultures. *Am. J. Vet. Res.*, **21**, 104–108.

PAN I.C., TRAUTMAN R., HESS W.R., DE BOER C.J., TESSLER J., ORDAS A., SANCHEZ BOTIJA C., OVEJERO J. & SANCHEZ M.C. (1974). African swine fever: comparison of four sero tests on porcine serums in Spain. *Am. J. Vet. Res.*, **35**, 787–790.

PASTOR M.J., ARIAS M. & ESCRIBANO J.M. (1990). Comparison of two antigens for use in an enzyme-linked immunosorbent assay to detect African swine fever antibody. *Am. J. Vet. Res.*, **51**, 1540–1543.

PASTOR M.J., LAVIADA M.D., SANCHEZ VIZCAINO J.M. & ESCRIBANO J.M. (1989). Detection of African swine fever virus antibodies by immunoblotting assay. *Can. J. Vet. Res.*, **53**, 105–107.

SÁNCHEZ-VIZCAÍNO J.M. (1987). African swine fever diagnosis. In: African Swine Fever, Becker Y., ed. Martinus Nijhoff, Boston, USA, 63–71.

SÁNCHEZ-VIZCAÍNO J.M. (2006). African swine fever. In: Diseases of Swine, Ninth Edition, Straw B., D'Allaire S., Mengeling W., Taylor D., eds. Iowa State University, USA, pp. 291–298.

SÁNCHEZ-VIZCAÍNO J.M., CROWTHER J.R. & WARDLEY R.C. (1983). A collaborative study on the use of the ELISA in the diagnosis of African swine fever. In: African Swine Fever. (CEC/FAO Research Seminar, Sardinia, Sept. 1981). Wilkinson P.J., ed. Commission of the European Communities Publication EUR 8466 EN, 297–325.

SANCHEZ-VIZCAINO J.M., MARTINEZ-LOPEZ B., MARTINEZ-AVILES M., MARTINS C., BOINAS B., VIAL L., MICHAUD V., JORI F., ETTER E., ALBINA E. & ROGER F. (2009). Scientific Review on African Swine Fever. CFP/EFSA/AHAW/2007/02, pp: 1–141.

VINUELA E. (1985). African swine fever. *Curr. Top. Microbiol. Immunol.*, **116**, 151–170.

WARDLEY R.C., ABU ELZEIN E.M.E., CROWTHER J.R. & WILKINSON P.J. (1979). A solid-phase enzyme-linked immunosorbent assay for the detection of African swine fever antigen and antibody. *J. Hyg.*, **83**, 363–369.

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NB: There are OIE Reference Laboratories for African 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 and reagents for African swine fever

A. INTRODUCTION

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Paragraph

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