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# Comparative evaluation of novel African swine fever virus (ASF) antibody detection techniques derived from specific ASF viral genotypes with the OIE internationally prescribed serological tests

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ABSTRACT

The presence of antibodies against African swine fever (ASF), a complex fatal notifiable OIE disease of swine, is always indicative of previous infection, since there is no vaccine that is currently used in the field. The early appearance and subsequent long-term persistence of antibodies combined with cost-effectiveness make antibody detection techniques essential in control programmes. Recent reports appear to indicate that the serological tests recommended by the OIE for ASF monitoring are much less effective in East and Southern Africa where viral genetic and antigenic diversity is the greatest. We report herein an extensive analysis including more than 1000 field and experimental infection sera, in which the OIE recommended tests are compared with antigen-specific ELISAs and immuno-peroxidase staining of cells (IPT). The antibody detection results generated using new antigen-specific tests, developed in this study, which are based on production of antigen fractions generated by infection and virus purification from COS-1 cells, showed strong concordance with the OIE tests. We therefore conclude that the lack of success is not attributable to antigenic polymorphism and may be related to the specific characteristics of the local breeds African pigs.



## 1. Introduction

African swine fever (ASF) is a complex and lethal viral disease of swine with significant socio-economic impact in the developed and developing world. The disease has a major negative effect on national, regional and international trade and constrains pig production by livestock farmers in affected areas in Sub-Saharan Africa (Penrith et al., 2004) and in the Caucasus region, where the disease was first identified in 2007 (Rowlands et al., 2008). The devastating acute form of the disease is characterized among others by functional and congestive-haemorrhagic disorders of the digestive and

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respiratory systems, causing to 100% mortality of infected pigs.

African swine fever virus (ASFV), the causative agent of ASF, is a large double-stranded DNA virus and the only member of *Asfaviridae* family, genus *Asfivirus* (Dixon et al., 2000, 2005). Transmission of ASFV can occur in a sylvatic or in a domestic pig cycle, with or without tick involvement. Depending on the presence or absence of wild suids and arthropod vectors and the type of pig production system, the epidemiology varies substantially between countries, regions and continents. In East and South Africa the disease has been maintained, since the first description in 1920s (Montgomery, 1921), in a sylvatic cycle involving asymptomatic wild African pigs (*Phacochoerus* and *Potamochoerus* spp.) and soft ticks of the genus *Ornithodoros*, mainly *O. porcinus* (Plowright et al., 1969; Wilkinson et al., 1988; Kleiboeker and Scoles, 2001; Penrith et al., 2004; Penrith, 2009; Jori and Bastos, 2009; Costard et al., 2009). In contrast, in West Africa the virus appears to be maintained by transmission between domestic pigs and the existence of a sylvatic cycle has never been demonstrated except for a single record in Sierra Leone (Penrith et al., 2004; Vial et al., 2007). Outside Africa, wild boar (*Sus scrofa*) and feral pigs are susceptible to ASFV and show similar clinical signs and mortality to domestic pigs. Long-term persistence of ASFV caused by the presence of the soft tick vector *Ornithodoros erraticus* was also reported in the Iberian Peninsula (Oleaga et al., 1990; Oleaga-Pérez et al., 1990; Boinas et al., 2011).

ASF is endemic in most sub-Sahara Africa with an increase of intermittently reported ASF outbreaks from the late-2000s onwards that continue to this day. In Europe, until 2007 and since the eradication of the disease in the Iberian Peninsula, ASF has been confined to Sardinia (Italy). In April 2007 its remarkable potential for trans-boundary spread was demonstrated by the appearance of the virus in the Republic of Georgia with subsequent outbreaks in Armenia, Azerbaijan and Southern Russia. Currently ASF is considered to be established in the southern part of the Russian Federation with sporadic reported outbreaks in Georgia and Armenia (Penrith, 2009; FAO, 2009a, 2010; World Organization for Animal Health, OIE, 2007–2012). The endemicity of ASF in the Caucasus threatens Europe, central Asia and even China, which has the largest pig population in the world.

Since there is no vaccine available, rapid and specific diagnostic procedures are an essential component of a control strategy in affected countries. Available conventional and Real Time PCR assays have been shown to be highly sensitive as diagnostic tools in acute and subacute forms of the disease caused by current circulating ASFV isolates in Europe and Africa (Aguero et al., 2003; King et al., 2003; Zsack et al., 2005; McKillen et al., 2010; James et al., 2010; Ronish et al., 2011; Tignon et al., 2011; Fernández-Pinero et al., 2012). Based on long term observations of p72 genotype I ASFV infections in European and West African pigs, it is apparent that antibodies to ASFV persist for long periods after the infection. Due to the absence of vaccine currently deployed for control of ASFV these antibodies are a good indicator of infection. Therefore the detection of seropositive animals is the most important and cost effective method for the control of the disease even when chronic or apparently asymptomatic pigs are present in the field, as it was demonstrated during the Spanish and Portuguese eradication programs (Bech-Nielsen et al., 1993a,b; Arias and Sanchez-Vizcaino, 2002). However recent investigations have indicated a low incidence of sero-positive ASFV infected virus-positive animals in East African countries, such as Kenya and Uganda, using recombinant antigens and OIE-approved tests based on crude antigen extracts (Perez-Filgueira et al., 2006; Gallardo et al., 2009b, 2011a). The comparison of the gene sequences of ASFV antigenic proteins p30 and p54 revealed higher levels of genetic variation located in areas of the proteins containing predicted antigenic determinants in the eastern African ASFV isolates comparing with sequences obtained from viruses circulating in West Africa and Europe (Perez-Filgueira et al., 2006; Sun et al., 1995). A similar pattern was observed by the genotyping of ASFV viruses using the C-terminal end of the ASFV p72 structural protein. This enabled discrimination of 22 different ASFV p72 genotypes in eastern and southern African regions. By contrast the West African, American and European ASFV isolates has been traditionally classified in a single p72 genotype I (Lubisi et al., 2005; Boshoff et al., 2007; Gallardo et al., 2009a, 2011a,b; Giammarioli et al., 2011). This historically broad distribution of genotype I viruses in Europe was altered in 2007 in Georgia when the first non-genotpye I

**Table 1**
ASFV isolates employed in this study.

| ASFV isolate | Origin | | Host species | Year collection | P72 genotype | Virulence | Haemadsorbing | Reference |
|---|---|---|---|---|---|---|---|---|
| | Country | Town/province | | | | | | |
| E70[a] | Spain | Pontevedra | Pig | 1970 | I | vir | + | Zsak et al. (2005) |
| E75 | Spain | Lerida | Pig | 1975 | I | vir | + | de Villiers et al. (2010) |
| Moz64[a] | Mozambique | NK | Pig | 1964 | V | vir | + | Gallardo et al. (2009a,b) |
| MwLil 20/1[a] | Malawi | Chalaswa | Tick | 1983 | VIII | vir | + | Haresnape et al. (1988) |
| Ken06.Bus[a] | Kenya | Busia | Pig | 2006 | IX | vir | + | Gallardo et al. (2009b) |
| Ken05/Tk1 | Kenya | Machakos | Tick | 2005 | X | mod vir | + | Gallardo et al. (2011a) |
| Ken08/Tk2.1[a] | Kenya | Machakos | Tick | 2008 | X | mod vir | + | Gallardo et al. (2011a) |
| Arm07[a] | Armenia | Dilijan | Pig | 2007 | II | vir | + | Unpublished CISA-INIA |
| SS08/47223 | Italy | Sardinia | Pig | 2008 | I | vir | + | Giammarioli et al. (2011) |
| NH/P68 | Portugal | | Pig | 1967 | I | att | – | Gil et al. (2003) |

att: attenuated; vir: virulent; mod vir: moderately virulent.
[a] ASF viruses selected for ASFV genotypes-specific-antigens production.

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virus was the responsible of an outbreak outside the endemic area in Africa, and was classified within the South East African ASFV genotype II (Rowlands et al., 2008; FAO, 2009b). Since the viral antigens included in the current OIE-approved tests derive from European ASFV viruses belonging to p72 genotype I (OIE 2012), the Georgian outbreak clearly demonstrated that it will increasingly important to take account of how viral genetic complexity of the virus in the endemic areas of Africa may affect serological detection of ASFV infections.

Therefore, within this study and in order to test the hypothesis that antigenic variation affects the sensitivity and specificity of the OIE approved serological diagnostic test for ASFV, we have developed novel ELISA assays using antigens derived from several different specific viral genotypes and validated the data using an immunoperoxidase assay. We also report herein comparative analysis of the performance of these assays relative to the OIE approved serological diagnostic tests.

## 2. Methods

### 2.1. Viruses

The ASFV stocks selected in this study are shown in Table 1 and include highly virulent, moderately virulent, attenuated, haemadsorbing or non-haemadsorbing strains, representing both African and European isolates. The ASFV isolates were obtained from the collections of the European Union Reference laboratory for ASF (CISA-INIA), the Italian National reference laboratory (IZSUM) and the Faculdade de Medicina Veterinária, Universidade Técnica de Lisboa (FMV-UTL).

For preparation of soluble cytoplasmic antigens (Cp-Ag) in COS-1 cells, six ASFV isolates were selected on the basis of genome variability criteria and date collection comprising: (i) the most variable isolates within p72 genotypes X, IX, VIII and V so far identified in East African countries (Ken08/Tk2.1; Ken06.Bus, Moz64 and MwLil20/1), (ii) a current p72 genotype II from Armenia in the Caucasus region (Arm07) and (iii) a historical European isolate belonging to p72 genotype I (E70).

For the experimental *in vivo* studies eight ASFV isolates belonging to p72 genotype X (Ken05/Tk1 and Ken05/K2), II (Arm07) and I (NH/P68, L60, E75 $CV_14$, E70 and SS08/47223) were used.

### 2.2. Cell cultures and virus propagation

The ASFV isolates were propagated (through 2–6 passages) in porcine blood monocytes (PBM) recovered from naive domestic pigs (Carrascosa et al., 2011). Titrations of ASFV stocks were performed using a haemadsorption assay to monitor end point dilution of ASFV isolates into PBM. Titres were estimated using the method of Reed and Muench (1938) and expressed as 50% haemadsorbing doses per ml ($HAD_{50}$/ml) per sample except for ASFV NH/P68 isolate which was titrated by plaque assay (Carrascosa et al., 2011).

**Table 2**
Panel of field sera samples collected from pigs in ASFV-infected areas.

| Country | Region | Date collection | Host | Total sera | OIE antibody detection[a] | |
|---|---|---|---|---|---|---|
| | | | | | Positives | Negatives |
| Kenya | Central Kenya | July 2005 | Domestic pig | 86 | 0 | 86 |
| | Western Kenya | September 2006 | Domestic pig | 26 | 0 | 26 |
| | Western Kenya | April–May 2008 | Domestic pig | 37 | 0 | 37 |
| | Central Kenya | February 2009 | Domestic pig | 12 | 0 | 12 |
| | Central Kenya | October 2008 | *Phacochoerus africanus* | 11 | 11 | 0 |
| | Central Kenya | February–March 2009 | *Phacochoerus africanus* | 32 | 32 | 0 |
| | North Kenya | 2006–2008 | *Phacochoerus aethiopicus* | *72* | 71 | 1 |
| | Western Kenya | October 2008 | *Potamochoerus larvatus* | *2* | 0 | 2 |
| Tanzania | Serengueti National Park | 2004 | *Phacochoerus africanus* | 31 | 31 | 0 |
| | Manyara | November 2004 | Domestic pig | 35 | 0 | 35 |
| | Dodoma Region | December 2004 | Domestic pig | 35 | 0 | 35 |
| | Arusha Region | December 2004 | Domestic pig | 28 | 0 | 28 |
| Uganda | Western Uganda | May-03 | Domestic pig | 5 | 0 | 5 |
| | Central Uganda | November–December 2003 | Domestic pig | 74 | 0 | 74 |
| | The Whole Country | 2006 | Domestic pig | 242 | 1 | 241 |
| | Central Uganda | August 2007 | Domestic pig | 28 | 0 | 28 |
| Mozambique | Whole country | 2008 | Domestic pig | 174 | 3 | 171 |
| Nigeria | South-south | March–October 2006 | Domestic pig | 16 | 14 | 2 |
| | South-south | September–November 2008 | Domestic pig | 7 | 5 | 2 |
| | North-central | March–December 2008 | Domestic pig | 27 | 27 | 0 |
| | North-west | December 2008– | Domestic pig | 2 | 2 | 0 |
| | North-east | December 2008 | Domestic pig | 18 | 18 | 0 |
| | South-west | September 2008–April 2009 | Domestic pig | 8 | 6 | 2 |
| | North-east | April 2009 | Domestic pig | 2 | 0 | 2 |
| Benin | Collines | June 2009 | Domestic pig | 1 | 1 | 0 |
| Republic of the Congo | Bouenza | August 2009 | Domestic pig | 3 | 3 | 0 |
| | Niari | August 2009 | Domestic pig | 1 | 1 | 0 |
| Italy | Sardinia | 2009 | Domestic pig | 47 | 37 | 10 |
| | | Total | | 1062 | 263 | 799 |

[a] Positive and negative results by a first screening using the OIE-indirect ELISA following by the confirmation by OIE immunoblotting assay.

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The African green monkey kidney COS-1 cell line was originally obtained from the American Type Culture Collection (CRL-1650, Manassas, VA 20108, USA). COS-1 cells were grown in Dulbecco's modified Eagle medium (DMEM) supplemented with 2 mM L-glutamine, 100 U of gentamycin per ml, and non-essential amino acids. Cells were cultured in a humidified atmosphere containing 5% $CO_2$ at 37 °C in medium supplemented with 10% heat-inactivated foetal bovine serum (Hurtado et al., 2010).

For ASFV stock propagation in COS-1 cells, pre-confluent cultures were infected with the six ASFV isolates selected for antigen production at a multiplicity of infection (m.o.i.) of 1–3 pfu per cell. After 2 h of adsorption the remaining virus was washed away, and cultures were incubated in fresh medium supplemented with 2% heat-inactivated foetal bovine serum for 72 h in a humidified atmosphere containing 5% $CO_2$ at 37 °C. Supernatants from COS cell cultures infected with stock viruses were collected and viral suspensions were titrated by end point dilution on PBM cells as described above.

The MS stable monkey kidney cell line (ECACC, 91070510) was used for conventional soluble cytoplasmic antigen production after the infection with the ASFV MS adapted-E70 isolate (E70 MS 48) as it is described in the OIE Manual of diagnosis (OIE 2012). The CV-1 cell line, derived from monkey kidneys (ATCC, CCL 70) were used for the titration by end point dilutions of the partially E75 $CV_1$4, attenuated virus employed for experimental infection (Ruiz Gonzalvo et al., 1986).

### 2.3. Test sera

#### 2.3.1. Porcine sera from field ASFV-infected areas

A panel of 1062 field sera collected between 2003 and 2010 from domestic and wild pigs in endemic areas of sub-Saharan African regions and Sardinia (Italy) were used in this study (Table 2). The Sardinian field samples were provided by IZSUM; the Mozambique field samples were provided by the ARC-Onderstepoort, South Africa; the remaining samples were originated from the collection of CISA-INIA, through their collaborations with the specific countries, and with the International Livestock Research Institute (ILRI).

All sera were previously tested using the OIE prescribed serological tests (ELISA and immunoblotting) (OIE 2012) and classified as 263 positives and 799 negatives.

#### 2.3.2. Porcine sera from field ASFV-free areas

Two hundred and twenty one negative field sera from domestic pigs and forty from wild boar were collected during surveillance programs in Spain and employed in the comparative study.

#### 2.3.3. Porcine sera from ASFV experimental studies

A total of 214 porcine sera collected at various stages from six independent experimentally infected pigs were included in this study. Animal experiments, conducted at the BSL3 animal facilities at CISA-INIA and IZSUM, were performed in accordance with the EC Directive 86/609/EEC following the accommodation and care of animals used for experimental and other scientific purposes, as described in the recommendation 2007/526/EC. A short description of the experimental design is shown below:

*Experimental infections with ASF viruses belonging to p72-genotype I*: (I) Four Landrace × Large White pigs were inoculated intramuscularly with $10^2$ 50% tissue culture infectious doses per ml ($TCID_{50}$/ml) of the attenuated ASFV Spanish isolate E75 $CV_1$4. Animals #1, #2 and #3 were euthanized from 14 up to 23 days post infection (dpi). Animal #4 was re-inoculated at 30 dpi with $10^3$ $HAD_{50}$/ml of the homologous virulent Spanish E75 isolate and then given a third challenge at 45 dpi with the heterologous virulent Spanish isolate E70 ($10^3$ $HAD_{50}$/ml). The animal was humanely killed at 50 dpi. (II) Serum samples collected at 0, 7, 14, 21 and 29 dpi(s) from four Landrace × Large White pigs inoculated intramuscularly with 10 $HAD_{50}$/ml of the Sardinia ASF isolate SS08/47223 were included in this study. Animals either died or humanely slaughtered from 14 up to 29 dpi(s). (III) Four Landrace × Large White pigs were inoculated intramuscularly with $10^5$ $TCID_{50}$/ml of the attenuated and non-haemadsorbing Portugal ASFV isolate NH/P68 (NHV). At 29 dpi the animals were re-inoculated with $10^5$ $HAD_{50}$/ml of the homologous virulent Portugal ASFV L60 isolate. Serum samples collected at 0, 6, 12, 19, 29, 35, 42 and 48 dpi(s) from pigs #13 and #18 slaughtered at 48 dpi were included in this study.

*Experimental infections with ASF virus belonging to p72-genotype II*: four Landrace × Large White pigs (C1, C2, C3 and C4) were inoculated intramuscularly with 10 $HAD_{50}$/ml of the haemadsorbing ASFV Arm07 isolate. Two untreated pigs (CC5 and CC6) were maintained in contact, housed in the same box as the inoculated animals. Inoculated and contact animals developed acute forms of clinical disease and were slaughtered or died as a result of the infection, between days 7 and 9 (inoculated pigs) or day 16 (contact pigs) post infection. Serum samples collected from contact animals at 0, 5, 9, 12, 15, 16 dpi(s) were included in this study.

*Experimental infections with ASF virus belonging to p72-genotype X*: (I) Serum samples collected at different intervals from 0 up to 70 dpi(s) from two pigs (#7 and #8) inoculated intramuscularly with 10 $HAD_{50}$/ml of the moderately virulent Kenyan ASFV isolate Ken05/Tk1 were used in this study. (II) Ten Landrace × Large White pigs were inoculated by the oro-nasal route with 10 $HAD_{50}$/ml of the moderately virulent Kenyan ASFV Ken05/K2 isolate. The infected animals either died or were humanely slaughtered between days 12 and 36 post infection. Serum samples collected at 0, 3, 7, 10, 14, 21, 23 and 36 dpi(s) were analysed in this study.

### 2.4. Optimization of ASFV cytoplasmic soluble antigen (Cp-Ags) production

The six selected ASFV virus isolates (Ken08/Tk2.1, Ken06.Bus, Moz64, MwLil20/1, Arm07 and E70), which had been grown in COS-1 cells, were used for antigen production. To determine the optimal conditions for the expression of ASFV antigens, COS-1 cells cultured in the presence of 2% heat-inactivated foetal bovine serum were inoculated with each ASFV isolate at a m.o.i. of 10 and

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harvested by centrifugation at 650 × *g* for 5 min at 0, 24, 48, 72 and 96 h post infection (h.p.i.). The cytoplasmic soluble antigens (Cp-Ags) were obtained by semi-purification of the ASF virions in a sucrose cushion (20%) according the procedure described in the OIE Manual of diagnosis (OIE 2012). The sediments obtained after the centrifugation of the COS-1 ASFV-infected cell cultures harvested at different times post infection were washed with 0.34 M sucrose in 5 mM tris–HCl (pH 8), resuspended in 0.067 M sucrose and held at 0 °C for 10 min to allow the cells to swell. Cells were lysed by adding the detergent Nonidet P40 at a final concentration of 1% (w/v) and held for 10 min at 0 °C. A 1/7 volume of 64% (w/v) sucrose to 0.4% tris–HCl (pH 8) was added, and nuclei were pelleted at 1000 × *g* for 10 min at 4 °C. The supernatants (cytoplasmic fraction) were treated with 2 mM ethylene-diamine–tetra acetic acid, 0.05 M 2-mercaptoethanol and 0.5 M NaCl. After 15 min at 25 °C, the mixtures were centrifuged at 100,000 × *g* for 1 h at 4 °C over a 20% (w/w) sucrose cushion in 50 mM tris–HCl (pH 8). The fractions eluted from the sucrose layers were used as soluble cytoplasmic antigens (Cp-Ag) in an indirect ELISA test for initial standardization and further validation. The Cp-Ags extracts obtained were quantified following the instructions supplied with a commercial kit (protein assay; Bio-Rad).

### 2.5. Determination of ASF cytoplasmic soluble antigens (Cp-Ags) viral genotypes

ASFV DNA was extracted from 200 μl of each Cp-Ag(s) in a final volume of 50 μl using a High Pure Viral Nucleic Acid kit (Roche) following the manufacturer's instructions. A PCR assay was set up using the ASFV genotyping primers p72U/p72D which generate an amplicon of 478 bp within the C-terminal end of p72 protein according as described in Bastos et al. (2003). Amplicons of the predicted size were excised and purified by Quiaex gel extraction (QUIAGEN) and directly sequenced using an automated "3730 DNA sequence analyzer" (Applied Biosystems). The analysis of sequence data was performed with Chromas software (www.technelysium.com.au), BioEdit (www.mbio.ncsu.edu/BioEdit/BioEdit.html), and ClustalX version 1.83 (www.clustal.org). The sequence obtained of each of the p72 amplicons was compared with homologous sequences representatives of the 22 p72 ASFV described genotypes (Boshoff et al., 2007) to confirm the genotypes of the Cp-Ag(s) obtained in this study.

### 2.6. Indirect ELISA test with the new soluble cytoplasmic antigens (Cp-Ag)

Microtitre plates (Polysorp Immunoplates Nunc) were incubated at 4 °C overnight with 100 μl/well of each Cp-Ags at the optimized working dilution in coating buffer (0.1 M carbonate buffer, pH 9.6). The coated plates were washed four times with PBS-T [Phosphate Buffer Saline, pH 7.5, containing 0.05% (v/v) Tween 20] and used immediately, or stored at −20 °C until use. The plates were subsequently blocked with PBS-M [PBS-T; 5% (w/v) skimmed milk] for 1 h at 37 °C. Then, the sera were added at a dilution 1:30 in PBS-M and incubated for 1 h at 37 °C. Duplicate wells were used for positive, limit and negative OIE-reference standards sera included in each plate. The plates were washed four times with PBS-T and the HRPO labelled protein A (PIERCE) was added diluted 1:5000 in PBS-M. The plates were incubated for 1 h at 37 °C. After washing the plates, 50 μl of the substrate o-phenylene-diamine (OPD-Sigma) was added per well. The reaction was stopped by the addition of 50 μl of 3 N $H_2SO_4$, and the optical density (OD) was measured at a wavelength of 492 nm in ELISA microplate reader (Multiskan EX; Thermo Electron Corp.) after incubation for 20 min at room temperature. Results were valid when the OD in positive control wells was over 1.5 OD units and the OD of the negative control wells was below 0.350 OD units. To determine the positive and negative samples the CO was established for each plate applying the formula: CO = (OD positive control × 0.2) + OD negative control). Sera with OD up to the CO + 0.1 were considered as positive samples. Sera with an OD of less to the CO − 0.1 were considered as negative samples. Sera with an OD with the range CO ± 0.1 were classified as "doubtful" samples. To enable standardization and intra-assay sensitivity it was established that the OD of the limit control sera was within the CO range defined by the positive and negative controls. All results were confirmed by Indirect Immunoperoxidase Technique (IPT).

### 2.7. Indirect Immunoperoxidase Technique (IPT)

*Preparation of antigen plates for IPT test*: Confluent monolayers of COS-1 cells were grown in fresh medium without serum into 96-well tissue culture grade microtitre plates. The plates were infected at a m.o.i. of 20 with each of the COS-1 cell-propagated ASF viruses selected for the Cp-Ag production and incubated in a humidified atmosphere containing 5% $CO_2$ at 37 °C for 18 h. After incubation, the inoculums were removed by vacuum suction and the ASFV-infected cell sheets were fixed with a cold solution containing 70% methanol and 30% acetone for 10 min. Finally the plates were washed with PBS for 10 min, sealed with tape and stored at −20 °C until used.

*IPT test*: For performing the IPT test, antigen plates taken from the freezer were warmed for 30 min at room temperature before the tape was removed to prevent the cell sheets from detaching from the plastic wall during washing. Then, the plates were blocked with PBS-M for 1 h at 37 °C. After incubation, the sera were added at a dilution 1:80 in PBS-M and incubated for 1 h at 37 °C in continuous agitation. Duplicate wells were used for positive, limit and negative OIE-reference standards sera included in each plate. The plates were washed for 10 min three times with PBS-T and the HRPO labelled protein A (PIERCE) was added diluted 1:5000 in PBS-M. The plates were incubated for 1 h at 37 °C. After washing, 50 μl of the substrate "3-aminoethyilcarbazol (AEC-Sigma)-dimethylformamide" diluted in acetate buffer was added per well. The reaction was stopped by the addition of 50 μl of PBS after 10–15 min of incubation at room temperature and the results were recorded using an optical microscope. The sera producing an intensive red

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cytoplasmic colouration in the cells were considered as positive samples and those with absence of red-colour were classified as negative samples.

### 2.8. OIE-ELISA and immunoblotting assays (OIE-approved analysis)

Both conventional ELISA and immunoblotting assays (IB) were performed using a lysate of MS cells infected with ASFV E70MS48 as the antigen and protein-A conjugated to HRPO as the indicator. Both procedures were carried out following the protocols described in the OIE Manual of diagnosis (OIE 2012).

### 2.9. Data analysis

Sensitivity and specificity were calculated by using the results of the OIE approved assays as a reference. All sera with a "doubtful" result according to the Cp-Ags ELISA (those with results in the cut off interval) were considered positive. The concordance between each ELISA (using the new Cp-Ag(s) based assays) plus IPT as a confirmatory technique and the OIE approved assays was calculated as the overall percent agreement between the results of the two assays by using two-by-two contingency tables. Kappa Coefficient ($\kappa$) statistics were used to evaluate the significance of the level of concordance between results in excess of that expected by chance, with $\kappa$ values of 0.81–1.00 representing almost perfect agreement, values of 0.61–0.80 representing substantial agreement, values of 0.41–0.60 representing good agreement, values of 0.21–0.40 representing moderate agreement, values of 0.01–0.20 representing slight agreement, and values of 0.00 representing no agreement (Everitt, 1989).

## 3. Results

### 3.1. Production of the ASFV cytoplasmic soluble antigens (Cp-Ags) and standardization of based Cp-Ags ELISAs

Optimization of the production of Cp-Ags in infected COS-1 cells was established by ELISA tests in which the plates were coated at 1:1600 dilution with the each of the Cp-Ags collected at 0, 24, 48, 72, and 96 h.p.i. and tested using the OIE-reference positive and negative standard sera. The absorbance values were found to be optimal against ASF positive sera at 48 h.p.i. confirming that the highest level of expression of ASF antigenic viral proteins occurred at this time. At 72 and 96 h.p.i. the cell cultures showed severe cytopathic effect and the ASF viral protein accumulation was reduced, as indicated by the decreasing absorbance values obtained relative to the positive control. The specificity of the ASFV p72 genotypes expected for each of the soluble ASFV antigens E70 (I), Arm07 (II), Moz64 (V), MwLil20/1 (VIII), Ken06.Bus (IX) and Ken08/Tk2.1 (X) obtained was confirmed by direct sequencing of PCR fragments derived from the C-terminal end of the p72 ASFV major capsid protein (data not shown).

Once the conditions for optimal Cp-Ags production in infected COS-1 cells had been established, we determined the optimal antigen dilution to use in coating ELISA plates using the OIE-reference positive and negative standard sera. The absorbance values of wells tested by ELISAs with the Cp-Ags were found to be optimal when the microtitre plates were coated with 0.2–0.9 μg of the Cp-Ag per well which correlated with a 1:1600 working dilution. We determined that at this antigen concentration and using a single dilution of sera at 1:30, the absorbance corresponding to positive reference sera was 10 times greater than that corresponding to the negative reference serum (data not shown).

### 3.2. Comparison of OIE-reference and novel antibody detection techniques based on Cp-Ags ELISA and IPT, using pig sera obtained in different field conditions

*Serum samples from pigs in ASF endemic areas*: One thousand and sixty two field samples collected between 2003 and 2010 in endemic areas of Africa and Sardinia were analysed in parallel using the six newly developed ELISA and IPT tests based on Cp-Ags. The specificity and sensitivity of each Cp-Ag-ELISA was determined by comparative analysis of 797 negative and 265 ASFV positive field sera classified using the OIE prescribed assays, ELISA combined with immunoblotting (IB), as gold standards.

The number of false positives out of the 797 negative sera tested was 1 with the Arm07-ELISA (99.6% specificity: [95% confidence interval [CI], 98.8–100]), 4 with the MwLil20/1-ELISA (99.5% specificity: [95% CI, 99–99.9]), 5 with the E70-ELISA (99.4% specificity: [95% CI, 98.8–99.9]),

**Table 3**
Specificity (Sp) and sensitivity (Se) results obtained using the Cp-Ag(s)-based ELISAs analysing a total of 930 East African serum samples comprising 782 from domestic pigs (negatives = 778; positives = 4) and 148 from wild African pigs (positives = 145 from warthogs, negatives = 2 from bushpigs and 1 from warthog).

| | Domestic pig sera | | African wild pig sera | | Performance of the assays[a] | |
|---|---|---|---|---|---|---|
| | % Sp (no. false positive out of 778) | % Se (no. false negative out of 4) | % Sp (no. false positive out of 3) | % Se (no. false negative out of 145) | % Specificity [95% CI] | % Sensitivity [95% CI] |
| Ken08/Tk2.1-ELISA | 98.8% (9) | 75% (1) | 100% (0) | 100% (0) | 98.8 [98.1–99.6] | 98.6 [96.8–100] |
| Ken06/Bus-ELISA | 98.3% (13) | 100% (0) | 100% (0) | 99.3% (1) | 98.3 [97.4–99.2] | 98.6 [96.8–1] |
| Arm07-ELISA | 99.8% (1) | 75% (1) | 100% (0) | 98.6% (2) | 99.8 [99.6–100] | 97.3 [94.7–99.9] |
| Moz64-ELISA | 99.2% (6) | 100% (0) | 100% (0) | 97.9% (3) | 99.2 [98.6–99.8] | 97.3 [94.7–99.9] |
| E70-ELISA | 99.2% (4) | 100% (0) | 100% (0) | 97.4% (4) | 99.49 [98.9–99.9] | 96.6 [93.7–99.5] |
| MwLil20/1-ELISA | 99.4% (5) | 100% (0) | 100% (0) | 99.3% (1) | 99.36 [98.8–99.9] | 98.6 [96.81–100] |

Se: sensitivity; Sp: specificity.
[a] Sp and Se values for each assay from the analyses of the total 930 serum samples obtained from domestic and wild pigs in East Africa.

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7 with the Moz64-ELISA (99.1% specificity: [95% CI, 98.2–99.6]), 9 with the Ken08/Tk2.1-ELISA (98.9% specificity: [95% CI, 98.1–99.6]) and 13 with the Ken06.Bus-ELISA (98.4% specificity: [95% CI, 97.4–99.2]).

The number of false negative sera detected with the 265 ASF positive sera was 1 with the Ken06.Bus-ELISA (99.6% sensitivity: [95% CI, 98.8–100]), 4 with the Ken08/Tk2.1-ELISA (98.5% sensitivity: [95% CI, 97–99.9]), 7 with Moz64, Arm07 and MwLil20/1-ELISA (s) (97.4% sensitivity: [95% CI, 95.4–99.2]) and 13 with the E70-ELISA (95% sensitivity: [95% CI, 92.4–97.6]). It is important to note the results obtained from the analysis of the 930 sera collected in endemic areas in East Africa originated from Kenya ($n = 278$), Tanzania ($n = 129$), Uganda ($n = 349$) and Mozambique ($n = 174$). Using the OIE tests as a "gold standard" 778 (99%) of the 782 domestic pig sera, 1 (0.7%) of the 148 warthog sera (*Phacochoerus* spp.) and the 2 bushpig (*Potamochoerus* spp.) sera analysed were negative. The high specificity values between 98.3% for the Ken06.Bus-ELISA (lowest specificity) and 99.8% for the Arm07-ELISA (highest specificity) obtained when the East African negative sera were tested using the Cp-Ag-based ELISA(s) showed complete concordance ($\kappa$ values $\sim 0.96$) with the results obtained using the reference tests. Similarly, the sensitivity percentage values from 97.3% to 98.6% analysing the 151 positive sera (warthog = 147, domestic pig = 4), showed high concordance with the OIE reference tests results (Table 3).

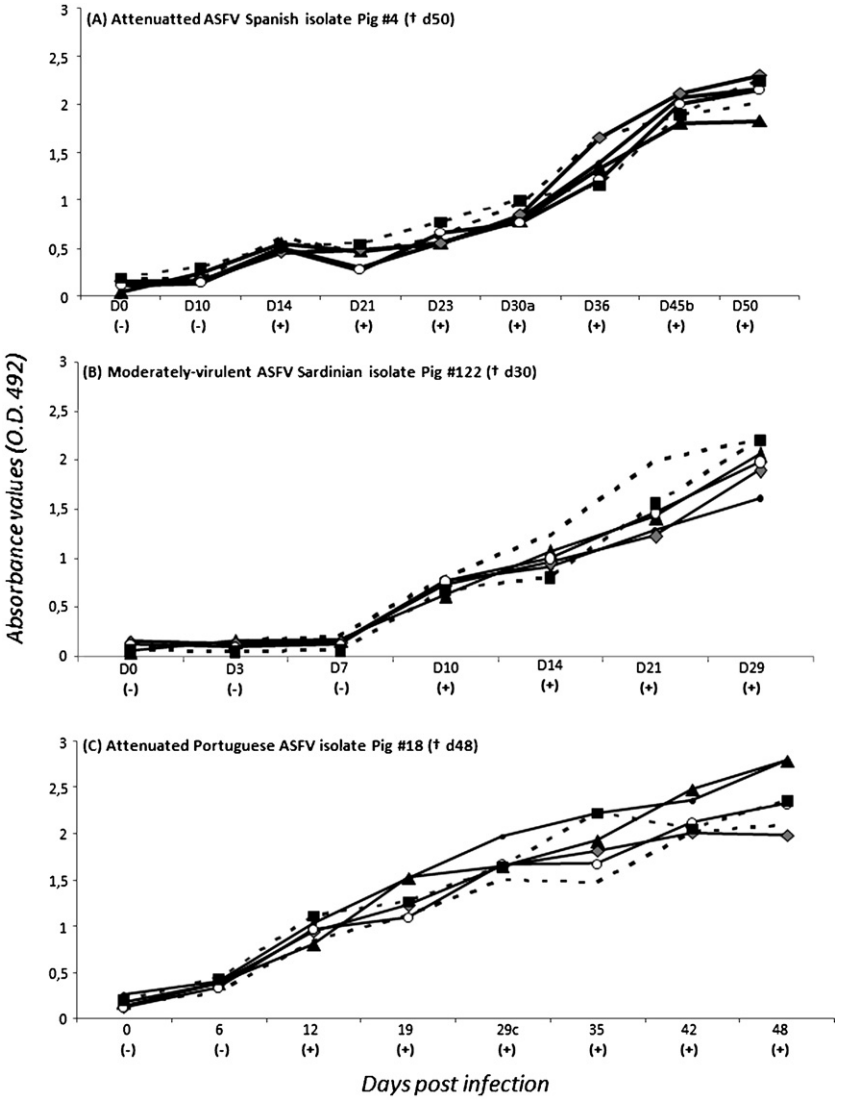


**Fig. 1.** Detection of ASFV-specific antibodies using isolate-specific Cp-Ag-ELISAs expressed in OD values from three representative pigs experimentally infected with ASFV isolates belonging to p72 genotype I including (A) the ASFV Spanish isolates, (B) the ASFV Sardinia isolate and (C) the Portuguese ASFV isolates. Positive and negative results obtained with OIE prescribed assays are indicated by (+) and (−) signs; slaughtering (†) or death (‡) dates are indicated. In (A), date of infection with the homologous virulent E75 strain is noticed by "a" and with the heterologous E70 strain by "b". In (C), date of infection with the homologous virulent L60 strain is marked with "c". Ken08/Tk2.1-ELISA (−▲−), Ken06.Bus (- - -), Arm07-ELISA (−●−), Moz64-ELISA (−◆−), E70-ELISA (−○−) and MwLil20/1-ELISA (−■−).

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All the sera included in this study were analysed in the IPT(s) assay based on each of the six antigen preparations. All the false positive results obtained in the first screen using the indirect Cp-Ag-based ELISAs were confirmed as negative by the ASFV genotype-specific-IPT test exhibiting 100% correlation with the OIE reference test results. The number of false negative results was 2 with the MwLil20/1-IPT, 3 with the Arm07 and Moz64-IPTs and 4 for Ken06.Bus and Ken08/Tk2.1-IPTs. No false negative results were obtained using the E70-based IPT test. The $\kappa$ values obtained were between 0.97 and 1 and show perfect agreement between the results of the OIE reference tests and the results observed with the new developed IPT tests (data not shown).

*Serum samples from pigs in ASF free areas*: Negative field samples from domestic pigs and wild boar, collected in ASFV-free regions (Spain), did not present specific antibody responses in the Cp-Ag-ELISA(s) or in the IPT, confirming the specificity of the OIE reference tests.

### 3.3. Comparison of OIE and newly developed antibody detection techniques (Cp-Ags ELISA and IPT) using pig serum samples obtained in experimental studies

The ability of the new Cp-Ags-based ELISA and IPT tests to detect ASF during acute, sub-acute and chronic infections was assessed in independent experimental infections with ASFV isolates belonging to p72 genotypes I, II and X. The results were compared with those obtained using the OIE-serological prescribed procedures (ELISA plus IB). Representative results from each experimental infection are shown in Fig. 1 (genotype I), Fig. 2 (genotype II) and Fig. 3 (genotype X). Complete data is available with the online journal in the supplementary data files.

Using sera collected from pigs inoculated with the attenuated Spanish isolate E75 $CV_1$4 (genotype I), specific antibody response was detected using each of the Cp-Ags based ELISAs, with confirmation using IPT, at 14 dpi(s) in all of the animals. The antibodies were detectable until 50 dpi in the re-inoculated animal #4. Same result was obtained with the OIE antibody detection techniques (Supplementary Fig. S1 panel A).

In samples collected from animals infected with the moderately-virulent Sardinian isolate (genotype I), antibody response was detected between 10 and 14 dpi(s) in 3 out of 4 pigs tested using the Cp-Ags ELISAs and IPT tests developed, and also using the OIE approved assays. One pig which died at 11 dpi had not yet seroconverted (Supplementary Fig. S1 panel B).

During the chronic infection developed in the inoculated animals with the attenuated Portuguese isolate NHV/P68, belonging to genotype I, all of the antibody detection techniques employed in this study detected a specific antibody response from 12 dpi(s) which was maintained until the end of the experiment (Supplementary Fig. S1 panel C).

The analysis of sera collected from two pigs kept in contact with inoculated animals using the ASFV Arm07 isolate (genotype II), revealed a detectable antibody response at 16 dpi using all of the Cp-Ags in both ELISA and IPT techniques. The same result was observed using the OIE approved assays (Fig. 2).

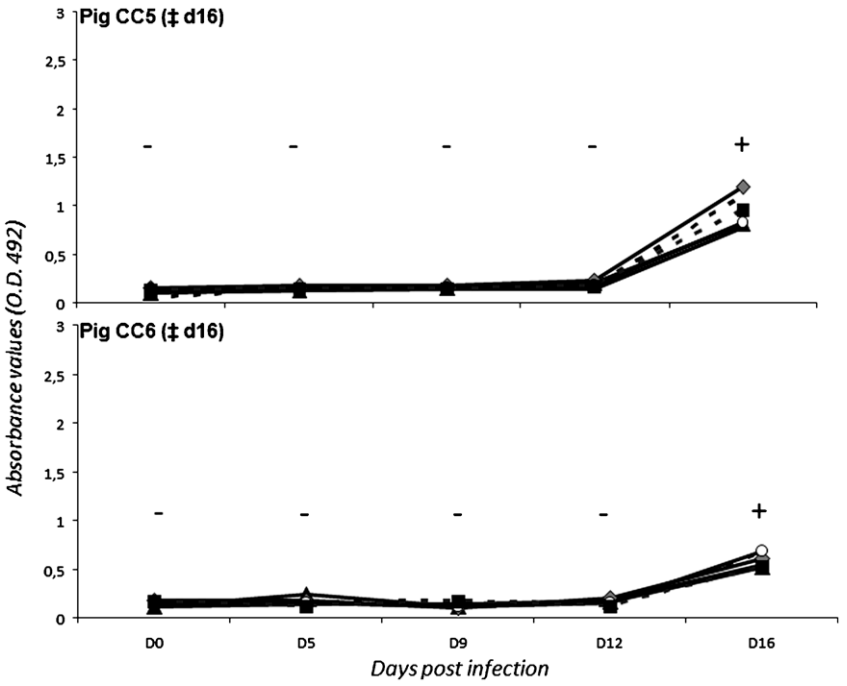


**Fig. 2.** Detection of ASFV-specific antibodies using isolate-specific Cp-Ag-ELISAs expressed in OD values from pigs experimentally infected with the virulent ASFV isolate Arm07 belonging to p72 genotype II. Positive and negative results obtained with OIE prescribed assays are indicated by + and – signs; slaughtering (†) or death (‡) dates are also indicated. Ken08/Tk2.1-ELISA (–▲–), Ken06.Bus (- - -), Arm07-ELISA (–●–), Moz64-ELISA (–◆–), E70-ELISA (–○–) and MwLil20/1-ELISA (–■–).

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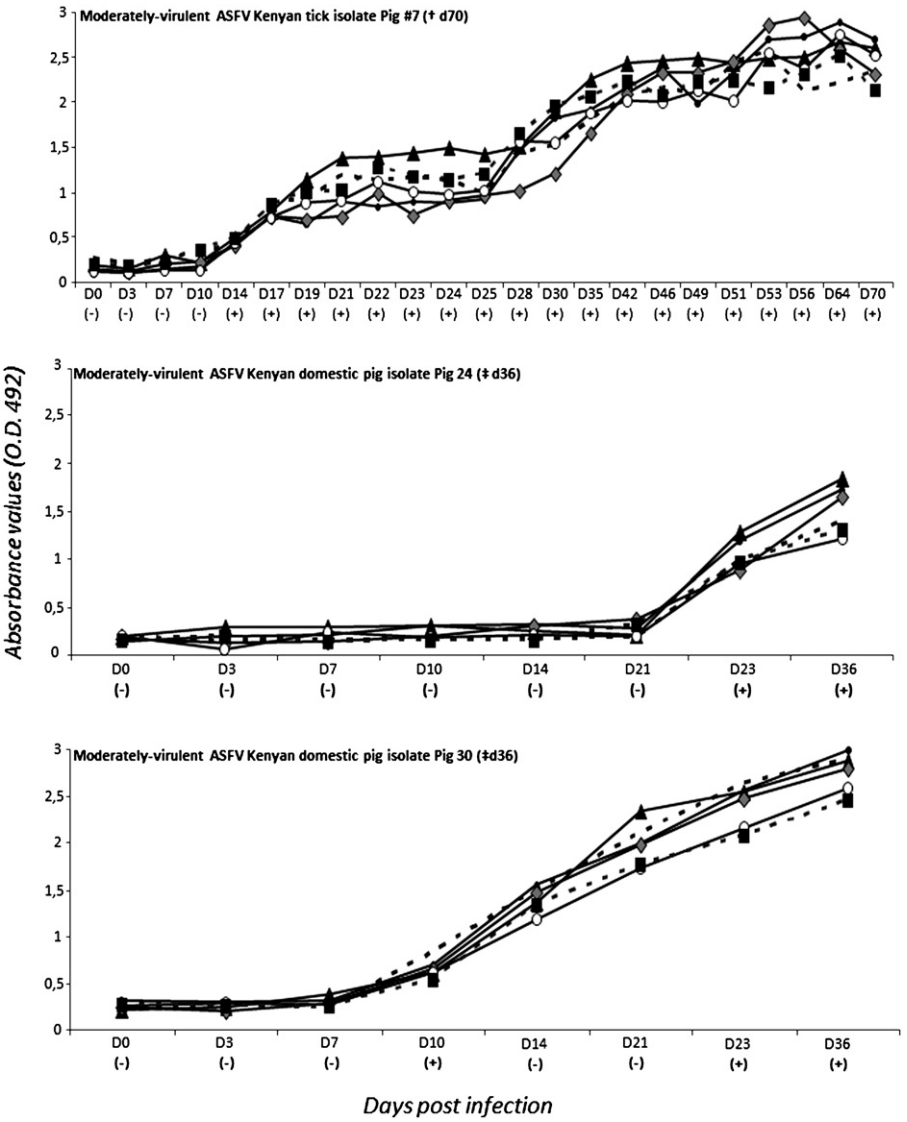


**Fig. 3.** Detection of ASFV-specific antibodies using isolate-specific Cp-Ag-ELISAs expressed in OD values from three representative pigs experimentally infected with the moderately virulent ASFV Kenyan isolates belonging to p72 genotype X. Positive and negative results obtained with OIE prescribed assays are indicated by (+) and (−) signs; slaughtering (†) or death (‡) dates are also indicated. Ken08/Tk2.1-ELISA (−▲−), Ken06.Bus (- - -), Arm07-ELISA (−●−), Moz64-ELISA (−◆−), E70-ELISA (−○−) and MwLil20/1-ELISA (−■−).

In the experimental direct inoculation of pigs with the two Kenyan viruses belonging to the most variable ASFV genotype X, the timing of a detectable antibody response was variable depending on the animals but independent of the antigen preparation used. The animals inoculated with the moderately virulent ASFV tick isolate Ken05/Tk1 developed antibody response on days 14 of bleeding (#7) or 21 (#8) that were detected with all of the assays employed (Supplementary Fig. S3 panel A). Among the ten animals oro-nasally infected with the moderately virulent ASFV domestic pig isolate Ken05/K2, highly variable antibody responses were observed between individual animals. Three pigs did not exhibit detectable antibody responses with any ELISA and/or IPT or IB tests, whilst in the remaining animals the antibody response initiated between 10 and 23 dpi(s) (Supplementary Fig. S3 panel B).

## 4. Discussion

An essential feature of ASFV infection is the absence of discernible serotypes, which may be correlated with the failure of ASFV infection to induce neutralizing antibodies. This fact together with the early appearance and long-term persistence of antibodies and the lower cost compared to PCR-based methods, emphasize the relevance of antibody detection techniques in the control of the disease in affected countries. The current ASF serological OIE endorsed assays consist of an initial screening of sera using an enzyme-linked immunosorbent assay (OIE-

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ELISA), followed by an immunoblotting assay (OIE-IB) or Indirect Immunofluorescence test (IIFT) as a gold standard to confirm the ambiguous ELISA results (OIE 2012). However, recent investigations in eastern Africa have documented a low incidence of detectable serological responses to ASFV infection using the OIE-approved assays in ASF virus positive pigs (Gallardo et al., 2009b, 2011a). Comparative studies of the eleven whole ASFV genome sequences corresponding to different African and European isolates, have demonstrated that the eastern African ASFV isolates are more genetically distant when compared with those historically circulating in Europe, America and West Africa classified within p72 genotype I. The amino acid identity scores of the p72, p54 and p30 antigenic proteins in relation to p72 genotype I decreases to 95%, 89% and 85% respectively for the Eastern isolate Kenya 1950 for which this information is available for the genes encoding the antigenic proteins (Perez-Filgueira et al., 2006; Chapman et al., 2008, 2011; de Villiers et al., 2010). Recently introduction of a virus closely related to the South East African p72 genotype II in Europe (Rowlands et al., 2008) and the detection of a genotype IX virus previously known only from East Africa in western central Africa (Gallardo et al., 2011b) have raised concerns about the capacity of the available diagnostic assays to detect ASF specific antibodies induced by infection with all known genotypes. Since there is evidence indicating that genetic variability exist between isolates from various geographical locations, the question is to whether a virus isolate from each location should be used in preparing antigen or whether the single representative isolates belonging to p72 genotype I used in the OIE serological reference tests could be used universally.

To answer this question, we have developed in this study new serological assays (ELISA and IPT) focused on the use of six new ASFV genotype-specific-antigens including the most variable ones circulating in East Africa and genotype II virus currently circulating in the Caucasus. The development of new ELISA and IPT tests has been based on the recently described ability of the ASFV isolates to grow in COS-1 cell monolayers (Hurtado et al., 2010). The ASFV genotype-specific-antigens used in the ELISA tests were the cytoplasmic soluble fraction (Cp-Ag) of COS-1 infected cells obtained by semi-purification of the ASF virions in a sucrose layer at 48 h post infection. Indirect ELISAs were standardized using each of the Cp-Ags obtained against the positive, limit and negative ASF reference sera. A comparative study was performed analysing one thousand and sixty two field samples collected from endemic areas in Europe (Sardinia) and Africa. The sensitivity and specificity values obtained which ranged from 95% to 99.6% and 98.4% to 99.6%, respectively, were in agreement with the results obtained using the OIE "gold standard" tests (ELISA and IB tests). The high concordance observed in the analysis of the 778 negative sera collected from domestic pigs in the endemic East African countries Tanzania, Kenya, Uganda and Mozambique using the genotype specific antigen assays reaffirmed the high level of accuracy obtained with the official OIE assays.

The concordance was confirmed by the analysis of 214 experimental sera collected from six independent experimental infections performed in European pig breeds using East African and European ASFV isolates including the Armenian p72 genotype II. The newly developed Cp-Ag-ELISAs were able to detect specific antibody response against ASFV following the same temporal pattern in the infection cycle as those obtained using the OIE sanctioned serological assays. During the course of the chronic infections induced by the attenuated strains belonging to p72 genotype I, antibody responses were detectable from 12 days post infection until the termination of the experiment. From animals inoculated with the moderately virulent ASFV strains belonging to p72 genotype I or X which induced a sub-acute form of the disease, high variability of the serological response was observed depending on the individual animal, but independent of the viral genotype used for the inoculation as well as the source of the antigen used to coat the ELISA plate. All the Cp-Ag-ELISAs were consistently able to detect antibodies in the acute form of ASF induced in animals infected with ASFV genotype II (Arm07 isolate) at 16 days post infection.

In this study the IPT test was developed as an alternative ASF serological confirmatory technique for each of the virus isolates. Although the IB assay has been widely demonstrated be sufficiently sensitivity and specific to perform ASF diagnosis during eradication and surveillance programs for disease control (Alcaraz et al., 1990; Bech-Nielsen et al., 1993a,b; Arias et al., 1993), the production of the antigen strips is laborious, requires specialized staff and only allows the analysis of a limited number of sera. The new developed IPTs were in full agreement with the reference test data, exhibiting specificity and sensitivity close to 100%. Furthermore, the technique makes interpretation of the data easier and allows the analysis of serum sample on a large scale.

Our data suggests that substitution of serological tests based on antigen extracts derived from ASFV isolates originating from different geographical sources and propagated in heterologous primate (COS) cells does not improve sensitivity and specificity relative to widely used OIE-approved assays (ELISA and IB) for detection of anti-ASFV antibodies in infected pigs. The OIE serological test uses only antigen extracts generated from the E70 Spanish reference strain (classified within p72 genotype I). These findings support the contention that, although there is genetic variability in the sequence of the antigenic proteins when different viruses are compared, the antibody response against different isolates of ASFV is directed against multiple shared virus determinants and there may be a core of epitopes that are recognized regardless of the virus isolate and antigenic polymorphism in the individual immunodominant proteins (Kollnberger et al., 2002).

In conclusion, this study has widely demonstrated, using newly developed antigen-specific assays for independent verification, that the OIE approved serological tests for ASF have high specificity and sensitivity to allow confident diagnosis of ASF independent of the viral genotypes circulating in a particular region. The reason for low seropositivity in infected pigs in East African regions remains unknown. An alternative explanation may reside in the pig host rather than the virus. Different pig breeds could have different immunological repertoires,

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clonal deletions, swine leukocyte antigen types, or innate immune mechanisms. There is evidence that domestic pigs from East African countries such as Kenya exhibit some introgression of genetic material from the Asian centres of wild boar domestication (Ramirez et al., 2009) and are therefore not identical to European and West African pig breeds. However more "*in vivo*" experimental studies using East Africa "indigenous pigs" are required to confirm this theory.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.vetmic.2012.08.011.

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