

## High level expression of the major antigenic African swine fever virus proteins p54 and p30 in baculovirus and their potential use as diagnostic reagents

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### Abstract

At present, the eradication of African swine fever (ASF) in affected countries is based only on an efficient diagnosis program because of the absence of an available vaccine. The highly antigenic ASF virus proteins p54 and p30, encoded by genes *E183L* and *CP204L* respectively, were expressed in baculovirus for diagnostic purposes. A sequence comparison analysis of these genes from different field virus strains which are geographically diverse and isolated in different years, revealed that both genes are completely conserved among the isolates. Partially purified baculovirus-expressed proteins were used in ELISA and Western blot for ASF antibody detection in sera from experimentally inoculated pigs and field sera from ASF innapparent carriers. These comparative analyses showed that p54 presents better reactivity than p30 in Western blot. However, recombinant p30 was more efficient for antibody detection by ELISA, improving the discrimination between positive and negative sera by this technique. These data suggest the convenience of using p30 as ELISA antigen, while p54 should be the selected antigen for ASF virus antibody detection by Western blot. The combined use of both antigens for serodiagnosis of ASF disease will improve the sensitivity of innapparent carriers detection, facilitating also the interpretation of the tests, and eliminating the use of ASF virus in antigen production. Copyright © 1997 Elsevier Science B.V.

*Keywords:* African swine fever; ELISA; Serodiagnosis; Antigen production

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### 1. Introduction

African swine fever (ASF) virus is an icosahedral cytoplasmic deoxyvirus that infects domestic pigs and soft ticks of the *Ornithodoros* genus. This

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virus is presently the sole member of an unnamed family of animal viruses which produces different forms of a disease, ranging from highly lethal to subclinical (DeTray, 1957; Plowright et al., 1969). The lack of a vaccine makes diagnostic procedures the only methodology that can help to plan the complete eradication of the disease in affected countries.

In most cases, ASF disease is diagnosed by the detection of antibody, due to the presence of strains of reduced virulence that result in low mortality (Hess, 1971; Bech-Nielsen et al., 1993). The incidence of innapparent carriers in enzootic areas is of importance in the eradication of the disease. Several studies indicated that more than 4% of Spanish farms surveyed in the affected area had seropositive animals (unpublished data). Long-term ASF virus persistent infection occurs in a high percentage of pigs surviving acute infection (DeTray, 1957). Recently, a study with experimentally inoculated pigs has demonstrated the presence of viral DNA in peripheral blood mononuclear leukocytes at more than 500 days postinfection (Carrillo et al., 1994).

Due to the importance of the presence of seropositive animals to both ASF affected and unaffected countries, false positive and false negative results must be minimized. For the last seven years, new diagnostic tests based on ASF antibody detection, essential for the continued progress towards eradication, have been developed (Escribano et al., 1989; Pastor et al., 1989, 1992; Alcaraz et al., 1990). The general procedure for antibody detection in the eradication program of Spain and other affected countries consists of a first screening of sera by ELISA, the positive results being confirmed by Western blot using nitrocellulose strips containing the ASF virus-induced proteins ranging in molecular weight between 23 and 35 kDa. Frequently, poorly preserved sera produce false positive reactions in ELISA (Alcaraz et al., 1990), or false negative results due to loss of antibodies against viral epitopes represented in the ELISA technique (Arias et al., 1993). In both cases, the Western blot technique proved to be a valuable alternative to confirm ELISA results (Alcaraz et al., 1990; Arias et al., 1993).

The use of recombinant proteins as reagents in the serological tests provide many advantages when compared to antigen production based on the obtention of antigens from infected cell extracts. These recombinant antigens provide a simpler interpretation of the tests, eliminate false-positive reactions produced by cellular compounds that contaminate the antigens, avoid the use of live virus in antigen production, and allow for a better standardization in antigen production. Recently, a modification of the conventional Western blot was developed for ASF diagnostic confirmation in which the *E. coli*-expressed recombinant protein p54 has been used with excellent results (Alcaraz et al., 1995).

We describe the expression in a baculovirus system of two of the most antigenic ASF virus-induced proteins, p54 and p30 (encoded by genes *E183L* and *CP204L* in Ba71V virus, Yáñez et al., 1995). These baculovirus-expressed proteins reacted in the serological tests in use for ASF detection, improving in some cases the antibody detection in innapparent carrier pig field sera and in sera from experimentally inoculated pigs with attenuated viruses.

## 2. Methods

### 2.1. Viruses and sera

A Spanish strain of ASF virus isolated in 1970 (E70) was adapted to grow in monkey stable (MS) cell line and used for antigen production after 81 passages (E70 MS81). A second Spanish strain of ASF virus isolated in 1975 (E75), attenuated by four passages on CV<sub>1</sub> (ATCC, CCL 70) cell line (E75 CV<sub>1</sub>-4) was used for oronasal inoculation of four pigs with 10<sup>5</sup> 50% tissue culture infectious doses (TCID<sub>50</sub>) as previously described (Pastor et al., 1990). The attenuated viruses 608 and 1207 adapted to growth in Vero cells and also attenuated by cell culture passage (13 and 15 passages, respectively), were also used for pig inoculation. Of 608VR13 virus 10<sup>3</sup> TCID<sub>50</sub> were intramuscularly inoculated in seven 3-month old pigs, and 10<sup>6</sup> TCID<sub>50</sub> of 1207VR15 virus were inoculated by the same route to one pig with the same charac-

teristics. Pigs were bled on postinoculation day (PID) 20 and sera from all pigs were pooled and used as hyperimmune ASF serum. The wild-type virus isolates E70, E75, 608 and 1207 growing in pig macrophages were used for p54 and p30 genes amplification by PCR and sequence analysis.

Fourteen field pig sera diagnosed previously as ASF positive sera by ELISA and collected between 1990 and 1994, were obtained from inapparent carrier pigs from southwest geographic areas of Spain (enzootic areas during these years). These sera were used in a comparative analysis by using the conventional and the recombinant antigens in serological tests. A pool of ten negative ASF virus sera was used as negative control serum.

A pig serum against the amino terminal part of p54 protein (109 amino acids from first ATG) from E75 virus isolate, expressed in *E. coli*, was obtained after three intramuscular inoculations scheduled every 15 days. A 200 µg dose of the protein in complete Freund's adjuvant was used for the first inoculation and the same protein amount in incomplete adjuvant for the next two. The inoculated pig was bled 15 days after the last inoculation. Monoclonal antibody 174F11.8 recognizing an epitope of the ASF virus protein p30 (Afonso et al., 1992; Ramiro-Ibáñez et al., 1995), was kindly provided by Dr Rock (PIADC, USDA, NY).

## 2.2. DNA sequencing

DNA from wild-type viruses obtained from infected pig macrophage supernatants (Blasco et al., 1989), was used as template in polymerase chain reactions (PCR) with AmpliTaq DNA polymerase (Perkin-Elmer Cetus) to amplify both p54 and p30 genes. The specific primers used for PCR amplifications included the recognition sequences for the *EcoRI* and *BamHI* restriction enzymes to facilitate gene cloning in pUC19 plasmid (Clontech) according with standard procedures (Sambrook et al., 1989). The cloned genes were sequenced by the dideoxynucleotide chain termi-

nator method (Sanger et al., 1977) with M13-specific primers and internal p54 or p30 gene primers.

## 2.3. Transfection and selection of baculovirus recombinants expressing the ASF virus proteins p54 and p30

DNA amplification of the p30 and p54 genes from the 1207VR15 ASF virus strain, was carried out by PCR using the following primers for p30: (i) 5'-GCGCGGATCCATGGATTTTATT TAAATAT (5' primer) and (ii) 5'-GCGCTC-TAGACAATCATATAAGAATAACAT (3' primer), containing the recognition sequences for *BamHI* and *XbaI* enzymes, respectively. For p54 gene amplification the primers used were: (i) 5'-GCG CGGATCCATGGATTCTGAATTTTT CAACC (5' primer) and (ii) 5'-GCGCGCGGA TCCGTAGCTAATAAGCTCTGAGC (3' primer), containing the recognition enzyme sequence for *BamHI*. The PCR products were digested with the appropriate restriction enzymes and cloned into pBacPAK8 (Clontech) transfer vector under the control of the polyhedrin promoter of baculovirus, which had been digested previously with the same enzymes.

Then, recombinant baculoviruses expressing the p30 or p54 proteins were constructed and selected. Briefly, Sf cells were transfected with a mixture of the transfer recombinant vector (pBacPAKp30 or pBacPAKp54), and the purified non infectious BacPAK6 DNA, *Bsu36I* digested, which contains the  $\beta$ -galactosidase gene under the control of the polyhedrin promoter of baculovirus (Clontech). After 4 days incubation at 28°C, supernatants were tested for plaque formation in Sf monolayers. Selection of recombinant baculoviruses was made by negative chromogenic dye staining due to the replacement of the  $\beta$ -galactosidase gene by the newly introduced foreign gene. The selected viruses were purified by three consecutive plaque assays in Sf cells. The recombinant baculoviruses expressing the p30 or p54 genes were denominated Bacp30 and Bacp54, respectively. The cloned genes were sequenced to check for possible sequence changes introduced by the PCR amplification.

#### 2.4. Preparation of conventional and recombinant antigens

Cytoplasmic soluble antigen was prepared from ASF virus-infected monkey stable (MS) cells harvested 48 h post infection as described (Escribano et al., 1989).

Recombinant proteins p54 and p30 were solubilized from baculovirus-infected cells by using an hypotonic buffer containing 25 mM NaHCO<sub>3</sub> pH 8.3. After 15 min of incubation with lysis buffer on ice, cell debris were sedimented by centrifugation, and the supernatants were diluted in PBS to be used in the serological tests.

#### 2.5. Enzyme-linked immunosorbent assay (ELISA)

The ELISA was performed as described (Pastor et al., 1990). Briefly, 100  $\mu$ l each of cytoplasmic soluble antigen or recombinant proteins diluted in PBS at a previously determined optimal dilution (maximum antigen dilution that shows the maximum O.D. value with a hyperimmune pool of sera, consisting on five sera from experimentally inoculated pigs) were used to coat wells of microtitration plates. After 12 h incubation at 4°C, plates were washed with PBS-0.05% Tween 20 and used immediately or stored at -20°C until use. Then, 100  $\mu$ l of a 1:30 dilution of the test serum were added to the wells and incubated for 1 h at 37°C. Wells were washed, and protein A conjugated with horseradish peroxidase (Sigma) was added at 1:1000 dilution. Plates were incubated for 1 h at 37°C, then washed again and developed with 3-methylaminobenzoic acid (DMAB)-methyl-2-benzothiazolinone hydrazone (MBTH) substrate (Sigma, St. Louis, Mo). The reaction was stopped by the addition of 50  $\mu$ l of 3 N H<sub>2</sub>SO<sub>4</sub>. Finally, results were read spectrophotometrically at O.D.<sub>620 nm</sub>. Sera with absorbance values > 0.25 were considered as positive results; negative controls had absorbances around 0.1.

#### 2.6. Western blot assay

Conventional and recombinant Western blot assays were performed as described (Pastor et al.,

1989) with minor modifications. Cytoplasmic soluble antigen proteins or recombinant proteins, at a optimal concentration determined by previous Western blot assays using the above mentioned hyperimmune pool of sera, were resolved in 17% acrylamide gels and transferred to a nitrocellulose filter. The portion of the filter containing the proteins with molecular weights between 23 and 35 K were cut into 4.0 mm wide strips that were then incubated with 2% nonfat dry milk as blocking solution for 15 min and reacted with test sera diluted to 1:30 for 30 min. The immunocomplexes were detected using peroxidase-labeled protein A of *Staphylococcus aureus* and 4-Chloronaphthol as substrate.

### 3. Results

#### 3.1. Comparison of the sequence of the genes encoding for proteins p54 and p30 from field virus isolates

DNA from wild-type ASF virus isolates E70, 608 and 1207 was used as template for p30 gene PCR amplification, while DNA from viruses 608 and 1207 was used for p54 gene amplification. The amplified genes were cloned into pUC19 and at least two independent clones for each virus isolate were sequenced. We also compared the deduced amino acid sequence of these viruses with that determined previously for other virus isolates (Afonso et al., 1992; Prados et al., 1993; Rodriguez et al., 1994). Both genes displayed 100% conservation both at nucleotide and amino acid level among these geographically diverse viruses isolated in Spain at different years.

#### 3.2. Construction, selection, and characterization of recombinant viruses expressing proteins p54 and p30

Transfer vectors pBacPAKp54 and pBac-PACp30 were constructed as described in the methods section, and used to generate recombinant baculoviruses expressing p54 (Bacp54) and p30 (Bacp30), respectively. Five viral clones were selected from every cotransfection and purified in

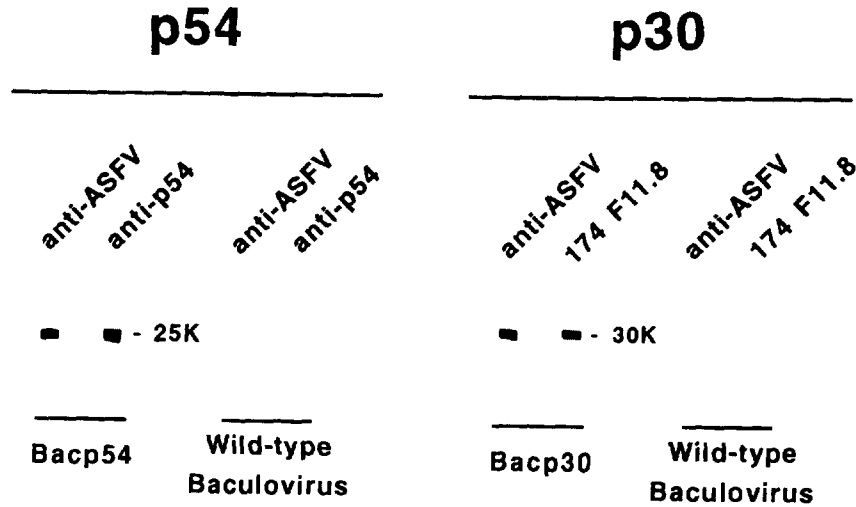


Fig. 1. Characterization of the ASF virus proteins expressed by the recombinant baculoviruses Bacp54 and Bacp30. Western blot analysis of extracts from insect cells infected with Bacp54, Bacp30 or wild-type baculoviruses, and reacting with anti-ASF virus (ASFV), anti-p54 serum or monoclonal antibody against p30 (174 F11.8).

three consecutive plaque assays in Sf cells. The purified viruses were titrated and used for infecting Sf cells at a multiplicity of infection of 5. At various times postinfection the cells were harvested and subjected to Western blot analysis using an anti-ASF virus serum, a monospecific antiserum against p54 or a MAb against p30 (Fig. 1). Wild-type baculovirus-infected cell lysates did not show reaction with specific antibodies (Fig. 1). A time course experiment showed maximum protein expression level at 48 h postinfection for both Bacp54 and Bacp30 baculoviruses (Fig. 2A). Protein p54 was expressed as a major band of 25 kDa, and a minor band of 17 kDa. Both bands are also detected in ASF virus-infected cells with the same electrophoretic mobility. Protein p30 was expressed as a unique immunoreactive band with the same electrophoretic mobility than the induced protein in ASF virus-infected cells (30 kDa).

Although the amount of recombinant proteins p54 and p30 detected in infected cells reached a plateau at 48 h postinfection, only p30 was detected in the medium at this time, remaining the recombinant p54 cell-associated (Fig. 2B). More than 60% of both recombinant proteins were easily solubilized from baculovirus-infected cells by

using an hypotonic treatment with 25 mM  $\text{NaHCO}_3$  pH 8.3 (Fig. 2C), or by treatment with 0.5% of the nonionic detergent NP-40 (data not shown).

### 3.3. Use of baculovirus-expressed p54 and p30 in ASF virus antibody detection by ELISA and Western blot

A panel of 12 sera from pigs inoculated with the attenuated viruses E75CV<sub>1-4</sub>, 608VR13 and 1207VR15, showing antibody titers between 1:100 and 1:200 determined by a conventional ELISA, were analyzed by Western blotting in order to compare their reactivity to both the ASF virus-induced and recombinant proteins (Fig. 3A). All sera reacted with the virus-induced p54, while only six sera (4, 5, 6, 7, 10 and 12) recognized the protein p30, and only three of them reacted in Western blot with the major structural protein p72 (sera 7, 10 and 12). In contrast, recombinant proteins p30 and p54 were recognized in Western blot by all sera, giving many of them a weaker reaction with p30 than with p54.

In order to compare the reactivity of field sera from inapparent carrier pigs with ASF virus-induced and recombinant proteins, 14 sera from

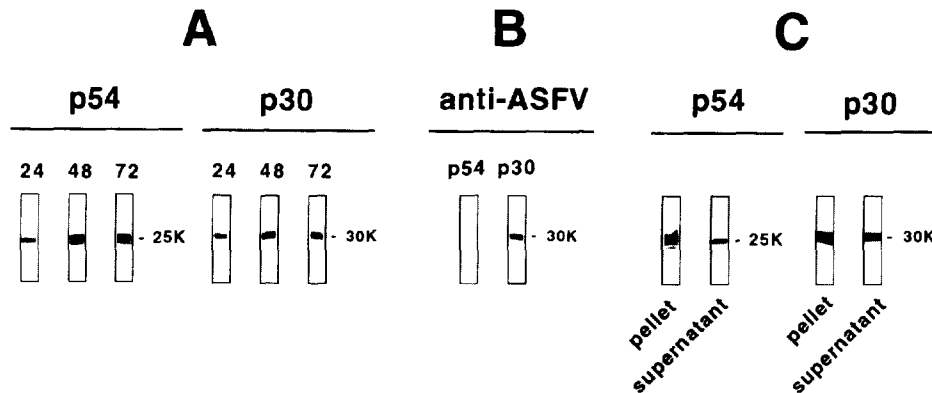


Fig. 2. A. Time-course of p54 or p30 recombinant proteins expression. Western blot analysis of extracts from Sf cells infected with Bacp54 or Bacp30 baculoviruses at 24, 48 or 72 h post infection, and reacting with anti-p54 serum or a monoclonal antibody against p30. B. Presence of recombinant p54 or p30 proteins in the extracellular medium. Western blot analysis of culture media of insect cells infected with Bacp54 or Bacp30 baculoviruses after centrifugation and recovered at 48 h post infection. Recombinant proteins were detected by using an anti-ASF virus polyclonal serum. C. Cell extraction of recombinant p54 or p30 proteins. Sf cells at 48 h post infection with Bacp54 or Bacp30 baculoviruses were subjected to hypotonic bicarbonate treatment and centrifugation. The pellet and supernatant fractions were analyzed by Western blot using anti-p54 serum or monoclonal antibody against p30.

enzootic areas of Spain in past years were also reacted in Western blot (Fig. 3B). Nine sera reacted with protein p72 (sera 2, 3, 4, 5, 6, 7, 8, 9 and 10), 10 sera reacted with the virus-induced p30 (sera 1, 2, 4, 5, 7, 8, 9, 10, 12 and 13), and 12 sera recognized the ASF virus-induced p54 (sera 1–10, 12 and 13). By using recombinant p30, 12 field sera reacted with this protein in Western blot (sera 11 and 14 failed in recognizing this protein). Finally all sera reacted with the baculovirus-expressed p54 by this technique, although sera 11 and 14 shown a weak reactivity.

After the analysis of reactivity of recombinant proteins in Western blot presented by sera obtained after experimental or natural infections, we analyzed the behavior in ELISA test of these antigens. ELISA microtiter plates coated with the previously determined optimal dilution of recombinant and conventional antigens (maximum dilution of antigen that yields the maximum OD value with a pool of anti ASF virus sera), were used for specific antibody detection. As a result of this comparative analysis with sera from experimentally-inoculated pigs, recombinant p54 and crude antigen presented similar results, showing slight differences in OD values ( $< 0.3$ ) (Fig. 4A). However, six sera (4, 5, 6, 7, 10 and 12) showed with

the baculovirus-expressed p30 higher ELISA values ( $> 0.3$  OD) than with conventional crude antigen or recombinant p54. Sera 11 was poorly detected by this technique with the three analyzed antigens.

The comparative analysis by ELISA of field sera from innaparent carrier pigs revealed an important increase in sensitivity of antibody detection by using the recombinant p30 with respect to conventional crude antigen or recombinant p54 (Fig. 4B). Nine field sera presented higher ELISA values ( $> 0.3$  OD) with the recombinant p30 than with conventional antigen (sera 1–7, 9 and 10). Additionally, serum 14, in the limits of antibody detection by ELISA, was clearly positive when recombinant p30 was used as antigen. With respect to recombinant p54, the baculovirus-expressed p30 showed better reactivity with at least eight sera. Conventional crude antigen showed better results in ELISA than recombinant p54 in at least three sera (1, 2 and 9), while only one serum (3) was better discriminated as positive with the baculovirus-expressed p54 than with the conventional antigen.

Regarding the yield of recombinant antigens production, about  $35 \times 10^6$  Bacp30-infected Sf cells provided sufficient antigen for coating ten

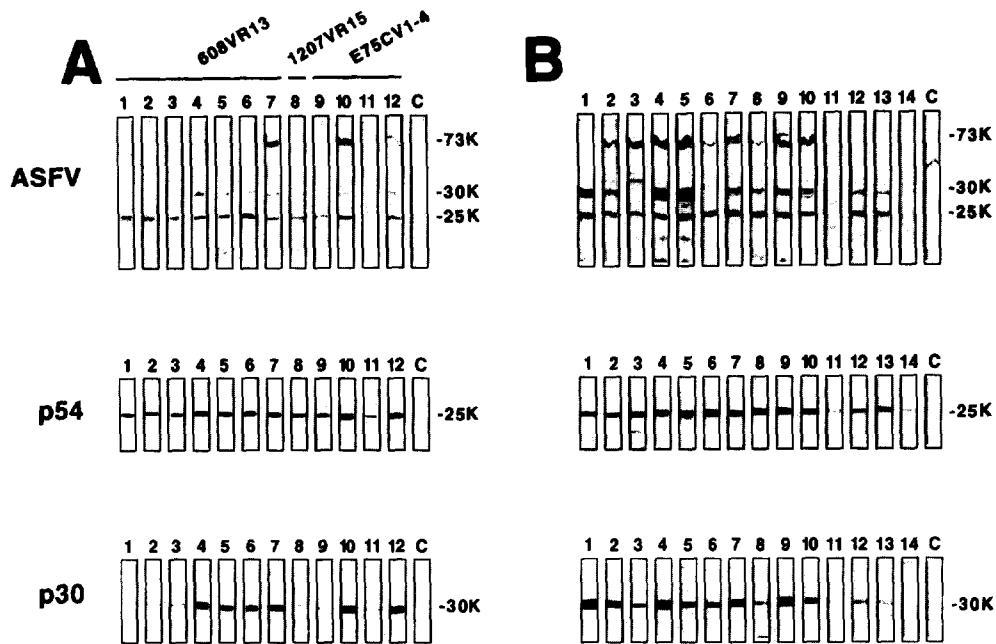


Fig. 3. Protein pattern recognized in Western blot by sera from experimentally ASF virus inoculated pigs or field sera from innaparent carriers. Sera were reacted with nitrocellulose strips containing ASF virus-induced proteins (ASFV) or recombinant proteins p54 or p30. A. Western blot of a panel of 12 sera from pigs inoculated with the attenuated ASF viruses 608VR13 (sera 1–7), 1207VR15 (serum 8), or E75CV<sub>1</sub>-4 (sera 9–12) and a control preimmune serum (serum C). B. Western blot of a panel of 14 field sera from innaparent carrier pigs (sera 1–14) and a control preimmune serum (serum C). All sera were used at 1:30 dilution. The  $M_r$  of protein p72 (73 kDa), p30 (30 kDa) and p54 (25 kDa) induced by ASF virus are indicated in the figures.

standard 96-well ELISA plates. In the case of recombinant p54,  $48 \times 10^6$  infected Sf cells were necessary for obtaining the antigen for coating ten ELISA plates. For Western blot analysis,  $1.25 \times 10^5$  and  $2.5 \times 10^5$  infected Sf cells producing p30 and p54, respectively, were needed for obtaining 25 nitrocellulose strips for routine diagnosis containing the optimal concentration of every recombinant antigen.

### 3.4. Discussion

Western blot tests and a novel ELISA were developed for ASF virus antibody detection based on the use of baculovirus-expressed viral proteins p54 and p30, respectively. These modifications of previous adapted tests for ASF diagnosis (Escribano et al., 1989; Pastor et al., 1989) provide essentially a number of advantages. First, they eliminate the use of infectious virus in the antigen

production. Second, the Western blot test is easier to interpret since only one band reacts with positive sera. Third, the recombinant tests reduce the possibility of false-positive reactions due to cellular proteins contained in the antigen used for the conventional ELISA and Western blot tests.

Additionally, the antigens based on ASF virus-infected cell extracts are difficult to standardize. The use of different batches of antigen (different multiplicity of infection, virus strain used, cell line, etc.) may produce differences in the relative proportion of the immunoreactive proteins included in the antigen. This feature makes interpretation of the test difficult, especially when low antibody titer sera are tested. The baculovirus-expressed proteins provide a solution to this problem, allowing an easy standardization of the antigen production.

Although more studies are needed to assess that recombinant proteins p54 and p30 can be used for

routine diagnosis, it is clear that both proteins discriminate ASF positive sera better than the conventional antigen in the different serological tests. The sera used in this study are representative of low titer sera that are frequently found in innaparent carrier pigs. These poorly reactive sera represent the major problem in the serological diagnosis of ASF. Any antigen that increase the sensitivity of serological tests is of great importance for the control of the disease. The available sequences of p54 and p30 genes from different virus isolates from Europe and Africa show a high degree of conservation at amino acid level (more than 90% and 87% of identity between p54

and p30 genes, respectively; unpublished results). This antigenic conservation suggests that the expressed proteins described in this work may be generally applicable for ASF diagnosis.

The advantages of using p30 for ELISA and p54 for Western blot is probably conditioned by the number of linear and conformational epitopes contained in these proteins. While p54 seems to contain mainly linear epitopes (reacts better with antibodies in Western blotting than in immunoprecipitation), protein p30 contains mainly conformational epitopes. The differences in the configuration of the epitopes in these proteins might explain the different reactivities found for p30 and p54 in ELISA and Western blot with sera from ASF virus-infected pigs. Interestingly, some sera analyzed in this study reacted better with recombinant proteins than with the ASF virus-induced p30 or p54 proteins. A possible reason for that is the relative representation of both antigens (recombinant or ASF virus-induced) in the nitrocellulose strips.

When the results obtained with all tested sera were compared with the recombinant p54 expressed in *E. coli* (Alcaraz et al., 1995) or in baculovirus systems, we did not observe differences in the sensitivity and specificity provided by both proteins in Western blot (data not shown). The unique difference is that p54 is expressed in *E. coli* as two forms of the fusion protein of 32 and 35 kDa, while the baculovirus expressed protein consists in only a immunoreactive majority band of 25 kDa and a minority band that can be easily eliminated from the strips used for routine diagnosis.

Protein p30 and p54 are two of the most antigenic proteins of ASF virus (Alcaraz et al., 1990). Both proteins are incorporated to the viral particle and mediate virus neutralization (Gómez-Puertas et al., 1996). Additionally, these proteins have a high degree of antigenic conservation among virus strains isolated in different geographical areas, and they induce antibodies in all natural or experimentally infected pigs that we have analyzed. These features make p54 and p30 two of the most interesting viral proteins to be used for the serological ASF diagnosis.

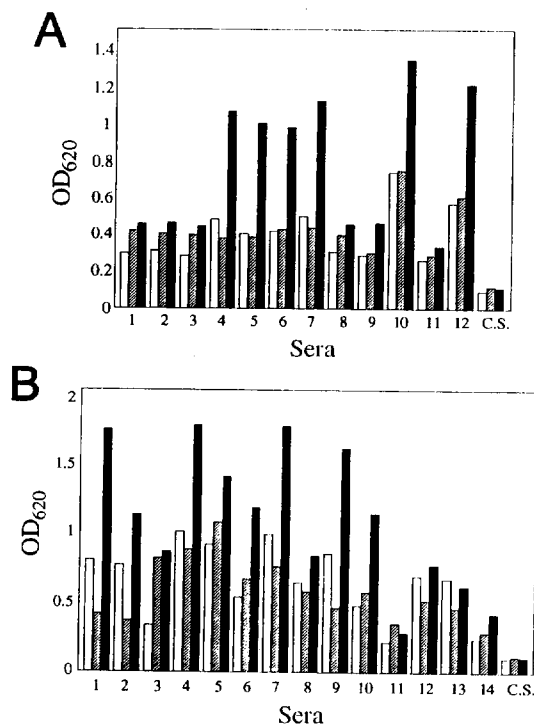


Fig. 4. Comparative analysis of reactivity in ELISA of recombinant and conventional antigens. A. OD<sub>620</sub> values obtained with a panel of 12 sera from pigs inoculated with the attenuated ASF viruses 608VR13 (sera 1–7), 1207VR15 (serum 8), or E75CV<sub>1</sub>-4 (sera 9–12) and a control preimmune serum (C. S.). B. OD<sub>620</sub> values obtained with a panel of 14 field sera from innaparent carrier pigs (sera 1–14) and a control preimmune serum (C. S.). All sera were analyzed at 1:30 dilution on microtitration ELISA plates coated with conventional crude antigen (white bars), or recombinant p54 (dashed bars) or p30 (black bars) proteins.



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