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Review

African swine fever virus serodiagnosis: A general review with a focus on the analyses of African serum samples

Carolina Cubillos[a,1], Silvia Gómez-Sebastian[b,1], Noelia Moreno[a], María C. Nuñez[b], Leopold K. Mulumba-Mfumu[c], Carlos J. Quembo[d,e], Livio Heath[e], Eric M.C. Etter[f], Ferran Jori[f,g], Jose M. Escribano[h], Esther Blanco[a,*]

[a] Centro de Investigación en Sanidad Animal, INIA, Valdeolmos 28130 Madrid, Spain
[b] Alternative Gene Expression S.L. (ALGENEX), Centro empresarial, Parque Científico y Tecnológico de la Universidad Politécnica de Madrid, Campus de Montegancedo, 28223 Pozuelo de Alarcón, Madrid, Spain
[c] Central Veterinary Laboratory, Avenue Wangata Hospital, General de Reference P.O. Box 8842, Kinshasa 1, Democratic Republic of Congo
[d] Laboratório Regional de Veterinária em Chimoio – Centro Zonal de Investigação Agrária da Zona Centro – Instituto de Investigação Agrária de Moçambique, P.O. Box 42
[e] Agricultural Research Council–Onderstepoort Veterinary Institute, Onderstepoort, South Africa
[f] Centre International de Recherche Agronomique pour le Developpement (CIRAD), UR 22 Animal and Integrated Risk Management, Montpellier, France
[g] Mammal Research Institute, University of Pretoria, 0002 South Africa
[h] Departamento de Biotecnología, INIA, Autovía A6 Km 7, 28040 Madrid, Spain

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ABSTRACT

African swine fever (ASF) is an infectious disease that causes heavy mortality in domestic pigs. At present there is no vaccine against ASF, and eradication in countries where the disease is endemic is based only on competent diagnosis programs and the sacrifice of infected animals. Due to the presence of natural attenuated strains, certain infection conditions may result in reduced mortality. In these situations, the disease can be diagnosed by detection of specific antibodies. The use of classical and validated diagnosis assays, such as ELISA and Indirect Immunofluorescence or Immunoblotting, allowed the eradication of ASF in the Iberian Peninsula in the 1990s. However, given that conventional tests include the use of antigens obtained from ASF virus (ASFV)-infected cells, they have several disadvantages, such as difficulties to achieve standardization and also the risks associated with the manipulation of live virus. Such drawbacks have led to the development of alternative and more robust systems for the production of ASFV antigens for use in anti-ASFV antibody detection systems. In the present review, we provide an update on current knowledge about antigen targets for ASFV serodiagnosis, the significant progress made in recombinant antigen production, and the refinement of ASF serological diagnostic assays. Moreover, we describe the accuracy of an ELISA developed for the serodiagnosis of ASFV in Africa. This assay is based on a novel p30 recombinant protein (p30r) obtained from an Eastern African viral isolate (Morara strain), which shares 100% amino acid sequence identity with the Georgia virus isolate. That study included the analyses of 587 field sera collected from domestic pigs and warthogs in Senegal (West Africa), the Democratic Republic of Congo (Central Africa), Mozambique (South-East Africa), and South Africa. The results revealed that the novel p30r-based ELISA allows the accurate detection of antibodies against ASFV, independently of the geographical origin of the sera.

Contents

* Corresponding author. Tel.: +34 916202300; fax: +34 916202247.
E-mail address: blanco@inia.es (E. Blanco).
[1] C. Cubillos and S. Gómez-Sebastián have contributed equally to the unpublished results presented in this paper.

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1. Introduction

Classified as a notifiable disease by the World Organisation for Animal Health (OIE), African Swine Fever (ASF) is a highly contagious disease of domestic and wild pigs and causes major economic losses for the pig industry in affected countries. In addition, in Africa, where the disease is endemic in most sub-Saharan countries, ASF also has serious implications for food safety, thus limiting the availability of an important source of human dietary protein (Costard et al., 2009). Since there is no vaccine available for ASF, the detection of specific antibodies against the virus are indicators of historic infection, or current infection only if the presence of antibodies coincides with the presence of viable virus. Therefore, rapid serodiagnosis procedures can contribute to the complete eradication of the disease in certain affected regions. For example, the strategy for eradication of the ASF in the Iberian Peninsula was carried out by detection and slaughter of seropositive pigs. This strategy cannot be recommended for Africa, where the countries are not able to compensate for pigs that are culled. However, in these areas the great value of highly sensitive and specific serological tests is to improve the understanding of the disease and on that bases be able to identify high risk areas and to develop appropriate recommendations for prevention of the disease.

ASF virus (ASFV) causes inapparent persistent infections in its natural hosts, namely warthogs (*Phacochoerus africanus*), bushpigs (*Potamochoerus porcus, P. larvatus*), and soft ticks (*Ornithodoros moubata*) (Anderson et al., 1998; Kleiboeker et al., 1999). In domestic pigs, ASF was originally described to cause an acute hemorrhagic fever, leading the death of all animals infected. However, less virulent isolates have emerged during the circulation of the virus in domestic pigs, thereby increasing the prevalence of subacute and inapparent infections (De Kock et al., 1940; Mebus and Dardiri, 1980; Bech-Nielsen et al., 1995; Penrith et al., 2004). Pigs that survive natural infection usually develop antibodies against ASFV from 7 to 10 days post-infection which persist for long periods. Therefore, the detection of specific antibodies against ASFV should be performed for the diagnosis of subacute and inapparent forms of the disease.

In this context, the present review focuses on current knowledge about ASFV serological tests and immunodeterminant antigens used for disease control. The progress made in these fields may have great impact on the development of more reliable and accurate serological assays for the diagnosis of ASFV infections. Moreover, we describe the accuracy of an enzyme-linked immunoabsorbent assay (ELISA), based on a novel recombinant p30 (p30r) protein obtained from a viral isolate from East Africa (Madagascar), for ASFV serodiagnosis in Africa and potentially in Europe. Serum samples from domestic and sylvatic hosts, collected in Senegal (West Africa), the Democratic Republic of Congo (Central Africa), Mozambique (South-East Africa), and South Africa, were successfully analyzed by this ELISA. The novel p30r-based assay allowed the accurate detection of antibodies against ASFV, independently of the geographical origin of the sera. This has special relevance taking into account that ASFV isolates from Europe and West Africa are closely related to each other (Genotype I), while South and East Africa isolates are more diverse (21 different genotypes).

2. ASF serodiagnostic protein targets

Knowledge of the protein composition of ASFV virion structures is crucial because some might have immunological significance. Furthermore, identification of the most antigenic viral proteins is highly relevant for the improvement of serological diagnostic tests. ASFV is the only known DNA arbovirus and the only member of the Asfarviridae family (Dixon et al., 2011). ASFV is a large virus showing tropism for macrophages and monocytes, where it induces approximately 100 polypeptides (Alcaraz et al., 1992). About 40 of these molecules have been reported to be incorporated into the viral particle (Carrascosa et al., 1985). The viral DNA encodes for a number of novel genes not present in other virus families. The capacity of ASFV to persist in its natural hosts and in domestic pigs recovered from infection and carrying low virulence isolates shows that the virus has effective mechanisms to evade host defense systems (Dixon et al., 2004). The virion core is composed of a nucleoid, enclosed by a protein layer (core shell) which contains several viral proteins (Andrés et al., 1997, 2002). Surrounding the core are two lipid bilayers, called the inner membrane and, outside this membrane, the capsid. Additional complete or disrupted membranes, acquired during virus budding, can also be found in the viral particle. The major component of the viral capsid is protein p72, one of the first viral proteins identified as responsible for the induction of antibodies after a natural infection (Tabarés et al., 1980). The development of a semi-purification process for this protein from viral particles first led to the use of ELISA for antibody screening, thus reducing the number of false positive reactions found with previously developed antigens (Tabares et al., 1981). In addition, the two structural proteins p30 (also denominated p32) and p54 were clearly identified as highly antigenic during infection (Pastor et al., 1989; Afonso et al., 1992; Alcaraz et al., 1990, 1995; Oviedo et al., 1997; Kollnberger et al., 2002; Gallardo et al., 2006). Furthermore, antibodies against these three proteins are involved in virus neutralization, inhibiting the attachment (p72 and p54) and internalization (p30) (Borca et al., 1994; Gómez-Puertas et al., 1996, 1998) of virus. However, despite the potential of p72, p54 and p30 as serodiagnostic targets, these ASFV proteins are not sufficient for antibody-mediated protection against different virus strains (Gómez-Puertas et al., 1998; Neilan et al., 2004).

The recognition of ASFV p54 and p30 by porcine field sera collected in Spain has been compared to that of polyprotein pp62 (encoded by the gene CP530R) (Gallardo et al., 2006). Sera from infected pigs recognized the three recombinant proteins (p54r, p30r and pp62r) by immunoblotting (IB). The similar reactivity of these three proteins was also found when they were used as antigens in ELISA. In this assay p54 requires solubilization in 7 M Urea to reach optimal signals. This observation suggests that antibodies induced against this protein during ASFV infection recognize mainly linear epitopes. Polyprotein pp62 is the precursor protein of mature products p35 and p15, structural proteins localized at the core shell (Andrés et al., 2002). Unpublished data from our lab at CISA identify p15 as the mature protein responsible for the antigenicity of pp62.

Interestingly from the point of view of ASF serodiagnosis, the recognition of pp62 by sera from ASFV-infected pigs was maintained even in poorly preserved sera, while less reactivity was detected against p30 and p54 (Gallardo et al., 2006). These results

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may indicate that antibodies against pp62 are more stable or display higher affinity than others. More experiments with field sera are needed to confirm this hypothesis. This property of pp62 could be very useful to overcome one of the disadvantages of using crude preparations from infected cells as antigens in ELISA, namely the lack of analytical reliability for poorly preserved samples (Arias et al., 1993).

The identification of other new serological immunodeterminants of ASFV was achieved by screening a viral cDNA expression library of the ASFV Ba71V isolate, using immune antisera from infected domestic pigs and from bush pigs (Kollnberger et al., 2002). That study identified 14 viral open reading frames (ORFs) encoding antigenic epitopes of the virus. Five of these corresponded to the following structural proteins: the previously identified p30 (CP204L), p54 (E183L) and p72 (B646L), the bacterial histone-like protein (A104 R), and p10 (K78R). In addition, 3 non-structural (F334L, K196R and NP419L) and 4 unassigned proteins (B602L, C44L, Cp312R and K205R) also showed significant reactivity with immune antisera. Strong reaction of convalescent pig serum has been described against a protein yielded from the in vitro translation of ORF B602L (Irusta et al., 1996). In a longitudinal antibody response analysis against the 12 abovementioned recombinant proteins using sera from experimentally infected pigs, strong and sustained antibody titers were confirmed against 4 of them: p54, K205R, A104R and B602L (Reis et al., 2007). Later on, the antigenicity of these recombinant proteins was further confirmed by testing porcine serum from animals naturally infected with ASFV (Gallardo et al., 2009a).

3. Conventional procedures for ASFV antigen production and serological assay formats

One of the earliest serological assays used for laboratory diagnosis of ASF was the immunoelectroosmophoresis (IEOP) test (Pan et al., 1972). This assay was much more sensitive than the agar gel double-diffusion precipitation test and even more sensitive than the complement fixation technique (Ferris et al., 1980), and was soon established as a screening test. The conventional antigen used in the IEOP test was prepared from extracts of Vero cells infected with ASFV (Pan et al., 1974).

Nevertheless, in infectious diseases where diagnosis is based on antibody determination, confirmatory tests are usually required to confirm positive reactions, especially when crude antigens are used in the diagnostic techniques. In this regard, the ASF serodiagnosis strategy used in the past included IEOP as a screening test and indirect immunofluorescence (IIF) to confirm positive reactions (Botija, 1970; Pan et al., 1974). This confirmatory assay is run with cell cultures in which only 10–20% of cells are infected with the virus. Therefore, typical positive reactions can be seen as fluorescent intracellular corpuscules, which correspond to viral factories. Positive and false positive reactions are easily distinguished when analyzing pig serum against infected and uninfected cell cultures. Although these assays played a critical role in ASF serosurveillance and eradication programs (Pan et al., 1974), they were laborious and not readily adaptable to large-scale surveys.

Therefore, the IEOP assay was soon replaced by the ELISA, considered then the most sensitive and suitable method available for testing large numbers of sera. This test is the most widely used technique for the diagnosis of subacute and inapparent ASF carriers (Wardley et al., 1979; Tabares et al., 1981; Pastor et al., 1990). ELISA shows greater sensitivity than the IEOP test, although in both assays the quality of the antigen preparation influences the final sensitivity and specificity of the test. Indeed, the use of crude antigens in ELISA, such as those described for the IEOP test, did not provide sufficient specificity to be included in an acceptable routine diagnostic test for ASF. Thus, Tabares et al. (1981) described the preparation of a semi-purified ASFV major capsid protein VP73 (p72), which greatly increased the reliability of ELISA when it was used as antigen.

Later on, the production of ASF antigen for ELISA was improved in order to make the technique economically feasible in large-scale surveys. At present, the cytoplasmic soluble antigen used in this assay (Escribano et al., 1989) is obtained from MS cells (monkey kidney cell line), grown in the presence of pig serum infected with an ASFV isolate passaged 48 times on MS cells. The use of pig serum in cell cultures instead of bovine serum circumvented antigen contamination with albumin from the latter, which was the main factor responsible for false positive reactions in ELISA until then (Escribano et al., 1989). The soluble protein fraction from infected cells is prepared by cell disruption, elimination of nuclei, and sedimentation of cellular debris by a 20% (w/w) sucrose cushion. The supernatant above the sucrose layer is used as the ELISA antigen. This crude antigen is currently recommended as a detector reagent in the prescribed screening test for international trade (OIE, 2012).

Despite the sensitivity of ELISA, one of the disadvantages continues to be the number of false positive reactions obtained with field sera and the standardization of the technique in laboratories. The main consequence of these limitations is that positive serum samples require confirmation by a second serological test. Although the IIF test can be used for this purpose, it has been replaced by IB, which provides increased specificity and a similar sensitivity to that of ELISA. Additionally, it has been demonstrated that sera lose reactivity in ELISA earlier than in IB (recognition of linear epitopes instead of conformational), thereby allowing improved detection of antibodies in poorly preserved sera (Arias et al., 1993). Furthermore, IB presents a simple and objective interpretation of the results (Pastor et al., 1989; Alcaraz et al., 1990; OIE, 2012). This test is performed using the same antigen as in ELISA and described above. The antigenic proteins are resolved in 17% acrylamide gels and transferred onto a nitrocellulose filter. Strips approximately 4 cm long, containing proteins of 23–35 kDa, constitute the antigen strips used for individual sera samples. This test has the additional advantage that the filter strips show stability throughout storage and transportation at room temperature in a dry atmosphere. No observable loss in the reactivity of transferred proteins is observed in at six months (Pastor et al., 1989). Another alternative confirmatory test to IIF and IB is the indirect immunoperoxidase plaque-staining method (IIPS) (Pan et al., 1982). This technique shows sensitivity and specificity comparable to that of the IIF test, but it is more suitable for large-scale analyses.

Despite the satisfactory performance of the classical assays described here (based on the use of virus-induced proteins in infected cells as antigen reagent), they have several limitations, such as the standardization of techniques and the need to manipulate the infectious agent and thus the consequent requirement of approved facilities for the biocontainment of Groups 3 and 4 pathogens (OIE, 2012).

4. Recombinant serological tests for ASF

Advances in molecular biology have greatly improved opportunities to upgrade the selection and production of immunoreagents and their application for the development of new assays. In this regard, during recent years extensive research has focused on the development of recombinant antigens to be introduced into serological diagnostic tests for ASF.

The use of recombinant proteins as reagents offers many advantages over antigen production based on virus-infected cells. In the case of ASFV, the use of recombinant proteins circumvents the

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need to manipulate potentially dangerous live viruses and allows the standardization and scale-up of their production. In addition, these antigens improve the homogeneity of the results obtained in different laboratories and may increase sensitivity and specificity, thus reducing the false positive reactions produced by cellular culture compounds that contaminate antigens (Escribano et al., 1989). In some cases, such as IB, recombinant antigens also facilitate the interpretation of diagnostic results (Alcaraz et al., 1995).

Many potentially useful virus protein candidates in diagnosis have been described; however, only a small number have been tested and validated in various techniques. One of the first recombinant assays described for ASFV was a Western blot technique to confirm positive results obtained in ASV antibody detection by ELISA (Alcaraz et al., 1995). This confirmatory test was based on the use of protein p54 expressed in *Escherichia coli* fused to the N-terminus of the MS2 polymerase. The recombinant Western blot assay was highly specific and equally sensitive for the detection of pigs with antibodies to ASFV as the conventional Western blot using crude antigens obtained from infected cells and described above.

The same prokaryotic expression system, *E. coli*, was used by Reis et al. (2007) to produce 12 viral proteins previously identified as serological targets (Kollnberger et al., 2002). Serum samples from pigs infected experimentally with a non-fatal ASFV isolate and collected at various days post-infection (d.p.i.) were analyzed by ELISA using the 12 recombinant proteins individually. Strong antibody responses were found to 4 of them (p54, Histone-like, pK205R and pB602L), all showing 100% sensitivity at 21 days d.p.i. Interestingly, that study found that recombinant p73 expressed in bacteria is a not an adequate serological target for ASFV serodiagnosis as it failed to detect serologically positive pigs with inapparent symptoms.

The suitability of these four candidate proteins as diagnostic tools was further evaluated with European and African porcine field sera (Gallardo et al., 2009a). Purified *E. coli*-derived recombinant protein-based ELISAs proved effective for the analysis of pig sera from Europe when proteins p54 and pB602L were used as antigens, obtaining high sensitivity and specificity (among 95 and 98%, respectively). Therefore, these recombinant ELISAs performed as well as the OIE-approved diagnosis technique (conventional ELISA plus confirmation by Western blot).

Serological differentiation of ASF field isolates is not possible due to the lack of discernible serotypes. However, the sequence variability reported among viral isolates from various geographic locations can affect the recognition of specific antigens in serological tests. The accurate selection of ASFV antigens with a low ratio of antigenic variability among virus isolates is prerequisite for their use in serological assays. Although a limited number of positive sera from West Africa were tested, the 9 positives identified by OIE-approved assays in that study were confirmed by the p54- and pB602L-based recombinant ELISAs. Given their performance, these recombinant assays would also be suitable for testing sera samples from West Africa. Regarding sera from East Africa, since very few ASF-positive samples were analyzed by these recombinant ELISAs, the sensitivity of the test was not calculated (Gallardo et al., 2009a). However, the frequency of positive sera detected by these assays was lower than achieved by the OIE-approved tests. This observation may thus indicate that these recombinant ELISAs provide unacceptable sensitivity for diagnosis purposes in East Africa.

In addition to the analyses of porcine serum samples, the studies of sera collected from wild animals, such as the warthog (*Phacochoerus africanus*), are particularly interesting in East and Southern Africa, were wild pigs have been described as reservoir hosts of ASFV. Therefore the control of the disease in warthogs is highly relevant for the management of sporadic outbreaks in domestic pigs in contact with these wild animals and for determining the exposure status of warthogs to ASFV, where the sylvatic cycle plays a crucial part in the epidemiology of the disease (Lubisi et al., 2005; Jori and Bastos, 2009). A small number of warthog samples collected in Uganda were tested by the recombinant ELISAs using the proteins expressed in *E. coli*. A total of 23 out of 26 positive sera by OIE-approved approaches were also positive with the B602L- and p54-based ELISAs (Gallardo et al., 2009a). These results contrast with the low sensitivity found when testing domestic pig sera from East Africa. This discrepancy may be related to the distinct ASFV genotypes responsible for the induction of antibodies. ASFV isolates belonging to genotype IX have been reported in Uganda (Gallardo et al., 2009b), whereas mainly genotype II occurred in Mozambique in outbreaks in 1998–2005 (Bastos et al., 2004; Lubisi et al., 2005). Therefore if this hypothesis were confirmed, the recombinant ELISA results would suggest that the antigenicity of proteins B602L and p54 is conserved in ASFV isolates from genotype IX but not in those from genotype II.

Eukaryotic systems, such as the insect cell-baculovirus recombinant system, provide an alternative system for the production of recombinant antigens. The advantage of this expression system with respect to *E. coli* is that the proteins are likely to be produced in a native conformation as they can post-translationally modify the proteins that are expressed efficiently. ASFV proteins p30, p54 (Oviedo et al., 1997; Gallardo et al., 2006) and polyprotein pp62 (Gallardo et al., 2006) were produced as recombinant antigens expressed in insect cells (Sf9 or Hi5 cells) using a baculovirus expression system. These recombinant proteins were used in ELISA and Western blot for ASF antibody detection in sera from experimentally inoculated pigs and field sera from European inapparent ASF serologically positive animal. These analyses showed that the sensitivity and specificity of p30-r, p54-r and pp62-r based ELISAs, using the baculovirus-expressed proteins, were highly efficient for ASF serodiagnosis, with sensitivity and specificity ranging between 96 and 99%. Furthermore, in agreement with results obtained using p54 and pB602L proteins expressed in *E. coli* (Gallardo et al., 2009a), recombinant ELISAs using the baculovirus-expressed p30, p54 and pp62 showed improved sensitivity over than the conventional OIE-approved ELISA (based on cell extracts from infected cells) for the analyses of poorly preserved samples (Gallardo et al., 2006). Therefore, the use of these baculovirus-expressed proteins as reagents in ELISA reduces the number of false positives detected, thus allowing a more accurate diagnosis.

The use of insects as living biofactories is a cost-efficient alternative with respect to the baculovirus-based protein production in insect cells (Barderas et al., 2000; Pérez-Filgueira et al., 2007). This is highly relevant for developing countries. Heterologous protein production by the combination of recombinant baculovirus and *Trichoplusia ni* (*T. ni*) insect larva has been denominated improved baculovirus expression system technology (IBES®technology) and represents one of the best production alternatives based on baculovirus vectors. This inexpensive platform has been used to efficiently produce several recombinant antigens as diagnostic reagents for other diseases (Gomez-Sebastian et al., 2008; Pérez-Martín et al., 2008; Encinas et al., 2011; Todolí et al., 2009). Furthermore, ASFV p30 has been generated at very high levels by IBES®technology (Barderas et al., 2000; Pérez-Filgueira et al., 2006). ELISA and immunoblotting assays have been validated using insect extracts containing p30 without further purification. The insect-derived p30 presented very low levels of background reactivity when used as an ELISA reagent, discriminating accurately between positive and negative sera and reducing false positive reactions (Pérez-Filgueira et al., 2006). These results contrast with those obtained with p30 expressed in *E. coli*, which provided a very high background reactivity, even when the protein was extensively purified (Reis et al., 2007). Purification processes dramatically increase the production costs of any protein and cause considerable loss of recombinant protein yields. A single infected insect larva may produce enough p30 reagent to carry out more than 40,000 ELISA

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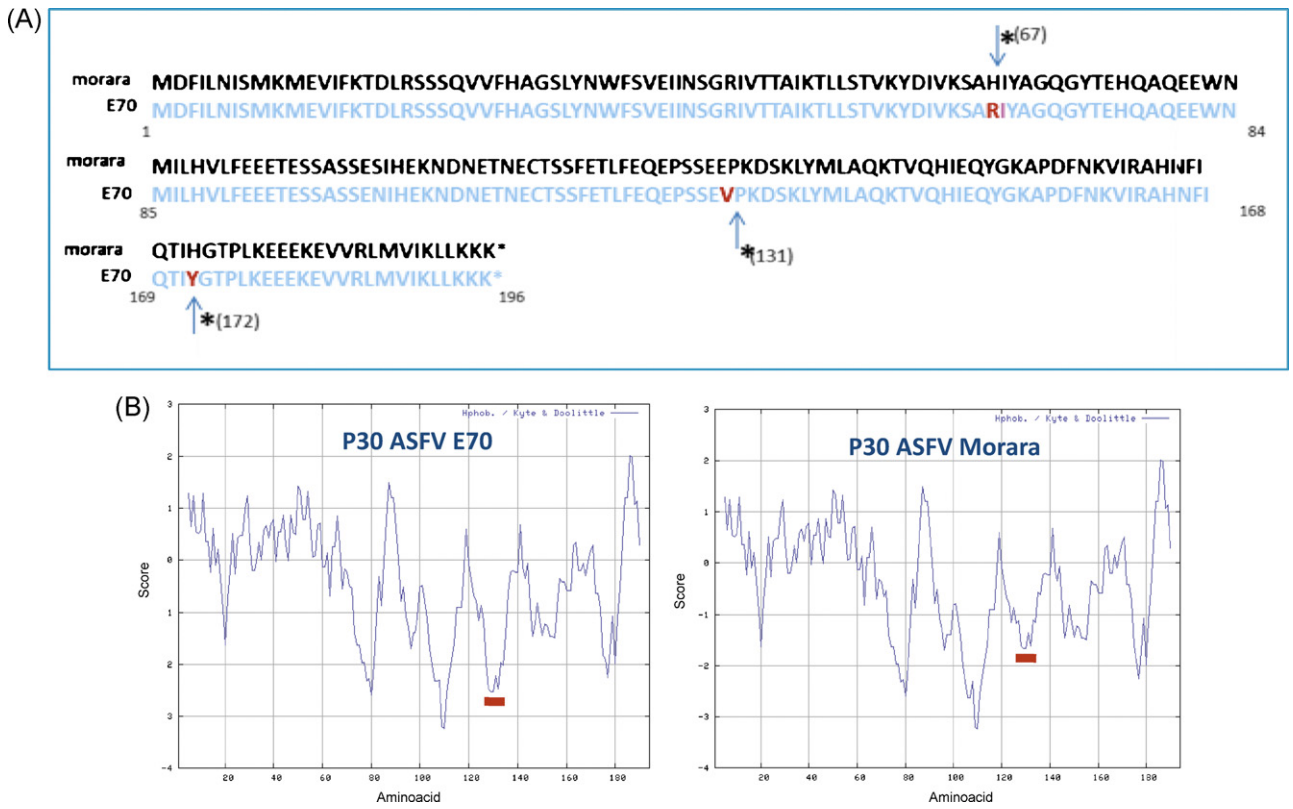

Fig. 1. Sequence analysis of p30 proteins from a Morara/Georgia ASFV or the E70 ASFV isolates. (A) Alignment of p30 sequences obtained from the two ASFV isolates. The residues in which they differed are indicated with an arrow. The number of the residue is also indicated in brackets. (B) Correlation between antigenicity, structure, and variability in the p30 protein from the two ASFV isolates. Charts display the variation of the antigenic index as a function of amino acid position for the Spanish E75 and the African isolate Malagasy. Horizontal black bars indicate regions of predicted intrinsic disorder.

determinations and 2000 confirmatory IB tests (Pérez-Filgueira et al., 2006).

The recombinant ELISA with p30 produced in *T. ni* insects was validated testing Spanish ASF porcine field sera. This assay showed 98.2% sensitivity, a value similar to that of the conventional OIE-approved ELISA (Escribano et al., 1989). However, the specificity with the recombinant protein was greatly improved compared to conventional ELISA (97.4% versus 87.8%).

5. Accuracy of a Morara/Georgia-derived p30r- ELISA for serodiagnosis of African sera of distinct geographical origin

The recombinant ELISA using p30 produced in larvae was also tested with serum samples from Africa. Recombinant proteins p54 and pB602L (Gallardo et al., 2009a) and the recombinant p30 produced in larvae all derived from a Spanish isolate. Insect-derived p30 was accurate when tested with a limited number of samples from West Africa but performed with less effectiveness with samples from East Africa (Pérez-Filgueira et al., 2006).

Comparison of the p30 sequences from Spanish isolates and isolates from West, Southern and East Africa, showed that identity scores decreased from West to East, with those of the Southern isolates falling between the two (Pérez-Filgueira et al., 2006). These results may indicate that antigenic divergences regarding p30 are responsible for the distinct performance of p30r-ELISA for African sera from various geographical origins.

On the basis of these results, we produced an additional version of p30r from an ASFV isolate more distant to genotype I (European, South American, Caribbean and Western Africa isolates), and used it as antigen in an ELISA. We selected the CP204L gene encoded by the virus strains Morara and Georgia (Morara/Georgia). DNA sequences of the CP204L genes from Morara/Georgia and Spanish (E70) ASFV isolates were translated, and alignments and identity scores were obtained using the Clustal-W program (European Bioinformatics Institute). Antigenicity profiles along the sequence were determined using the algorithm described by Hopp and Wood (1981), whereas the hydropathicity pattern was analyzed by the ProtScale tool, using the Kyte and Doolittle scale (Kyte and Doolittle, 1982). The amino acid sequence of the two p30r proteins (Morara/Georgia and E70) presented a high degree of identity (97%). However, the Morara/Georgia p30r protein had three amino acid changes compared to p30r from the Spanish isolate. These changes were found in position 67 (Histidine by Arginine), 131 (Glutamic by Valine) and 172 (Histidine by Tyrosine) (Fig. 1A) and were responsible for variations in the hydropathicity pattern of the African p30r protein (Fig. 1B).

The Morara/Georgia CP204L gene, which encodes for p30, was amplified from a plasmid kindly provided by Drs. E. Albina and V. Michaud (CIRAD, Montpellier, France). This gene was cloned into the pFastBac1™ (Invitrogen) under the control of the polyhedrin promoter for high protein expression, and a recombinant baculovirus was obtained following the Bac to Bac™ system (Invitrogen). The resulting recombinant baculovirus containing the gene encoding for Morara/Georgia p30 was used to express the recombinant protein by IBES®technology. The Morara/Georgia p30r was efficiently expressed in *T. ni* larvae and accumulated in the inoculated insects in a dose- and time-dependent manner (data not shown), as was found previously for p30r from the Spanish isolate E70 (Pérez-Filgueira et al., 2006). Total p30r protein extracts obtained from infected larvae with the baculoviruses expressing the p30 derived from E70 or Morara/Georgia viruses were analyzed by SDS-PAGE electrophoresis. Both recombinant proteins were clearly identified in Coomassie blue-stained gels as a single band,

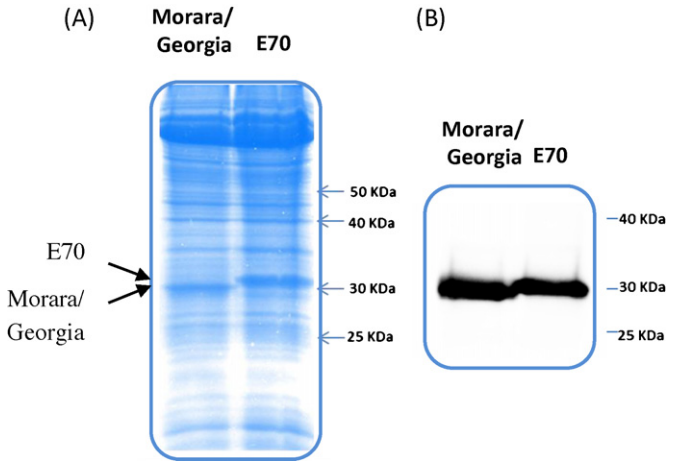

Fig. 2. Production of the Morara/Georgia recombinant p30 in *T. ni larvae*. Total protein extracts from larvae infected with the Bacp30 baculovirus expressing the Morara/Georgia or the E70 p30r proteins were analyzed by (A) Coomassie brilliant blue staining of SDS-page gels and (B) Western blotting using a positive reference pig serum as a probe. Red arrows indicate the position of the recombinant p30.

with slightly different electrophoretic moieties, but both around the expected size of 30 kDa (Fig. 2A). This distinct electrophoretic moiety of Morara/Georgia p30r could be attributable to its higher hydropathicity. Both p30r proteins reacted with a pool of sera from ASFV-seropositive swine used as probe in a Western blot (Fig. 2B). Total soluble protein extracts from insect larva were obtained basically as described by Pérez-Filgueira et al. (2006) (see details in Supplementary material) and tested to demonstrate improvement in ASF serodiagnosis in African countries, adapting the tools available so far for detection of this disease.

An initial panel of 92 pig serum samples collected in Mozambique were tested by OIE-approved assays (ELISA plus IB) as gold-standard, and against both Morara/Georgia and E70 p30r proteins by ELISA to compare the performance of the latter two. Twenty-one out of 92 samples were classified as ASFV-positive by OIE-approved tests. Pig sera from Mozambique did not present significant background reactivity against control (uninfected) larva protein extracts (OD values < 0.2), as also found for Spanish serum samples (Pérez-Filgueira et al., 2006). Therefore, the results were expressed as the ratio between the mean OD obtained for each sample against positive antigen (p30r protein) and negative antigen (larva extracts). (Details on ELISA procedures are given in Supplementary material.)

With an OD ratio of 2.5 as the cut-off level, 21 out of 92 pig sera were seropositive by Morara/Georgia p30r-ELISA, whereas only 19 were seropositive by E70 p30r-ELISA (data not shown). Furthermore, 2 out of the 19 seropositive samples detected by the E70 p30r-assay were in the cut-off interval. Positive sera in ELISA were all confirmed by p30r-Western blot (data not shown). Comparative results between p30r-ELISAs and OIE-approved tests indicated that the specificity of these recombinant proteins (Morara/Georgia and E70) for the analysis of pig sera from Mozambique was equivalent. However, differences were detected between Morara/Georgia and E70 p30r proteins with regard to sensitivity, which was slightly lower for the E70 p30r-ELISA (90.4% of seropositives detected) than for the Morara/Georgia p30r-ELISA (100% of seropositives detected). The results obtained by the Morara/Georgia p30r-ELISA for sera samples from Mozambique were expressed as ODs ratios and are shown in a Box-plot (Fig. 3).

The characteristics of the Morara/Georgia p30r-ELISA for ASF serodiagnosis of samples from Mozambique were calculated by evaluating the results of this assay by means of a Diagnostic test 2×2 table, using OIE-approved assays (ELISA + IB) as reference tests. The sensitivity of the test reached 100% (95% CI, 83.89% to 100%) and the specificity 97.18% (95% CI, 90.19% to 99.66%).

Given the satisfactory performance of the Morara/Georgia p30r-ELISA in the analysis of pig serum samples from Mozambique, we extended the study and evaluated this assay for the detection of antibodies against ASFV in sera from other geographical locations in Africa. For this purpose, we used the Morara/Georgia p30r-ELISA and OIE-approved tests to test domestic pig serum samples collected in the Democratic Republic of Congo (DRC) ($n = 303$) and in Senegal ($n = 109$). Ratio values obtained for sera from these two countries are shown in Figs. 4 and 5 respectively.

One hundred and thirty-four serum samples out of 135 DRC seropositive samples (tested by OIE-assays) were correctly diagnosed by the Morara/Georgia p30r-ELISA. Regarding the reactivity of OIE-negative sera to the recombinant ELISA, 166 out of 168 DRC serum samples were correctly detected. For samples from Senegal, 20 out of 22 OIE-seropositive samples were correctly detected by the Morara/Georgia p30r-ELISA, while all the samples from this country tested negative by OIE-approved assays were also negative by the recombinant ELISA.

Therefore, the sensitivity of the Morara/Georgia p30r-ELISA for the sera samples from DRC and Senegal was 97.78% (95% CI, 93.64% to 99.54%) and 90.91% (95% CI, 70.84% to 98.88%) respectively. With respect to the specificity of the recombinant test, it was 98.81% (95% CI, 95.77% to 99.86%) and 97.75% (95% CI, 92.12% to 93.73%) for samples from the DRC and Senegal respectively.

Together, the results obtained testing pig sera from Africa indicate that the Morara/Georgia p30r-ELISA has a specificity of around 98%, independently of the origin of the sera (East, Central or West Africa), whereas the sensitivity varied between 100% (East Africa)

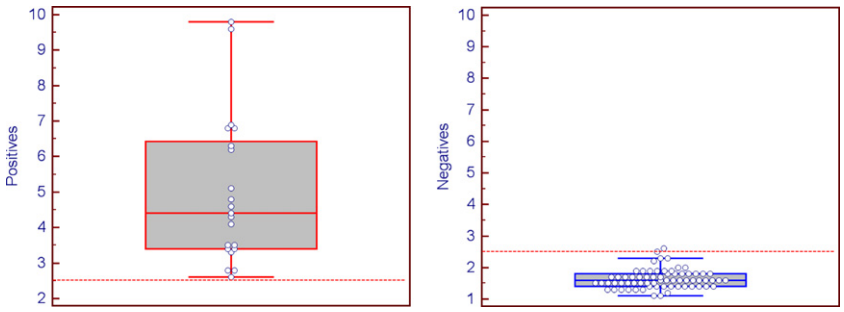

Fig. 3. Detection of ASFV-specific antibodies in pig serum samples from Mozambique by the Morara/Georgia p30r-ELISA. Ratio OD distribution of OIE ASFV-positive (red box at left) and -negative (blue box at right) sera are shown in a Box-and-whisker plot graph. Each dot corresponds to media of duplicate analyses of an individual sample. Cut-off value is shown as dotted line. The central box represents the values from the lower to upper quartile (25–75 percentile). The middle line shows the median and the vertical line extends from the minimum to the maximum value. Among seropositive sera detected by OIE-approved tests, the highest value recorded was 9.8.

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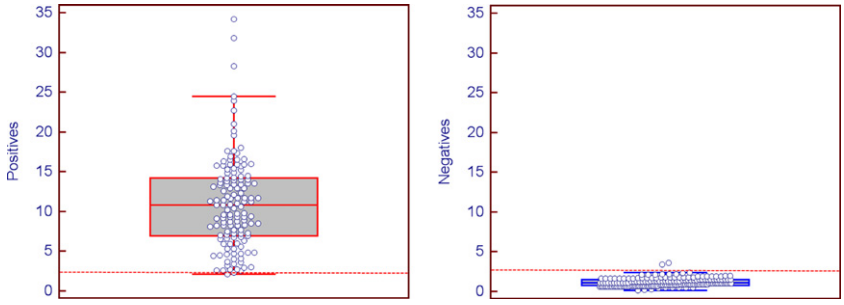

Fig. 4. Detection of ASFV-specific antibodies in pig serum samples from DRC by the Morara/Georgia p30r-ELISA. Results are expressed as explained in figure 4. Among seropositive sera detected by OIE-approved tests, the highest value recorded was 34, 2.

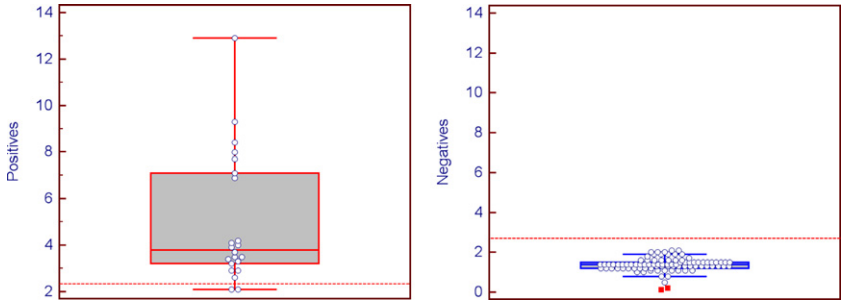

Fig. 5. Detection of ASFV-specific antibodies in pig serum samples from Senegal by the Morara/Georgia p30r-ELISA. Results are expressed as explained in Fig. 4. Among seropositive sera detected by OIE-approved tests, the highest value recorded was 12, 9.

and 90% (West Africa). These variations are probably due to the high genetic variability between ASFV isolates from these locations. However, these variations in sensitivity were clearly lower than those described previously using the E70 p30r protein, which provided sensitivity of 70% and 100% for samples from East and West Africa respectively (Pérez-Filgueira et al., 2006). Therefore, we can conclude that the Morara/Georgia p30r-ELISA provides acceptable specificity (97–98%) and sensitivity (90–100%) for ASF serodiagnosis in domestic pig samples from South-Eastern (Mozambique), Central (DRC) and Western (Senegal) African countries. A combination of p30r from E70 and Morara/Georgia would probably provide a universal ELISA test for ASF.

ASF diagnosis in wild species is especially relevant in Africa, where these animals are reservoirs of the virus. Thus, in this study we used the recombinant ELISA and OIE-approved tests to analyze warthog serum samples collected in Senegal ($n = 73$), the Gorongosa National Park (Mozambique) ($n = 5$), and the Kruger National Park (South Africa) ($n = 1$), and also from a Classical Swine Fever challenge trial in Mozambique ($n = 4$).

Six out of the 83 warthog samples tested were positive by OIE-approved tests and also by the Morara/Georgia p30r-ELISA, while the rest of the samples were negative by both analyses (Fig. 6). Unfortunately, due to the limited number of ASF-positive serum samples included in this panel, it was not possible to determine the characteristics of the recombinant assay for the ASF diagnosis in warthogs. However, since the six ASFV-positive serum samples were detected by the recombinant ELISA, we can expect that this assay has the capacity to provide acceptable sensitivity for warthog diagnosis.

In summary, this report describes a feasible and inexpensive serological test able to accurately detect antibodies against ASFV, independently of the geographical origin of the sera. It should be highlighted that, to the best of our best knowledge, this is the first evaluation of a recombinant ELISA test with such a high number of ASF-seropositive samples from the three main African locations in terms of virus variability (representative of South-Eastern, Central and Western regions).

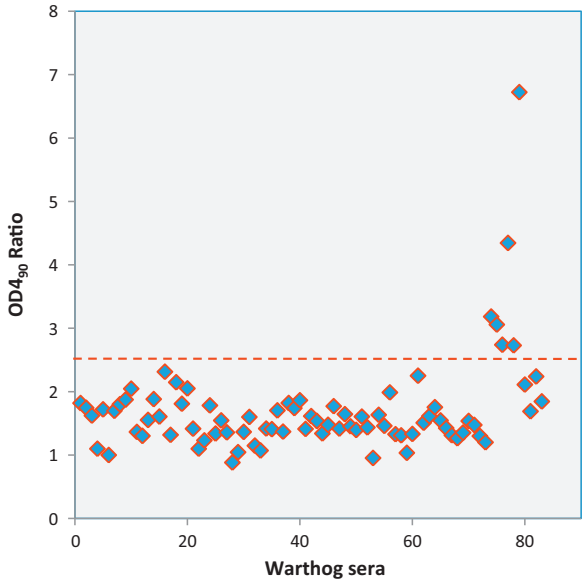

Fig. 6. Detection of ASFV-positive and -negative warthog serum samples using the Morara/Georgia p30r-ELISA. Results are expressed as ratio ODs where each dot corresponds to media of duplicate analyses of individual samples. Cut-off value is shown as dotted line.

6. Conclusions

ASF is a devastating disease caused by a large and complex virus. Given its extremely high potential for transboundary spread, escaping from Africa to Asia (Georgia, Armenia) and Europe (Russia, Ukraine), this virus is a threat to as yet unaffected African countries and other continents. In the absence of any vaccine, the control of ASF relies on rapid diagnosis and implementation of sanitary measures and domestic pigs movement restrictions. However, ASF diagnosis is complicated by the varying pathogeneses and

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epidemiological scenarios, as well as by its similarity to other hemorrhagic diseases, such as Classical swine fever.

The detection of ASF-specific antibodies is indicative of previous infection, and as antibodies are produced from the first week of infection and persist for long periods they are appropriate markers for the diagnosis of the disease. Classical assays such as ELISA or IB, based on the use of crude extracts from ASFV-infected cells, are very useful for the diagnosis of subacute or inapparent forms of the disease, but present several disadvantages, mainly related to biosecurity, diagnostic interpretation and standardization concerns.

Therefore, measurement of the presence serum antibody using a recombinant protein would be a reproducible and safe alternative to conventional methods, allowing the standardization of antigen production and eliminating the need for the manipulation of infectious material. Several studies focusing on the use of recombinant ASFV proteins for serological diagnosis have shown promising results. Furthermore, these proteins have the additional advantage that they simplify the interpretation of tests, improve the reproducibility of the assays, and provide high sensitivity for poorly preserved samples.

The utility of the newly developed tests for ASF diagnosis requires validation. As reflected by the studies discussed in this review, recombinant assays have shown differences in performance depending on the origin of the samples. Only the new Morara/Georgia p30r-ELISA appears to present adequate characteristics for ASF serodiagnosis throughout Africa. Furthermore, the Morara/Georgia (Genotype II) p30r sequence corresponds to the circulating viruses in Eastern Europe (Georgia 2007/1 isolate; Gene Bank accession number: FR682468.1; Rowlands et al., 2008), thus constituting the best diagnostic option for these regions. This protein also is recognized by all positive sera previously collected in Spain (data not shown).

Finally, the data shown here demonstrate that the production of ASFV proteins, such as p30, in insect larvae (IBES®technology) is a reliable alternative to other methods, especially considering that p30r antigen can be used in diagnostic tests without any previous purification. This advantage implies a reduction of cost and antigen loss during antigen production. Worthy of note, three companies are currently commercializing ELISA tests for ASF serodiagnosis, and two of these assays (SVANOVIR® ASFV-Ab assay from Boehringer Ingelheim Svanova and ID Screen® African Swine Fever Indirect ELISA kit from ID.vet) use p30 antigen as a reagent.

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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.virusres.2012.10.021.

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