SECTION 3.9.

SUIDAE

CHAPTER 3.9.1.

AFRICAN SWINE FEVER (INFECTION WITH AFRICAN SWINE FEVER VIRUS)

SUMMARY

African swine fever (*ASF*) *is an infectious disease of domestic and wild pigs of all breeds and ages, caused by ASF virus* (*ASFV*). *The clinical syndromes vary from peracute, acute, subacute to chronic, depending on the virulence of the virus. 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 ASFV. The virus is present in tick salivary glands and passed to new hosts* (*domestic or wild suids*) *when feeding. It can be transmitted sexually between ticks, transovarially to the eggs, or transtadially throughout the tick's life.*

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

Laboratory diagnostic procedures for ASF fall into two groups: detection of the virus and serology. 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 inoculation of pig leukocyte or bone marrow cultures, the detection of antigen in smears or cryostat sections of tissues by fluorescent antibody test and/or the detection of genomic DNA by the polymerase chain reaction* (*PCR*) *or real-time PCR. The PCRs are excellent, highly sensitive, specific and rapid techniques for ASFV detection and are very useful under a wide range of circumstances. They are especially useful if the tissues are unsuitable for virus isolation and antigen detection. In doubtful cases, the material is passaged in leukocyte 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 or moderate 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 the enzyme-linked immunosorbent assay* (*ELISA*)*, the indirect fluorescent antibody test* (*IFAT*)*, the indirect immunoperoxidase test* (*IPT*), *and the immunoblotting test* (*IBT*) *is available for antibody detection.*

Requirements for vaccines*: At present, there is no vaccine for ASF.*

A. INTRODUCTION

The current distribution of African swine fever (ASF) extends across more than 50 countries in three continents (Africa, Asia and Europe). Several incursions of ASF out of Africa were reported between the 1960s and 1970s. In 2007, ASF was introduced into Georgia, from where it spread to neighbouring countries including the Russian Federation. From there ASF spread to eastern European countries extending westwards and reaching the

European Union in 2014. Further westward and southern spread in Europe has occurred since that time. In all these countries, both hosts – domestic pig and wild boar – were affected by the disease. In August 2018, the People's Republic of China reported its first outbreak of ASF and further spread in Asia has occurred.

ASF virus (ASFV) is a complex large enveloped DNA virus with icosahedral morphology. It is currently classified as the only member of the *Asfaviridae* family, genus *Asfivirus* (Dixon *et al.*, 2005). More than 60 structural proteins have been identified in intracellular virus particles (200 nm) (Alejo *et al.,* 2018). More than a hundred infection-associated proteins have been identified in infected porcine macrophages, and at least 50 of them react with sera from infected or recovered pigs (Sánchez-Vizcaíno & Arias, 2012). The ASFV double-stranded linear DNA genome comprises between 170 and 193 kilobases (kb) and contains between 150 and 167 open reading frames with a conserved central region of about 125 kb and variable ends. These variable regions encode five multigene families that contribute to the variability of the virus genome. The complete genomes of several ASFV strains have been sequenced (Bishop *et al.,* 2015; Chapman *et al.,* 2011; De Villiers *et al.,* 2010; Portugal *et al.,* 2015). 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 3' terminal end of the B646L open reading frame encoding the p72 protein major capsid protein, which differentiates up to 24 distinct genotypes (Achenbach *et al*., 2017; Boshoff *et al.,* 2007; Quembo *et al.* 2018). To distinguish subgroups among closely related ASFV, sequence analysis of the tandem repeat sequences (TRS), located in the central variable region (CVR) within the B602L gene (Gallardo *et al.,* 2009; Lubisi *et al.,* 2005; Nix *et al.,* 2006) and in the intergenic region between the I73R and I329L genes, at the right end of the genome (Gallardo *et al.,* 2014), is undertaken. Several other gene regions such as the E183L encoding p54 protein, the CP204L encoding p30 protein, and the protein encoded by the EP402R gene (CD2v), have been proved as useful tools to analyse ASFVs from different locations and hence track virus spread.

ASF viruses produce a range of syndromes varying from peracute, acute to chronic disease and subclinical infections. Pigs are the only domestic animal species that is naturally infected by ASFV. European wild boar 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 (Costard *et al*., 2013; Sánchez-Vizcaíno, *et al.,* 2015).

The incubation period 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. Case fatality 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. Moderately virulent strains are recognised that induce variable disease forms, ranging from acute to subacute. Low virulence, non-haemadsorbing strains can produce 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 that have recovered from either acute, subacute or chronic infections may potentially become persistently infected, acting as virus carriers. The biological basis for the persistence of ASFV is still not well understood, nor it is clear what role persistence plays in the epidemiology of the disease.

ASF cannot be differentiated from classical swine fever (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 the inoculation of pig leukocyte or bone marrow cultures, the detection of genomic DNA by polymerase chain reaction (PCR) or the detection of antigen in smears or cryostat sections of tissues by direct fluorescent antibody test (FAT). Currently the PCR is the most sensitive technique and can detect ASFV DNA from a very early stage of infection in tissues, ethylene diamine tetra-acetic acid (EDTA)-blood and serum samples. The PCR is particularly useful if samples submitted are unsuitable for virus isolation and antigen detection because they have undergone putrefaction. Pigs that have recovered from acute, subacute 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. Virus isolation by the inoculation of pig leukocyte or bone marrow cultures and identification by haemadsorption tests (HAD) are recommended as a confirmatory test when ASF is positive by other methods, particularly in the event of a primary outbreak or a case of ASF.

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, particularly in subacute and chronic forms.

ASF epidemiology is complex with different epidemiological patterns of infection occurring in Africa and Europe. ASF occurs through transmission cycles involving domestic pigs, wild boar, wild African suids, and soft ticks (Sánchez-Vizcaíno *et al.,* 2015). 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 (Costard *et al*., 2013).

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

ASFV should be handled with an appropriate level of bio-containment, determined by risk analysis in accordance with Chapter 1.1.4 *Biosafety and biosecurity: Standard for managing biological risk in the veterinary laboratory and animal facilities*.

B. DIAGNOSTIC TECHNIQUES

Table 1. *Test methods available and their purpose*

Method	Purpose					
	Population freedom from infection	Individual animal freedom from infection prior to movement	Contribute to eradication policies	Confirmation of clinical cases	Prevalence of infection – surveillance	Immune status in individual animals or populations post-vaccination
Agent identification						
Virus isolation/ HAD test[1]	n/a	n/a	++	+++	++	–
FAT	n/a	n/a	++	++	+	–
ELISA for antigen detection	+	++	+	+	+	–
Conventional PCR	++	++	++	++	++	–
Real-time PCR	+++	+++	+++	+++	+++	–
Detection of immune response						
ELISA	+++	+++	+++	+	+++	–
IPT*	+++	+++	+++	+	+++	–
IFAT*	+++	+++	+++	+	+++	–
IBT*	++	++	++	+	++	–

Key: +++ = recommended for this purpose; ++ recommended but has limitations;
+ = suitable in very limited circumstances; – = not appropriate for this purpose.
HAD = haemadsorption; FAT= fluorescent antibody test; ELISA = enzyme-linked immunosorbent assay;
PCR = polymerase chain reaction; IPT= indirect immunoperoxidase test;
IFAT = indirect fluorescent antibody test; IBT = immunoblotting test.
*Recommended method as confirmatory serological test.

1 As some current ASF virus isolates are non-haemadsorbing, negative HAD results should be confirmed using other tests such as PCR.

1. Identification of the agent

Where ASF is suspected, the following samples should be sent to the laboratory: blood in anticoagulant (EDTA for PCR, heparin or EDTA for virus isolation), serum and tissues, mainly spleen, lymph nodes, bone marrow, lung, 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. Virus isolation

1.1.1. Sample preparation

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. Alternatively, specimens can be prepared by placing tissues in tubes containing buffer or medium and sterile beads or shards then homogenising using a homogeniser.

ii) Clarify the suspensions by centrifugation at 1000 ***g*** for 5 minutes. Use the supernatant for virus isolation.

1.1.2. Haemadsorption test

The haemadsorption (HAD) test (de León *et al.,* 2013) 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 have a HAD phenotype. A positive result in the HAD test is definitive for ASF diagnosis. A small number of 'non-haemadsorbing' viruses have been isolated, most of which are attenuated or avirulent, 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 from the blood of naive pigs or into alveolar macrophages cell cultures. It is essential to carry out all procedures in such a way as to prevent contamination of the cultures.

1.1.2.1 Test procedure 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. 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 ×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 Non-inoculated 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, subculture the supernatant up to three times into fresh leukocyte cultures. All isolations should be confirmed by PCR and sequencing.

1.1.3. Virus isolation in porcine bone marrow cells

In circumstances where a reliable or consistent supply of defibrinated pig blood cannot be achieved for the HAD test, cultures of primary bone marrow cells may be used as an alternative culture system for the isolation of ASFV. Infected cells are detected by indirect FAT. The advantage of this detection method is that it is not affected by HAD phenotype. This method can also be employed using porcine primary leukocyte or alveolar macrophages cultures, using the procedures described above, substituting HAD detection with pig erythrocytes with FAT.

1.1.3.1 Test procedure in primary bone marrow cell cultures

i) Seed a 25 cm^2 tissue culture flask with 5×10^7 cells/ml in culture medium containing 12.5% fetal calf serum (FCS) and antibiotics and incubate at 37°C for 3 days.

ii) Carefully remove the media. As the monolayer can be easily disturbed, it may be necessary to centrifuge the media (1000 ***g***, 3 minutes) to recover dislodged cells if significant disruption of the monolayer occurs. Inoculate the flask with 1 ml of test specimen and incubate for 1 hour at 37°C.

iii) Remove the inoculum and gently wash the monolayer with PBS. Perform an additional centrifugation step of the inoculum and PBS if significant numbers of cells were removed during this step.

iv) Add fresh media to the flask and incubate for 5–7 days at 37°C in a humidified incubator with 5% CO_2.

v) Harvest the cell culture supernatant by transferring into a centrifuge tube, centrifuge to remove cellular debris and freeze at –70°C for storage or subsequent subculture.

vi) Fluorescence antibody staining of ASFV antigens can be performed on infected cells from the flask that have been dried onto a positively charged microscope slide. First, scrape cells from the surface of the flask using a sterile cell scraper. Aliquot into a centrifuge tube, then pellet the cells at 1000 ***g***, 3 minutes.

vii) Resuspend the cell pellet in 1 ml cell culture media or PBS and deposit 300 µl onto a slide, air dry and fix with acetone for 10 minutes at room temperature.

viii) Wash by immersing slides 3–4 times in fresh PBS, then stain with fluorescein isothiocyanate (FITC)-conjugated anti-ASFV immunoglobulin at the recommended or pre-titrated dilution for 1 hour at 37°C in a humid chamber.

ix) Fix and stain positive and negative control slides similarly.

x) Wash by immersing slides 3–4 times in fresh PBS, mount stained cells in PBS/glycerol, and examine under an upright ultraviolet light microscope with suitable barrier and excitation filters.

xi) *Reading the results*

Test specimens are positive if specific cytoplasmic fluorescence is observed in inoculated porcine marrow cells.

1.2. Antigen detection by fluorescent antibody test

The FAT 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 detecting antibody conjugate (Sánchez-Vizcaíno & Arias, 2012).

1.2.1. 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.

1.3. 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 in use have been shown to be particularly useful for identifying viral 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. Due to its high sensitivity and specificity, together with the possibility for a high throughput application, the PCR is a recommended method for screening and confirmation of suspected cases.

A number of conventional and real-time PCR methods have been described (Agüero *et al.,* 2003; Basto *et al.,* 2006; Fernández-Pinero *et al.,* 2013; King *et al.,* 2003; Tignon *et al.,* 2011) and several commercial PCR kits are available for ASFV detection, including one formally registered with OIE[2]. Duplex RT-PCR techniques have also been described for simultaneous and differential detection of ASFV and CSFV (Agüero *et al.,* 2004; Haines *et al.,* 2013). Any PCR protocol used must have been validated as fit for the chosen purpose, in accordance with Chapter 1.1.6 *Principles and methods of validation of diagnostic assays for infectious diseases* and Chapter 2.2.3 *Development and optimisation of nucleic acid detection assays,* for further details on PCR techniques.

Three validated PCR procedures are described below (Agüero *et al.,* 2003; Fernández-Pinero *et al.,* 2013; King *et al.,* 2003), consisting 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.

1.3.1. Sample preparation procedure

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. Details are given in cited publications (Agüero *et al.,* 2003; Fernández-Pinero *et al.,* 2013; King *et al.,* 2003) and in current methods available from reference laboratories (see Table given in Part 4 of this *Terrestrial Manual*). The detection of ASFV can be performed in parallel to that for CSF virus (see Chapter 3.9.3 *Classical swine fever* for CSFV molecular detection methods).

Different samples can be processed for PCR analysis such as cell culture supernatants, EDTA-blood, serum and tissue homogenates. Blood swabs may be useful for sampling wild boar.

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

2 https://www.oie.int/en/scientific-expertise/registration-of-diagnostic-kits/background-information/

parallel (e.g. if organ homogenate looks turbid and/or is suspected to have excessive genomic DNA content.

Control samples for the DNA extraction step: at least one positive and one negative control should be included in each nucleic acid extraction run. The positive control sample should be ASFV-positive serum, EDTA-blood, or 1/10 tissue homogenates (of the same tissue type as the test samples). It is highly recommended that the positive control is prepared to be close to the detection limit of the technique to track the yield of the DNA extraction procedure (e.g. to give a cycle threshold [Ct] value of 32±2 in real-time PCR). The negative control could be water or ASFV-negative EDTA-blood, serum or tissue homogenate. Controls should be processed alongside the test samples. The quality of nucleic acid preparation may affect the efficiency of PCR amplification.

For DNA extraction, follow the kit manufacturer's instructions. Finally undertake the nucleic acid elution, preferably using molecular grade water (this will ensure that both DNA and RNA are recovered, in case an analysis of ASFV and CSFV is required). Use the eluted DNA immediately or store at –20°C for future use.

1.3.2. 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).

Control samples for the DNA amplification step: at least one positive and one negative control should be included in each PCR run. The positive control sample should be 2 µl of ASFV-positive DNA. It is highly recommended that the positive control should be close to the detection limit of the PCR technique to check the yield of the DNA amplification procedure (e.g. to give a Ct value of 32±2 in real-time PCR). The negative reaction control should be 2 µl of nuclease-free sterile water, or DNA extracted from ASFV-negative EDTA-blood, serum or tissue homogenate.

1.3.2.1 Stock solutions

i) Nuclease-free sterile water.

ii) Hot Start DNA polymerase, PCR Buffer, and magnesium chloride are commercially available.

iii) PCR nucleotide mix containing 10 mM of each dNTP is commercially available.

iv) Primers are prepared 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) Agarose 2% solution: agarose to prepare a 2% solution in TAE buffer 1× is commercially available.

viii) Molecular weight marker DNA: 100 base-pair ladder is commercially available.

ix) Double-stranded DNA intercalating dye is commercially available.

1.3.2.2. Conventional PCR protocol

i) In a sterile 1.5 ml microcentrifuge tube prepare the PCR reaction master mix for the number of samples to be assayed allowing for at least one extra sample.

ii) Prepare the PCR reaction mix to a final volume of 25 µl per sample, including the following reagents at the final concentrations indicated: 1 × PCR buffer, 2 mM magnesium chloride (this may be included in the PCR buffer), 0.2 mM dNTP mix, primer PPA-1, 20 pmol/µl (0.25 µl, final concentration 0.2 µM), primer PPA-2, 20 pmol/µl (0.25 µl, final concentration 0.2 µM), 0.625 U hot-start DNA polymerase and nuclease-free or sterile distilled water.

iii) Add 23 µl of the PCR reaction mix to the required number of 0.2 ml PCR tubes.

iv) Add 2 µl of extracted DNA 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.

Note: the thermocycling programme may vary depending on the DNA polymerase used, but the conditions indicated above serve as a reference and is the programme established in the original procedure (Agüero *et al.,* 2003).

vi) At the end of the programme, remove PCR tubes and add 2.5 µl of 10× loading buffer to each tube.

vii) Load all the samples in a 2% agarose gel in TAE buffer 1× containing DNA intercalating dye (suitable concentration depends on the dye used, follow manufacturer's instructions).

viii) Add marker DNA to one well on each of the gel.

ix) Run the gel at a constant voltage of 150–200 volts for about 30 minutes.

x) Gels are examined over a UV or blue 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 DNA marker. The PCR product of the positive control has a size of 257 base pairs. No bands should be seen in the negative control.

1.3.3. Real-time PCR procedure 1 (King *et al.,* 2003)

1.3.3.1. Stock solutions

i) Nuclease-free or another appropriate sterile water and PCR reaction master mix (2×).

ii) Primers are prepared at a concentration of 50 pmol/µl: Primer 1 sequence 5'-CTG-CTC-ATG-GTA-TCA-ATC-TTA-TCG-A-3' (positive strand); Primer 2 sequence 5'-GAT-ACC-ACA-AGA-TC(AG)-GCC-GT-3' (negative strand).

iii) Fluorescent-labelled hydrolysis probe is included at a concentration of 5 pmol/µl: (5'-[6-carboxy-fluorescein (FAM)]-CCA-CGG-GAG-GAA-TAC-CAA-CCC-AGT-G-3'-[6-carboxy-tetramethyl-rhodamine (TAMRA)]).

1.3.3.2. PCR amplification

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

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

ii) Add 22 µl PCR reaction mix to one well of an optical reaction plate for each sample to be assayed.

iii) Add 3 µl of extracted DNA 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 sequence detection system for PCR amplification (real-time PCR machine equipped with FAM fluorescence channel) 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 purpose-built 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 cycle threshold (Ct) 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 Ct value >40.0. Positive test samples and controls should have a Ct value < 40.0 (strongly positive samples have a Ct 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. One modification of this using a fast amplification protocol has been validated for diagnostic purposes by Fernández-Pinero *et al.,* 2010 (detailed protocol available in Fernández-Pinero *et al.,* 2013).

1.3.4. Real-time PCR procedure 2 (Fernández-Pinero *et al.,* 2013)

This real-time PCR method has been shown to exhibit the highest sensitivity for detection of ASFV DNA both at very early stages of infection and in long-term chronically infected animals where the viraemia level is usually quite low (Fernández-Pinero *et al.,* 2013; Gallardo *et al.,* 2015a).

1.3.4.1. Stock solutions

i) Nuclease-free sterile water.

ii) A range of real-time PCR kits is commercially available. Selected kits should be validated before use for diagnostic purposes.

iii) Primers are prepared at a concentration of 20 pmol/µl: primer ASF-VP72-F sequence 5'-CCC-AGG-RGA-TAA-AAT-GAC-TG-3' (forward primer); primer ASF-VP72-R sequence 5'-CAC-TRG-TTC-CCT-CCA-CCG-ATA-3' (reverse primer).

Note: the nucleotide code, R = A+G mixed base position.

iv) Labelled hydrolysis probe (10 pmol/µl, labelled with FAM reporter dye) is commercially available ready to use (see Fernández-Pinero *et al.,* 2013 for details).

Note: If access to the specific probe is not possible, it could be substituted by the following standard probe using identical concentration and reaction conditions: (5'-[6-carboxy-fluorescein (FAM)]-TCC-TGG-CCR-ACC-AAG-TGC-TT-3'-[black hole quencher (BHQ)])

1.3.4.2. PCR amplification

Control samples for the DNA amplification step: at least one positive and one negative control should be included in each PCR run. The positive control sample should be 2 µl of ASFV-positive DNA. It is highly recommended that the positive control should be close to the detection limit of the PCR technique to check the yield of the DNA amplification procedure (e.g. to give a Ct value of 32±2 in real-time PCR). The negative control should be 2 µl of nuclease-free sterile water.

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) The PCR reaction mixture includes: nuclease-free or sterile distilled water (7 µl), master mix 2× (10 µl), primer ASF-VP72-F, 20 pmol/µl (0.4 µl), primer ASF-VP72-R, 20 pmol/µl (0.4 µl), fluorescent-labelled hydrolysis probe 10 pmol/µl (0.2 µl).

iii) Add 18 µl of the PCR reaction mix to the required number of 0.2 ml optical PCR tubes.

iv) Add 2 µl of extracted DNA 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 a real-time PCR thermal cycler (equipped with FAM fluorescence channel) and run the following programme:

One cycle at 95°C for 5 minutes.

45 cycles at 95°C for 10 seconds, 60°C for 30 seconds. Programme the fluorescence collection in FAM channel at the end of each cycle.

Note: the incubation programme may vary depending on the DNA polymerase used, but the indicated one serves as a general one and it is the programme established in the original procedure publication (Fernández-Pinero *et al.,* 2013).

vi) Reading the results

The point where the fluorescence measurement is above the background signal and reaches the detectable level is called the cycle threshold (Ct), and this is determined automatically by the PCR equipment software. It will be the starting fluorescence point for considering a sample as positive.

In a positive sample, a sigmoid-shaped amplification curve will be obtained where the Ct value will be <40. Samples giving a Ct value ≥38 should be considered as doubtful if a sigmoidal plot is observed and the analysis should be repeated for confirmation. A negative sample will maintain the fluorescence profile under background fluorescence level and the equipment will not report any Ct value.

Samples reporting a Ct value >40 can be considered as negative unless the results of serological techniques or the epidemiological information suggest the possibility of ASFV infection. In this case, the analysis should be repeated to confirm the negative PCR result.

1.4. Antigen detection enzyme-linked immunosorbent assay

Antigen detection ELISA may be performed as an alternative method but, as its sensitivity is much lower than PCR or HAD, it should not be used as the only method for virus detection and results should be confirmed by PCR or HAD. A commercial kit is available for use on specimens of porcine blood, spleen or lymph nodes, in accordance with recommendations of the kit manufacturer. The kit uses a double antibody sandwich ELISA. An MAb specific for the virus capsid protein is coated on the plate and binds to ASFV in positive samples. After washing a second MAb specific to a different epitope on the capsid protein and conjugated to peroxidase is added. Binding of this second antibody to the captured antigen is detected by adding an appropriate substrate.

2. Serological tests

Serological assays are the most commonly used diagnostic tests due to their simplicity, relatively low cost and the fact that they require little specialised equipment or facilities. For ASF diagnosis, this is particularly relevant given that no commercial vaccine is available against ASFV, which means that the presence of anti-ASFV antibodies always indicates infection. Furthermore, anti-ASFV antibodies appear soon after infection (7–10 days) and persist for several months or even years. Domestic pigs and wild boar infected with virulent strains usually die before a specific antibody immune response is raised. In areas with a well established ASFV infection, where attenuated and low virulent virus isolates are also circulating, serological detection is crucial for identifying recovered and asymptomatically infected animals. There are no fully neutralising antibodies.

The most commonly used is the ELISA (Gallardo *et al*., 2015b; Sánchez-Vizcaíno, 1987), which is suitable for examining serum or plasma. Confirmatory testing of ELISA-positive samples should be carried out using an alternative test, such as the IFAT, IPT or immunoblotting (Gallardo *et al.,* 2015b; 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, A penside test (lateral flow assay) for antibody detection has been validated and is commercially available.

In recent years, extensive studies were carried out to assess the specificity and sensitivity of ASF serological tests in the different epidemiological scenarios of Africa and Europe. These studies included currently circulating ASFV genotype II isolates in Eastern Europe and the eastern African isolates exhibiting more variability. The results showed the OIE tests recommended to certify animals prior to movement (see Table 1) are able to detect the presence of antibodies to ASFV in all the epidemiological situations evaluated with accuracy and suitable sensitivity (Gallardo *et al.,* 2013; 2015b).

Where ASF is endemic, confirmation of suspected cases of disease can be done using a standard serological test (ELISA), combined with an alternative serological test (IFAT, IPT, IBT) and an antigen-detection test. In some countries, over 95% of positive cases have been identified using a combination of IFATs and FAT.

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 ELISA is a direct test that can detect antibodies to ASFV in pigs that have been infected by viruses of low or moderate virulence. Currently, several commercial ELISA kits, based on a competition or indirect format, are available for the detection of antibodies to ASFV, validated for use under different

epidemiological situations. A cheaper alternative is to prepare a soluble antigen for use in an indirect ELISA, and procedure using this soluble antigen is described below.

Carrying out a second confirmatory test such as the IBT, IFAT or IPT 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 cm^2 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 mMTris/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 96-well ELISA micro 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).

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 200 µl of substrate DMAB/MBTH to each well prepared as follows:

The volume required per plate is 10 ml of DMAB 80.6 mM Solution + 10 ml of MBTH 1.56 mM solution + 5 µl H_2O_2 30%.

a) DMAB/MBTH substrate preparation

DMAB – 3-dimethylaminobenzoic acid; MBTH – 3-methyl-2-benzothiazolinone hydrazone hydrochloride monohidrate.

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.

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.

Alternatively 0.04% orthophenylene-diamine (OPD) can be used instead of DMAB/MBTH. It is prepared in phosphate/citrate buffer, pH 5.0 at the rate of 10 µl/25 ml. Add 100 µl of substrate to each well. **Note:** when OPD substrate is used, the number of false positive reactions is increased.

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 600–620 nm (in case of DMAB/MBTH) or 492 nm (OPD substrate) in an ELISA reader. 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. 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.

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 × 1 + optical density positive serum × 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 ± 0.1 can be considered inconclusive and the result needs to be confirmed by the IPT, IFAT or IBT.

This indirect ELISA procedure has been improved and validated and has higher sensitivity than that obtained with the previous procedure for serum samples collected at earlier stages of infection, by adjusting the incubation time, incubation temperatures, buffers, concentrations of the antigen and the samples, as well as the type and concentration of the conjugate and substrate (Fernández-Pacheco *et al.*, 2016). Briefly, test and control sera are diluted 1/10 in blocking buffer (PBS containing 0.05% Tween 20, 2% skim milk and 2% normal porcine serum), and one well of the ELISA plate is left blank as a control well (100 µl of blocking buffer). Sera are incubated for 2 hours at 37±2°C on a plate shaker. Pre-titrated conjugate at a working dilution (suggested range 1:5000–1:20000 of protein A, 1 mg/ml) is maintained for 45 minutes at 37±2°C on a plate shaker. Finally, a new substrate is used, adding 100 µl/per well of substrate solution (ABTS [2,2'-azino-di(3-ethyl-benzothiazoline)-6-sulfonic acid]-diammonium salt) and incubating for 30 minutes at room temperature in the dark. The reaction is stopped by the addition of 100 µl/well stopping solution (1% sodium dodecyl sulphate) and results are obtained at 405 nm wavelength. For interpretation of the results, the test is validated when the mean of the optical density obtained in the blank (ODblank) is <0.250. A value cut-off (VCO) is calculated as mean OD NC (optical density negative control) × 2. The index cut-off (ICO) will define the result and is calculated as ICO = (mean OD sample)/VCO. Serum samples with ICO ≤1 are considered as negatives, ICO >1 ≤1.25 as doubtful, and serum samples with ICO >1.25 are considered as positives.

2.2. Indirect Immunoperoxidase test (IPT) (Gallardo *et al.,* 2015b)

The IPT is an immune-cytochemistry technique on fixed cells to determine the antibody–antigen complex formation through the action of the peroxidase enzyme. In this procedure, African green monkey kidney (Vero) or MS cells are infected with ASFV isolates adapted to these cell cultures. The infected cells are fixed and are used as antigens to determine the presence of the specific antibodies against ASF.

IPT 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. Given its superior sensitivity and its performance, this is the best test to test blood, fluids or exudate tissue samples (Gallardo *et al.,* 2015b).

2.2.1. Preparation of 96-well plates coated with fixed ASFV adapted viruses

i) Subculture MS or Vero cells at a 1:2 split ratio and distribute the diluted cell suspension into the required number of 96-microwell plates (growth area/well 0.32 cm^2).

ii) Incubate for 24 (Vero) or 48 (MS) hours at 37±3°C in a 5% CO_2 humidified incubator to get an 80–90% confluent plate. After 24 or 48 hours' incubation, carefully decant the medium of the cell cultures grown in the 96-microwell plates.

iii) In a separate bottle prepare the appropriate dilution (in culture medium without FCS) of the adapted ASFV to inoculate with an m.o.i. (multiplicity of infection) between 0.025 and 0.05.

The dilution factor is calculated using the following formula:

$$\text{Dilution factor} = \frac{0.7 \times \text{virus titre} \times \text{volume}}{\text{Number of cells} \times \text{m.o.i}}$$

iv) Inoculate the plates with 100 µl/well of the inoculum and incubate at 37±3°C in 5% CO_2 humidified incubator for:

a) 18±1 hours for Vero infected cell plates.

b) 24±1 hours for MS infected cell plates: In this case, after 2 hours at 37±3°C, complete the volume to 200 µl with medium +4% FCS (final concentration of 2% SFBi).

v) Fix the cells: the inocula are removed by vacuum suction and the ASFV-infected cell sheets are fixed with a cold solution containing 70% methanol and 30% acetone for 8±2 minutes at room temperature. Finally, the plates are washed with PBS 3–5 times for 5 minutes each, on a plate shaker.

vi) The fixed and dry IPT plates can be used directly or stored at <–10°C.

2.2.2. IPT procedure

i) Keep the ASFV IPT plates at room temperature (18–25°C) for 30 minutes after defrosting.

ii) Blocking step: Block the plates by adding 100 µl per well of blocking solution (PBS/0.05%Tween 20, pH 7.2 [±0.2]/milk 5%). Incubate for 1 hour at 37±2°C on a plate shaker.

iii) Sample pre-incubation: In a separate 96-well microtitre plate dilute at 1/40 the samples (sera, blood, fluid or exudate tissues) and controls (positive, limit and negative) in blocking solution (PBS/0.05% Tween 20, pH 7.2 [±0.2]/milk 5%) containing 2% SFB. Add 100 µl per well and incubate for 1 hour at 37±2°C on a plate shaker.

iv) Sample incubation: After 1 hour discard the blocking solution from the ASFV-IPT-plates and add 100 µl per well of the pre-incubated samples and controls. Incubate for 45 minutes at 37±2°C on a plate shaker.

v) Washing step: Wash three times with 100 µl/per well of PBS 1× for 5 minutes at 37±2°C on a plate shaker.

vi) Add 100 µl of protein A peroxidase conjugate per well diluted at 1/5000 in blocking solution. Incubate for 45 minutes at 37±2°C on a plate shaker.

vii) Washing step: Wash three times with 100 µl/per well of PBS 1× for 5 minutes at 37±2°C on a plate shaker.

viii) Add 50 µl/well of substrate solution and incubate 5–10 minutes at room temperature (18–25°C).

Substrate solution: The substrate solution must be prepared when it is going to be used. Mix 300 µl of stock solution in 5 ml of acetate buffer + 5 µl of H_2O_2 (this volume is recommended for one 96 well plate).

a) Stock solution (20 mg/one tablet AEC (3-amino-9-etilcarbazol) in 2.5 ml dimethylformamide (keep at 4 ±3°C in the dark).

b) Acetate buffer 74 ml solution A + 176 ml solution B. Store at room temperature. Expiry date 6 months.

1. Solution A: 0.2 N acid acetic glacial (1.155 ml acetic in 100 ml water). Store at room temperature.

2. Solution B: 0.2 M sodium acetate (2.72 g [±0.05] AcNa tri-hydrated in 100 ml water). Store at room temperature.

ix) Add PBS 1 × 100 µl/per well to stop the reaction.

x) Reading the results: In the wells with positive samples against ASF, an intense red cytoplasmic coloration will be observed in the ASFV-infected cells. The red cytoplasmic coloration is interpreted as a positive result against ASF and the absence as a negative result.

In some specific situations related to samples collected from vaccinated animals against other diseases, some slight background can be observed with a nonspecific red coloration in the wells. In these cases the samples must be analysed against non-infected cells in parallel with the infected cells.

2.3. Indirect fluorescent antibody test

This test (Sánchez-Vizcaíno, 1987) should be used as a confirmatory test for sera from areas that are presumed free from ASF and are positive in the ELISA, and for sera from endemic areas that give an inconclusive result in the ELISA.

2.3.1. 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.

2.4. Immunoblotting test (Pastor *et al.,* 1989)

This test should be used as an alternative to the IFAT and IPT to confirm equivocal results with individual sera. It gives suitable results for weak-positive samples for ASF antibody detection from the second week post-infection. 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.

2.4.1. 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 cm^2 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.

2.4.2. 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% H_2O_2 (hydrogen peroxide).

2.4.3. 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 commercially available vaccine for ASF.

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NB: There are OIE Reference Laboratories for African swine fever
(please consult the OIE Web site:
https://www.oie.int/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3).
Please contact the OIE Reference Laboratories for any further information on
diagnostic tests and reagents for African swine fever

NB: FIRST ADOPTED IN 1990. MOST RECENT UPDATES ADOPTED IN 2021.