

Analysis of ASF field samples

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1 Introduction

This document has been put together in collaboration of partners involved in the Wellcome Trust (WT) African swine fever (ASF) project and compiles experiences gained in previous projects on ASF virus. The document aims at giving guidelines of how to transport samples and lists laboratory protocols that are recommended to apply for the analysis of samples collected in the field in the context of the WT ASF project.

For most of the ASF diagnostic tests Sensitivity (Se) and Specificity (Sp) have never been established using a representative sample, a fact that hinders the interpretation of results in prevalence studies. Therefore one of the objectives is to evaluate the performance of these tests. To do so, samples from the same animal need to be analysed by independent methods. This may seem to be redundant at first sight, however we need to do this in order to have accurate data for further analysis and the quality of the interpretation of results will substantially be improved. Se and Sp of the various tests will be determined using the so called 'latent class analysis', a Bayesian based approach which is used in the absence of a gold standard. This approach allows the assessment of the performance of several tests when applied in two different populations, preferably with different disease prevalence. As we need to get as much data as possible to run this analysis in the first place, it is most of all important in the early stages of the project to analyse the samples with various methods. As soon as we know more on the performance of each of the diagnostic methods, the number of different tests that are run on each sample will be reduced. The list below gives the tests and the respective protocol we think are useful to perform. In each section the tests are listed according to priority. We strongly recommend that all partners use the same protocols as this will facilitate comparisons of the results and to determine the Se and Sp of the tests. Ideally of each sampled animal a blood sample, blood on filter paper and a tissue samples are taken.

We also plan to organise a ring test in autumn to assess the agreement between the laboratories involved. In addition, we recommend to laboratories in Madagascar, Senegal, Mozambique and DR Congo to send at least 10% of all samples to the Onderstepoort Veterinary Institute (OVI) (from Mozambique and Madagascar) and to the Institute for Animal Health (IAH) (from Senegal and DR Congo). The samples will be retested for test confirmation and p72 and 9RL (B602L0) of the positive samples will be sequenced for molecular epidemiological investigations.

The following protocols and the most recent version of this document can be downloaded from the ASF network website (www.asfnetwork.org)

2 Collection, transport and storage of samples

2.1 Blood samples

2.1.1 Blood (serum)

Collect 5ml blood in standard Vacutainers, if possible collect two samples to have sufficient serum for eventual retesting. Store upside down and leave at room temperature for 12-24h. Remove the blood clot and store the serum at 4°C (in a cold box surrounded

by ice packs) during transport to the lab. Once at the lab, the serum should be stored at -20°C or -80°C for long term preservation.

For wild pigs, it is also recommended to collect blood stored in tubes with anticoagulant (EDTA) (two tubes per animal). Store cool if possible during transport. Freeze at -70°C as soon as possible to retain virus infectivity. Infectivity is destroyed by storage at -20°C, but may be retained for weeks to months if stored at 4°C and antibodies and DNA should be detected over longer periods of possibly several months with storage at 4°C.

2.1.2 Filter papers

Whatman 3M filter paper. Collect ~ 100 µl (6-10 drops) blood on 1 X 5 cm strips and air dry for at least 15 minutes at room temp. Store in sealed plastic bags with silica gel desiccant. If possible, transport and store samples at 4°C (stable at up to 25°C for ~ 6 months). Take care to ensure that no moisture reaches the samples due to melting ice or condensation.

Whatman FTA filter paper. Collect ~ 100 µl (6-10 drops) blood on individual filter papers and dry for at least one hour (not in the sun). Follow the spotting and drying instructions in the protocol supplied with the filter papers. Use sealed plastic bags with silica gel for transport at 4°C. Store the filter papers in plastic bags containing silica gel preferably at 4°C, if not possible samples are stable at up to 25°C for ~ 6 months.

2.2 Tissue samples

If possible, collect spleen samples. Alternatively, take lymph nodes samples.

Collect samples of approximately 1cm diameter. Add a PBS solution containing antibiotics (100 µg/ml penicillin and streptomycin) and store at 4°C during transport. If antibiotics are not available, samples can be transported in 50% glycerol and 50% PBS, storage at 4°C. Freeze at -80°C as soon as possible to retain virus infectivity. Infectivity is destroyed by storage at -20°C, but may be retained for several weeks if stored at 4°C and antibodies and DNA should be detected over longer periods up to possibly several months with storage at 4°C.

2.3 Ticks

Ticks should preferably be kept alive by placing them in screw capped 50ml sample bottles/tubes with gauze placed under the cap that has holes punched on it to allow for air circulation. Soil from the burrow or a piece of filter paper should be included inside the sample bottle. For long term storage, keep live ticks in a cool place (maximum temperature 20 -25°C) where humidity can be maintained (e.g. by adding a piece of humid cotton in the tube).

To maintain infectivity, ticks are to be stored at -70°C (liquid nitrogen, dry ice or ultra-freezer -70°C). Avoid storing samples at -20°C as African Swine Fever virus does not keep well at this temperature.

Preservation in 100% alcohol should only be done if samples are only collected for morphological identification of the vector species. With alcohol storage, ASF virus detection is only possible by PCR. Storage in alcohol: in 1.5 ml nunc tubes with screw and plastic gasket cover, maintain no more than 1 volume of ticks for 9 volume of alcohol.

3 Test protocols

Working in the laboratory and labelling of samples: please follow the guidelines of sample labelling. We would like to encourage all laboratories involved in the ASF diagnosis to use the excel or access file `WT ASF samples` to record the samples and the respective test results. This approach allows a high level of harmonisation of the information recorded in the various laboratories and facilitates the data compilation for further analysis. Each animal or each tube with ticks gets a unique sample label with the format

XXYYZddmmyy.A000, where:

XX: country code (MO, MA, SE, CO)

YY: region code (3-8 codes per country)

Z: species of sampled animal (P=pig, B=bushpig, W=warthog, T=tick)

ddmmyy: sampling date

A: sample type (A=tick, B=blood, C=Whatman 3M filter paper, D=FTA filter paper,

E= tissue lymph node, F= tissue spleen, G= other tissue, H=other)

000: sample number of the day in the same region

For ticks the first digit of the 000 number is used to number the tubes, single ticks are given a unique identification upon testing in the laboratory using the last two digits of the 000 number.

We advice to use a labelling machine (for example Brother P-touch 2450 DX Label Maker) to label the sample tubes for long-term storage or transport. Make sure that the labels stick well.

3.1 DNA extraction

There are various useful kits for DNA extraction available. Known from experience, good results were obtained using the High Pure Viral Nucleic Acid Kit (Roche) where of 200µl of tick supernatant or virus suspension DNA is recovered in a final volume of 50-65µl, also the QIAamp DNA Mini Kit (Qiagen) or Amersham GFX Genomic Blood DNA Purification kit (Cat 27-9603-01) have been used for extraction of DNA from tick homogenates, tissues and from blood. Also recommended is the guanidium extraction method as described by Boom et al. (4) (for further details contact Wilna Vosloo, OVI: VooslooW@arc.agric.za).

To obtain tick homogenate, sterilise tick surface with a 10 % hypochlorite solution. Homogenise ticks using a syringe and needle in 0.5 ml RPMI medium supplemented with 20 % foetal calf serum and 50 U/ml of penicillin, streptomycin and fungizone. Freeze ticks at -70 °C. Individual homogenised ticks or organs/tissues are best grinded with sterile sand (10% w/v) and the supernatant collected after centrifugation.

For DNA purification of blood samples on filter papers use the protocol by Whatman: `stamp out` 2 discs of 2mm diameter with a Whatman Harris Micro Punch™, wash the discs 3 times with the FTA DNA purification reagent and rinse twice for 5 minutes with TE-1 buffer. The washed discs should not have any red colour left. Let the discs dry for 1 hour before use.

3.2 Virus detection

3.2.1 PCR on blood (serum) samples

There are various protocols that have been proved to be useful for virus detection. We propose to use the primers described by Basto et al. (2) as they have the advantage of generating a larger product which will make it easier to interpret when analysing the

products on an agarose gel. In addition this method has the advantage of having an internal control which allows assessment of the presence of inhibitors (protocol 3.2.2). A list of alternative protocols is given in section 3.2.6.

3.2.2 IC-PCR with internal control

The following protocol is adapted protocol from Basto et al. (2) and consists of a first-round PCR reaction with an Internal Control, using the primers 72ARs and 72ARas (specific for conserved regions of the VP72 gene B646L) followed by a nested PCR reaction using the primers 72Ns and 72Nas. The first-round PCR fragment obtained by amplification of ASFV DNA has a length of 370 bp. The Internal Control is a plasmid (p72AR-ICg) with the same primer recognition sequences as the viral DNA, but flanking a heterologous DNA fragment of larger size (498 bp). The nested PCR reaction amplifies a 243 bp fragment from the ASFV DNA. The Internal Control plasmid can be obtained from the IAH (linda.dixon@bbsrc.ac.uk).

Material needed:

- Nuclease-free water
- PCR Mastermix (we recommend to use the Eppendorf MasterMix 2.5x, it is stable, relatively cheap and easy to handle)
- Primers: prepare primers stock solutions at a concentration of 10 pmol/µl
- **First-Round PCR Primers:** 72ARs (forward): 5'-GAC GCA ACG TAT CTG GAC AT-3'
72ARas (reverse): 5'-TTT CAG GGG TTA CAA ACA GG-3'
- **Nested PCR Primers:** 72Ns (forward): 5'-TAC TAT CAG CCC CCT CTT GC-3';
72Nas (reverse): 5'-AAT GAC TCC TGG GAT AAA CCA T-3'
- **Internal Control:** before using the internal control, the lower amount of plasmid that proves to be consistently amplified in the PCR must be determined. For this purpose:
 - Prepare 10-fold serial dilutions of the plasmid in nuclease-free water.
 - Run PCR reactions under the conditions described below, using 0.5 µl of each plasmid dilution as template and substituting the sample template by water.
 - Load the PCR products on an agarose gel containing Ethidium Bromide. Examine the gel under a UV light source and observe which were the last two dilutions giving positive amplification.
 - Run new PCR reactions (same conditions) using as templates those last two plasmid dilutions that gave positive amplifications, but this time with 10 replicates of each one.
 - After running the PCR and the agarose gel, examine under UV light and observe which of the dilutions gave positive amplification in ALL the replicates. The highest dilution (lower concentration) of plasmid that proved to be consistently amplified is the right concentration to use as internal control in PCR reaction. If needed, dilute more plasmid at this concentration and store it at -20 °C.
- Marker DNA: marker DNA are commercial available. Take in consideration the size of the expected amplification products (498 bp for the internal control; 370 bp for the first-round PCR product and 243 for the nested PCR product).

a) First-round PCR with Internal Control

- Prepare a PCR mix in a sterile 1.5 ml micro centrifuge tube. This mix should contain the following reagents volumes multiplied by the number of samples to be assayed plus 3 or 2 times for control tubes (see note below), the total volume per reaction is 50 µl:
 - 20 µl Eppendorf MasterMix 2.5x,
 - 10.5 µl Nuclease-free water,
 - 2 µl Primer 72ARs 10 pmol/µl
 - 2 µl Primer 72ARas 10 pmol/µl
- Transfer 34.5 µl of the PCR mix to a 0.2 ml PCR tube, which will be the negative control. Add to this tube 15.5 µl of nuclease-free water.
- Add to the PCR mix prepared 0.5 µl of internal control (highest dilution of the plasmid that proved to be consistently amplified, as shown above), multiplied by the number of samples to be assayed plus 2 times for control tubes (see note below).
- Add 35 µl of the PCR mix to the required number of 0.2 ml PCR tubes (one for each sample to be assayed and 2, see note, for control tubes).

- Add 15 µl of extracted sample template to each PCR tube. For the positive control, use 2 µl of ASFV DNA and 13 µl of nuclease-free water. Run a negative control (15 µl nuclease-free water) to certify that the Internal Control solution is not contaminated by ASFV DNA (Negative Control for the Internal Control).

Note: since the control tube with only internal control (without ASFV DNA) is sufficient as positive control of the reaction, the only purpose of the positive control tube with internal control and ASFV DNA is to compare the size of the fragment obtained by amplification from the viral DNA (370 bp) with the size of amplification products obtained from a positive sample. In these circumstances, the control tube with internal control plasmid and ASFV DNA can be dispensed, if extra ASFV DNA manipulation is to be avoided.

- Place all the tubes in an automated DNA thermal cycler and run the following programme:
 - 1 cycle at 95°C for 3 minutes.
 - 35 cycles at 95°C for 30 seconds, 60°C for 30 seconds and 72°C for 30 seconds.
 - 1 cycle at 72°C for 10 minutes
 - Hold at 4°C.
- Load all the samples on an agarose gel containing Ethidium Bromide and examine the gel under a UV light source.
- Reading the results: calculate the size of the PCR products in the test samples and the positive control by reference to the standard markers.
 - A band of 498 bp, corresponding to the Internal Control, should be present in all the samples, except in the Negative Control. The PCR reaction of the samples where this band is not present was inefficient, probably as consequence of PCR inhibitors in the sample tested. Try to extract again the DNA or dilute the sample.
 - In the Positive Control, in addition to the band corresponding to the internal control, another band with 370 bp will be present, corresponding to the viral DNA.
 - In a positive sample, the same two bands observed in the Positive control will be present. If the amount of viral DNA is too high, it is possible that the band of the Internal Control is not visible.
 - In the Negative Control for the Internal Control, only the internal control band should be present. If the band corresponding to viral DNA is also observed it means that the plasmid solution is contaminated and the other positive results are not valid.
 - No bands should be seen in the negative control.

b) Nested PCR

- Prepare a PCR mix in a sterile 1.5 ml micro centrifuge tube. This mix should contain the following reagents volumes multiplied by the number of samples to be assayed plus 2 times for control tubes:
 - 20 µl Eppendorf MasterMix 2.5x
 - 21 µl Nuclease-free water
 - 4 µl Primer 72Ns 10 pmol/µl
 - 4 µl Primer 72Nas 10 pmol/µl
- Add 49 µl of the PCR mix to the required number of 0.2 ml PCR tubes, run a positive and a negative control.
- Transfer 1 µl amplification product of each first-round PCR tube to each nested PCR tubes.
- Place all the tubes in an automated DNA thermal cycler and run the following programme:
 - 1 cycle at 95°C for 3 minutes.
 - 35 cycles at 95°C for 30 seconds, 60°C for 30 seconds and 72°C for 30 seconds.
 - 1 cycle at 72°C for 10 minutes.
 - Hold at 4°C.
- Load the samples on an agarose gel containing ethidium bromide and examine under a UV light source.
- Reading the results: 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 243 base pairs
 - In a positive sample, a band will be present that should co-migrate with the PCR product of the positive control.
 - No bands should be seen in the negative control.

- A faint band of 498 bp, corresponding to the internal control amplified in first-round PCR, may be seen in all samples transferred from first-round PCR.

3.2.3 PCR on Whatman 3M or FTA filter paper

Having samples on both types of filter paper will allow assessing which one is better to use. Experiments carried out at the IAH indicated that ASF virus could be detected down to a virus titre of 10^3 /ml from both filter papers. Experiments at CIRAD showed that the highest test sensitivity was obtained by using the following protocol (virus titre of 10/ml was detected):

PCR reaction in a final volume of 50µl:

20 µl Eppendorf Mastermix 2.5x,

8 µl Nuclease-free water,

2 µl Primer VP72fwd (10 pmol/µl): 5'-TCggAgATgTTCCAggTAgg-3'

2 µl Primer VP72rev (10 pmol/µl): 5'-CgCAAAAggATTTggTgAAT-3'

Filter paper disc 2-5mm diameter

Add the filter paper discs into the PCR tubes (minimum 1 disc of 2mm diameter ~ 1µl of sample DNA) and run the PCR using the following cycles:

5min at 95°C

35 x 95°C 30sec / 55°C 30sec / 72°C 30sec

7 min at 72°C

Load the samples on an agarose gel containing ethidium bromide and examine under a UV light source.

Reading the results: 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 346 base pairs
- In a positive sample, a band will be present that should co-migrate with the PCR product of the positive control.
- No bands should be seen in the negative control.

The use of a real time PCR protocol and the detection of ASF antibodies from filter paper samples are currently being investigated at CIRAD and as soon as details and results are available, we will update this section.

3.2.4 PCR on whole ticks

After DNA extraction of homogenized ticks, use the protocol 3.2.2 (IC-PCR with internal control: Basto et al 2006 (2)).

3.2.5 PCR on tissue

After DNA extraction of tissue, use the protocol 3.2.2. (IC-PCR with internal control: Basto et al 2006 (2)).

3.2.6 Other PCR protocols

- Agüero et al. 2003 (1): Highly sensitive PCR assay for routine diagnosis of African swine fever virus in clinical samples
- Michaud et al., 2004, (9): protocol for direct PCR for ASFV from Whatman 3M filter paper:

With this protocol, blood-dried filter papers from pigs samples are directly processed in the PCR without any previous nucleic acid extraction. Pieces of 5 mm² are placed into 0.2 ml PCR tubes.

Reaction mix is then added in a final volume of 80 µl. The proof reading polymerase (Taq pol Pfu, Stratagene, Amsterdam) is used, what allows PCR products to be directly sequenced for molecular epidemiology. For the detection of ASF virus, the reaction mix consisted of 31.25 pmol of each primer [forward: 5' TCg gAg ATg TTC CAg gTA gg-3', reverse:5'-CgC AAA Agg ATT Tgg TgA AT-3'], 20 pmol dNTPn, 2.5 units of Pfu polymerase. After amplification (5 min at 95°C then 35 cycles - 30s at 95°C, 30s at 55°C and 30s at 72°C – and finally 7 min at 72°C), a DNA fragment of 346 base pairs is visualized on agarose gels.

- King et al. 2003 (8): Development of a TaqMan PCR assay with internal amplification control for the detection of African swine fever virus

3.3 Anti-ASF virus antibody detection

We recommend performing the two different ELISA tests described below on each sample, as this will allow the direct comparison of the two tests and henceforth a sound test evaluation. The Ingenasa ASF serological kit has been used extensively in the past and is commercially available, therefore allowing uniform supply to all laboratories. The p30-ELISA is new and has not been thoroughly tested on samples from different regions. However analysis of samples from Spain and West Africa was very promising and indicated that this test has a higher specificity. In addition the antigen can be adapted to local strains, which seems to be most of all interesting for Eastern African countries. For doubtful ELISA samples we recommend to use Western Blot Strips (delivered by Jose Escribano, see 3.3.3.)

3.3.1 Ingenasa ASF serological kit

The Ingezim PPA Compac 1.1.PPA K3 Elisa kit is a blocking enzymatic immunoassay (Blocking ELISA) using a purified protein extract from the virus (VP73) as antigen. Kits can be ordered from Ingenasa, Madrid (Spain), e-mail cvela@ingenasa.es. The delivered kit contains the following components:

96-well micro-titration coated plates, vials with inactivated positive control serum, vials with inactivated negative control serum, vials with peroxidase conjugate (100x concentrated), diluent (DEO1-01), substrate (TMB), stop solution.

Materials and reagent needed that are not provided with the kit: distilled or deionised water, micropipettes from 5 to 200 µl, disposable micropipette tips, washing plates device, test tubes from 50 to 250 ml, ELISA Reader (450nm filter).

Test procedure:

- All reagents (except conjugate) must be allowed to warm to room temperature before use.
- Add 50 µl of supplied diluent to each well. Add 50 µl of positive control sera to two wells (e.g. A1 and B1), and 50 µl of the negative control sera (e.g. A2 and B2). Add 50 µl of sera samples to test on each remainder wells. We recommend the use of two wells per sample. Seal the plate and **incubate for 1 h at 37°C or overnight (18 hours) at 18-25°C**.
- Empty the wells into a receptacle containing 0.1 M NaOH and wash 4 times using an automatic washing machine or a multi-channel pipetting device suitable for dispensing 300 µl on each well. Dispense a volume of 300 µl of washing solution on each well, shake the plate delicately avoiding contamination between wells, and brusquely pour over the plate to empty the wells. Prior to empty the content of the last washing step, verify that the next reagent to be added to the plate is ready to use. Do not maintain the plate dry longer than strictly needed. After the last washing step shake the plate turned over an absorbent filter paper.
- Add 100 µl of specific conjugate (prepared following previous instructions) to each well. Seal the plate and **incubate for 30 minutes at 37°C**.
- Wash 5 times as previously described
- Add 100 µl of substrate to each well, keep the plate for 15 min at room temperature.

- Add 100 µl of stop solution to each well.
- Read the OD of each well at 450nm

Reading and interpretation of the results:

The test can be considered valid when the OD of the negative control (NC) is higher than 0.7 and, at least 4 times higher than the OD of the positive control (PC): NC/PC >4

Cut-off calculation: Positive cut-off = $CN - [(CN - CP) \times 0.5]$
 Negative cut-off = $CN - [(CN - CP) \times 0.4]$

When running duplicates, the OD of the sample will be calculated as the arithmetic mean of OD values in both wells.

- Serum samples with an OD lower than the positive cut-off, are considered as positive to ASFV antibodies.
- Serum samples with an OD higher than negative cut-off, will be considered negative to ASFV antibodies.

Serum samples with OD values between both cut off are considered as doubtful. We recommend re-testing these animals one more time or applying a different technique to check this serum (Western blot, Indirect ELISA, etc.).

3.3.2 Algenex anti-rp30 ELISA

This protocol has been described by Perez-Filgueria et al. (11). Lyophilized recombinant p30 antigen in vials for 3-4 micro-titre plates can be obtained from Jose Escribano (email: escriban@inia.es). Coat the ELISA micro-plates (Polysorp, Nunc, Denmark) with 100 µl antigen (positive and negative) of a 1:3,000 dilution in 50 mM carbonate/bicarbonate buffer, pH 9.6 and incubate overnight at 4°C. The next day, wash the plates with PBST three times and use immediately or store at -20°C until use. The following incubations are for 1 h at 37°C under constant agitation. Incubate plates sequentially with blocking bufferr (PBST - 2% BSA, 50 µl/well) and pig serum samples diluted 1:200 in blocking buffer in duplicate wells. Each serum sample is tested against both Ag(+) and Ag(-). Wash plates four times with PBST and ad 50 µl/well of protein A-HRP conjugate (Sigma, Missouri, USA) diluted 1:2000. For the substrate reaction, wash the plates 4 times and ad 50 µl/well of substrate solution consisting of 1mM 2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) (KPL, USA), 35mM citric acid, 67 mM Na₂HPO₄ pH=5.0 and 0.015% H₂O₂. Allow peroxidase reaction to develop for 15 min at room temperature, stop reaction and read at 405 nm in an ELISA microplate reader (Multiskan EX, Thermo Electron Corp., USA). Sera titers are expressed as the ratio between the mean OD obtained for each sample in duplicate assays against Ag(+) and Ag(-) [OD Ag(+)/OD Ag(-)]. Ratios higher than 2 are considered positive.

Buffers required:

Carbonate/bicarbonate buffer 0.05M (pH 9.6)

Na ₂ CO ₃ (Merck 1.06392)	1.59 g
NaHCO ₃ (Merck 6329)	3.88 g
Distilled water to	1000ml

Store at 4°C. Check the pH before use.

Washing solution: PBS 1x pH 7.2 - 0.1% Tween-20 (PBST)

NaCl (Merck Ref. 1.06404)	8.0 g
KH ₂ PO ₄ (Merck Ref. 1.04873)	0.2 g
Na ₂ HPO ₄ 12 H ₂ O (Merck Ref. 1.06586)	2.9 g
KCl (Merck Ref. 1.04936)	0.2 g
Tween-20 (Merck Ref. 8.22184)	1 ml
Distilled water to	1000 ml

Check the pH before use it. Store at + 4°C.

Blocking solution:

2% (w/v) BSA in PBS 1x pH 7.2 - 0.1% Tween-20

Substrate: ABTS

Buffer solution 1x: citric acid 35 mM

Na₂HPO₄ 67 mM

5.0 pH

1mM ABTS in buffer pH 5.0 (dissolved immediately before being used) and 0.015% H₂O₂

Example: 15 ml buffer 1x + 6.75 µl H₂O₂ (al 33%) + 8.2 mg ABTS.

3.3.3 Western Blot**rp30-based immunoblotting assay**

Use Western blot strips provided by Jose Escribano (email: escriban@inia.es). Block strips for 1h with PBST 4% skim milk and subsequently incubate for 1 h at room temperature with pig sera diluted 1:50 and a protein A-horse radish peroxidase (HRP) conjugate (Sigma, USA) at a 1:2000 dilution in blocking buffer. Develop assays using 0.3% 4-chloronaphtol solution (Sigma, USA) as substrate and stop reaction with distilled water after 5 minutes.

Interpretation of results: Sera presenting a characteristic reaction with a protein band corresponding to protein p30 are considered positive to ASF antibodies.

Buffers required:

PBS buffer pH 7.2

NaCl (Merck 1.06404)	8.0 g
KH ₂ PO ₄ (Merck 1.04873)	0.2 g
Na ₂ HPO ₄ 12 H ₂ O (Merck 1.0686)	2.9 g
KCl (Merck 1.04936)	0.2 g
Distilled water to	1000 ml

Check the pH before use. Store at 4°C.

PBS 1x pH 7.2- 0.1% Tween 20 (PBST)

Detergent Tween 20	1 ml
PBS pH 7.2	1 L

PBST/Milk 4% buffer

Non fat dry milk (NESTLÉ- Sveltesse or Molico)	4 g
PBST pH 7.2	100 ml

Store at + 4°C. Do not use it after two days.

Substrate solution

a) Dissolve 12 mg of 4-chloronaphtol (Merck 11952) in 4 ml of Methanol (Merck 1.06009).

b) Add slowly 4-chloronaphtol/Methanol solution to 20 ml of PBS buffer pH 7.2, with vigorous agitation (a characteristic precipitate is formed).

c) Then, add 8 µl of H₂O₂ 30% (Panreac 131058) to the PBS/4-chloronaphtol solution.

3.3.4 ELISA using polyprotein pp62

The expression of the ASF virus polyprotein pp62 in the baculovirus expression system and its use for ASF virus diagnosis (ELISA and Immunoblotting) has been recently described (6). The ELISA using the pp62 performed very well in the diagnosis of poorly preserved sera.

3.4 Anti-tick antibody detection

The anti-tick ELISA determines if a pig has been bitten by *O. moubata* or *O. erraticus*. The antigen used in the test is prepared from tick salivary gland. The test can be useful to evaluate the importance of the tick cycle for the transmission in a pig population.

The protocol has been developed for the detection of anti-*O. erraticus* antibodies in pigs (5). Based on this protocol, an ELISA for anti-*O. moubata* antibodies can be carried out. The *O. moubata* antigen can be obtained from the IAH.

3.5 Genotyping

All sequences should be uploaded to the website maintained by EU Reference Laboratory for ASF in Valdeolmos, Madrid/Spain (<http://webainia.inia.es/cisa/asfv/index.asp>). The main objective of this database is to obtain epidemiological information of the ASFV isolates from all epidemics in or near the European Union, and from all over the world. In addition, the sequences from defined regions of the ASFV genome included in this database are a useful tool for genetic typing of new isolates. This can be very important for comparing a sequence to existing sequences in the database. The database allows searching sequences by the viral isolate name, country or continent of origin, year of collection, or by host species.

3.5.1 Sequencing of p72 (B646L)

Use the primers described by Bastos et al. (3) to amplify a 478bp C-terminal region of the vp72 (p72-D 5'-GGC ACA AGT TCG GAC ATG T-3', and p72-U 5'-GTA CTG TAA CGC AGC ACA G-3')

3.5.2 Sequencing of 9RL (B602L)

The sequencing of 9RL allows to distinguish within p72 groups: either use primers described by Irusta et al. (7) or Nix et al. (10) ORF9L-F2 (5'-CAT CCG GGC CGG TTT CTT GTA TAT-3') and ORF9L-R3 (5'-GGA GTT TG GTG ATT GCA TCA ATA TCG-3').

3.5.3 Optional genotyping methods

Sequencing of p30 (CP204L) or of p22 gives further information regarding the genotype (for protocol details contact Emmanuel.Albina@cirad.fr). The analysis of variable genome regions has also shown to be a useful approach, see Nix et al.(10).

4 References

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