African swine fever (ASF) virus (ASFV) is an icosahedral cytoplasmic DNA virus that infects pigs and soft ticks of the Ornithodoros genus. This virus is the sole member of the family Asfarviridae (6). ASFV has variable pathogenicity in domestic pigs, with infections ranging from being highly lethal to subclinical. Infection of wildlife mammalian hosts, the warthog and the bushpig, on the other hand, results in an unapparent, clinical. Infection of wildlife mammalian hosts, the warthog and the bushpig, on the other hand, results in an unapparent, and specific diagnostic procedures are an essential component of any control strategy. In addition, the presence of virus strains with reduced virulence and the resulting presence of asymptomatic infected animals (4, 11) make the serological diagnosis the only realistic basis for the control of the disease in affected countries. As a general rule, pigs that survive natural infection develop antibodies against ASFV from 7 to 10 days after infection. These antibodies persist for long periods of time (16), perhaps due to continuous antigenic stimulation by the frequent occurrence of persistent infection. Thus, antibody detection is a rational approach to the detection of the subacute and chronic forms of the disease.

The role of specific antibodies in immunity to ASFV infection in pigs has been controversial. The passive transfer of anti-ASFV antibodies delays the onset of clinical signs but does not consistently protect animals from eventual death (25, 26, 17). Similarly, vaccination with the putative protective proteins p30 and p54 conferred protection to only 50% of the tested animals (10). In a different study, in which no protection was observed after immunization against p54, p30, and p72, the only effects detected were a delay in the onset of clinical disease and a reduction in the level of viremia (15). Such observations emphasize the role of cell-mediated immune responses during ASFV infection. Indeed, a positive correlation was observed between the stimulation of NK cell activity and the absence of clinical symptoms after experimental infection, suggesting that NK cells play an important role in protective immunity (13). In addition to NK cells, CD8+ T cells may also play a role, as their depletion in vivo abrogates protective immunity to ASFV infection (18). Therefore, immunity to ASFV is likely to be due to a combination of both serological and cellular mechanisms. This complexity of the porcine immune response to ASFV has impaired the development of an effective vaccine but does justify diagnosis on the basis of the detection of antibodies.

Current Office International des Epizooties (OIE)-approved
assays for ASFV-specific antibody determination consist of an initial screening of sera by enzyme-linked immunosorbent assay (OIE-ELISA), followed by an immunoblotting assay to confirm the results for samples with doubtful and positive results. These OIE-approved tests are based on the use of live virus as the antigen and involve the requirement of level 3 biosafety facilities for the production and handling of the pathogen (16, 20, 21). The risk associated with the handling of live virus, together with the lack of reliability of the OIE-ELISA for the analysis of poorly preserved samples so often encountered in sera of African origin (1, 3), provides the stimulus for the development of alternative and more robust systems for the detection of anti-ASFV antibodies. Indeed, previous studies have demonstrated that recombinant viral proteins can give improved specificity and sensitivity when they are applied to the analysis of European field sera (9, 19, 22).

In previous studies, 12 serological immunodeterminants of ASFV were characterized by exhaustive screening of a representative lambda phage cDNA expression library of the tissue culture-adapted Ba71V isolate of ASFV for antibodies (12). These included four proteins encoded by previously unassigned open reading frames (ORFs) (B602L, C44L, CP312R, and K205R), as well as some of the more well studied structural proteins (pA104R, p10, p32, p54, and p73) and three enzymes (RNA reductase, DNA ligase, and thymidine kinase). The complete sequence of each of these proteins was then cloned into pGEX for expression in Escherichia coli, followed by purification of the recombinant proteins and testing against sera from experimentally infected animals. Four of these proteins (p54/E183L, histone-like/pA104R, pB602L, and pK205R) were recovered from the genomic DNA of isolate BA71V (see the next section) with specific primers and were cloned into the pGEX 4T-1 prokaryotic expression vector. The amplified ORFs A104R, E183L, B602L, and K205R were obtained by amplification from the genomic DNA of isolate BA71V (see the next section). Virus infection of MS cells was carried out with Dulbecco’s modified Eagle’s medium (DMEM) containing 2% fetal bovine serum.

### Experimental infections.

Experimental infections of Landrace × Large White pigs were carried out at a level 3 biosafety animal facility at CISA-INIA, Madrid, Spain. Two pigs (pigs 2U and 3U) were inoculated by the intramuscular route with $10^7$ HAD$	ext{_{50}}$/ml of the Ug03H ASFV isolate. The severely sick animals were painlessly killed at 9 days postinfection (dpi) (pig 3U) and 12 dpi (pig 2U). Two additional pigs (pigs 1 and 2) were inoculated by the intramuscular route with the attenuated ASFV E75 CV4, $10^5$% tissue culture infective doses/ml. One sick animal (pig 1) was painlessly killed at 21 dpi; and the other asymptomatic animal (pig 2) was reinoculated at 30 dpi with homologous virulent isolate E75 ($10^7$ HAD$_{50}$/ml); then a third challenge at 45 dpi with heterologous virulent isolate E70 ($10^7$ HAD$_{50}$/ml), and then painlessly killed at 51 dpi.

### Serum samples.

(i) Field serum samples. The pig and warthog sera used in this study consisted of a collection of samples obtained from different locations in Europe and Africa. The Eligible serum samples were validated by the OIE-approved tests (OIE-ELISA and immunoblotting) and contained 80 ASFV-positive serum samples collected during a series of ASF outbreaks in Spain between 1989 and 1992 and 119 ASFV-negative serum samples collected during the last 10 years in Spain and Germany. A total of 35 of the negative serum samples were previously confirmed to be antibody positive for the classical swine fever virus, and another 25 serum samples were previously confirmed to be antibody positive for swine vesicular disease. An additional 39 field serum samples that had initially tested positive by the OIE-approved tests were stored at 37°C for a month to generate poorly preserved sera for repeated analysis by the OIE-approved tests and the recombinant protein-based ELISAs.

The African serum samples were collected from domestic pigs in various regions of Africa during different epidemiological and surveillance studies performed in Uganda ($n=254$), Mozambique ($n=9$), Nigeria ($n=6$), and Burkina Faso ($n=20$).

Finally, 26 warthog serum samples were collected in Uganda.

(ii) Experimental serum samples. Sera were collected on days 0, 3, 6, 8, 10, and 12 postinfection from pigs 2U and 3U, which had been inoculated with ASFV isolate Ug03H. Sera were collected on days 0, 10, 15, and 21 postinfection from pig 1, which had been inoculated with attenuated ASFV isolate E75 CV4 ($10^5$% tissue culture infective doses/ml). Sera were collected from pig 2, which had first been inoculated with attenuated ASFV isolate E75 CV4, $10^5$% tissue culture infective doses/ml, reinoculated at 30 dpi with homologous virulent isolate E75 ($10^7$ HAD$_{50}$/ml) and then given a third challenge at 45 dpi with heterologous virulent E70 ($10^7$ HAD$_{50}$/ml).

(iii) Reference serum samples. Sera from pig 2, taken 7 days after the third challenge (51 dpi), was used as the positive reference serum. The ASFV-negative reference serum sample was obtained from a blood donor pig kept at CISA-INIA.

Conventional ELISA and immunoblotting assays (OIE-approved analyses). Both conventional ELISA and immunoblotting assays were performed with a lysate of MS cells infected with ASFV E70 MS 48 as the antigen and protein A conjugated to hors eradish peroxidase as the indicator. Both procedures were carried out by following the protocols described in the OIE Manual of Diagnostic Tests and Vaccines for Terrestrial Animals (16).

ELISA with recombinant proteins as antigen. Briefly, microtiter plates (Poly-sorb immunoplates; Nunc) were incubated at 4°C overnight with 50 μl/well of recombinant proteins (pA104R, p54, pB602L, pK205R) or the GST-negative control at a previously determined optimal concentration in coating buffer (0.1 M carbonate buffer, pH 9.6). The coated plates were washed four times with phosphate-buffered saline PBS, pH 7.5 containing 0.05% (vol/vol) Tween 20 (PBS-T) and used immediately or stored at −20°C until use. The plates were subsequently blocked with PBS (pH 7.5) containing 5% (wt/vol) skim milk (PBS-M) for 1 h at 37°C. Then, the porcine sera were added at a dilution of 1:200 in PBS-M and incubated for 1 h at 37°C. Positive and negative reference sera.
were included on each plate. The plates were washed four times with PBS-T, and
the horseradish peroxidase-labeled protein A diluted 1:5,000 in PBS-M was
added. The plates were incubated for 1 h at 37°C. After the plates were washed,
50 μl of o-phenylenediamine (Sigma) was added to each well. After incubation
for 20 min at room temperature, the reaction was stopped by the addition of 50
μl of 3 N H2SO4, and the optical density (OD) was measured at a wavelength of
492 nm. Titers were expressed as the ratio between the ODs obtained for each
sample against recombinant antigen-positive and GST negative-control proteins.

Calculation and data analysis. Sensitivity and specificity were calculated by
using the results of immunoblotting as the reference. All serum samples with
doubtful results by ELISA (those with results in the cutoff interval) were con-
sidered positive. The concordance between each ELISA (the conventional OIE-
approved assay and the proposed recombinant protein-based assays) and the
OIE confirmatory test (immunoblotting) was calculated as the overall percent
agreement between the results of the two assays by using two-by-two contingency
tables. χ statistics were used to evaluate the level of agreement between concor-
dant results in excess of that expected by chance, with χ values of 0.81 to 1.00
representing almost perfect agreement, values of 0.61 to 0.80 representing sub-
stantial agreement, values of 0.41 to 0.60 representing moderate agreement,
values of 0.21 to 0.40 representing fair agreement, values of 0.01 to 0.20 repre-
senting slight agreement, and values of 0.00 representing no agreement (8).

RESULTS

Standardization of recombinant protein-based ELISAs. The
optimal concentration of recombinant antigens used to coat
the ELISA microtiter plates was determined by using the pos-
tive and negative reference sera. The absorbance values of the
ELISAs with the recombinant antigens were found to be op-
timal when the microtiter plates were coated with a concen-
tration of 5.0 μg/ml of antigen pB602L, 2.5 μg/ml of antigen
p54, 2.0 μg/ml of antigen pA104R, or 1.3 μg/ml of antigen
pK205R. No reactivity (OD at 492 nm [OD492], less than 0.2)
was detected when the positive reference serum was tested
against the GST protein expressed in E. coli or when the
negative reference serum was incubated with the recombinant
proteins (data not shown). The appropriate dilution of sera for
the ELISA was determined by titration to be 1:200. At this
dilution, the absorbance corresponding to positive reference
sera was 10 times greater than that corresponding to the neg-
ative reference serum (Fig. 1).

Comparative study between conventional and recombinant
protein-based ELISAs with European field serum samples.
One hundred ninety-nine European porcine serum samples, all
from domestic pigs in the field established to be ASFV nega-
tive (n = 119) and ASFV positive (n = 80) by OIE-approved
tests (ELISA plus immunoblotting) were tested by the ELISAs
with the four purified recombinant virus proteins. On the basis
of previous results showing a low background reading for por-
cine sera against the GST protein (antigen negative), the re-
sults of the recombinant protein-based ELISAs are presented
as the ratio between the absorbance obtained for each serum
sample against the GST protein (antigen negative), the re-
sults of the recombinant protein-based ELISAs are presented
as the ratio between the absorbance obtained for each serum
sample against the GST-recombinant protein experimental
preparations and the GST-negative control preparations. A
cutoff value of 3 for the four recombinant protein-based
ELISAs was established by comparing the ratios for 119
ASFV-negative and 80 ASFV-positive field serum samples.
The numbers of serum samples among the 119 negative
control serum samples tested with false-positive results were 1
each by the pA104R-based ELISA (pA104R-ELISA) and the
pK205R-ELISA (99% specificity; 95% confidence interval
[CI], 97.5 to 100), 3 by the p54-ELISA (97% specificity; 95%
CI, 94.7 to 100), and 6 by the pB602L-ELISA (95% specificity;
95% CI, 91.0 to 98.9) (Fig. 2). A similar proportion of false-
positive results was detected by the OIE-approved ELISA (6 of
119), resulting in a specificity of 95% (95% CI, 91.0 to 98.9).

FIG. 1. Titration curves of reference sera by ELISAs with recombinant proteins pA104R, pB602L, p54, and pK205R. Sera were tested in serial
duplicate twofold dilutions ranging from 1:25 to 1:3,200. The results are expressed as the OD492 and correspond to the average of the values
obtained in at least three different analyses.
The numbers of false-negative serum samples detected among the 80 ASFV-positive serum samples were 17 by the pA104R-ELISA (79% sensitivity; 95% CI, 69.8 to 87.7), 7 by the pK205R-ELISA (91% sensitivity; 95% CI, 85.1 to 97.4), and 2 each by the p54- and pB602L-ELISAs (98% sensitivity; 95% CI, 94.1 to 100) (Fig. 3). By analysis of the seropositive samples by the OIE-approved ELISA, 75 of 80 serum samples were determined to be positive, achieving a sensitivity of 94% (95% CI, 88.4 to 99.1). The \( \kappa \) values showed a substantial to almost perfect agreement between the results of the reference test (immunoblotting) and the results of the pA104R-ELISA (\( \kappa = 0.81; 95\% \text{ CI, 0.72 to 0.89} \)) and almost perfect agreement between the results of the reference test and the results of the p54-ELISA (\( \kappa = 0.95; 95\% \text{ CI, 0.90 to 0.99} \)) and the pK205R-ELISA or the pB602L-ELISA (\( \kappa = 0.92; 95\% \text{ CI, 0.86 to 0.97} \)). Similar levels were observed for the OIE-ELISA (\( \kappa = 0.89; 95\% \text{ CI, 0.82 to 0.95} \)). A summary of the results obtained by each ELISA with the European field sera is shown in Table 1.

**FIG. 2.** Specificity of recombinant protein ELISAs. The 119 serum samples previously characterized as being ASFV negative by immunoblotting (OIE reference test) were tested by each recombinant protein ELISA. The ratios of the ODs obtained by each recombinant protein ELISA for the seronegative samples are shown. The dotted lines indicate the assay cutoff, which was established to be 3 for each recombinant protein ELISA. ID sera, serum sample number.

**Performance of recombinant protein-based ELISA with poorly preserved sera.** In the areas of Africa where ASFV is endemic, serum samples frequently reach the diagnostic laboratory after considerable time at ambient temperature and thus are in a degraded state. In order to assess the applicability of the new recombinant ELISAs to such sera, 39 field serum samples previously classified to be ASFV positive by OIE-approved tests were kept at 37°C for 1 month and were then tested by each recombinant protein-based ELISA. The results were compared with those obtained with the original untreated sera and are presented as a distribution chart in Fig. 4. As can be seen, there was a variable loss of reactivity, depending on which recombinant viral protein was used as the target of the ELISA. Although the heat-treated sera clearly functioned poorly by the pA104R-ELISA, most of the positive sera held for 1 month at 37°C remained positive by the p54- and pB602L-ELISAs. In fact, when the results obtained by the p54-, pB602L-, and pK205R-ELISAs are taken together,
only 1 of the 39 heat-treated serum samples that was previously positive converted to having a negative result. The sensitivities after heat treatment were 95% (95% CI, 87.9 to 100), 87% (95% CI, 76.7 to 97.7), 80% (95% CI, 66.8 to 92.2), and 31% (95% CI, 16.3 to 45.3) for the p54-, pB602L-, pK205R-, and pA104R-ELISAs, respectively, whereas the sensitivity of the OIE-ELISA was 87% (95% CI, 76.7 to 97.7) (data not shown).

Performance of recombinant protein-based ELISAs with serum samples from Africa. A total of 375 serum samples were obtained from domestic pigs in East Africa (Uganda, n = 254; Mozambique, n = 95) and West Africa (Nigeria, n = 6; Burkina Faso, n = 20), and 26 serum samples were collected from warthogs in Uganda. By taking the results of immunoblotting or the OIE-ELISA as the “gold standard,” most of the pig serum samples were negative, whereas all 26 warthog serum samples were positive (Table 2). The positive samples from West Africa (four from Nigeria, five from Burkina Faso) were positive by all four recombinant protein-based assays, and importantly, none of the four assays gave false-positive results for the negative sera from West Africa. Thus, with this limited number of serum samples, all four recombinant antigens were
acceptable and reproducible recombinant antigen targets for the diagnosis of ASF with sera from West Africa.

Similarly, high specificities were obtained when the four recombinant viral proteins were tested with the sera from East Africa (Uganda and Mozambique): for the pA104R-ELISA, 98% specificity (95% CI, 96.5 to 99.5); for the p54-ELISA, 99% specificity (95% CI, 97.3 to 99.8); for the pK205R-ELISA, 96% specificity (95% CI, 94.5 to 98.4); for the pB602L-ELISA, 99% specificity (95% CI, 97.7 to 100); and for the OIE-ELISA, 97% specificity (95% CI, 95.7 to 99.1). Given the few serum samples from East Africa tested, there was no justification for calculation of the sensitivities, but with the frequency of positive results observed (specifically, the pA104R-, p54-, pK205R-, and pB602L-ELISAs revealed that two of three,

![FIG. 4. Sensitivity of recombinant protein ELISA after incubation of 39 porcine serum samples for 1 month at 37°C. Each bar corresponds to the value of the ratio for each serum sample. The dotted lines indicate the assay cutoff, which was established to be 3 for each recombinant protein ELISA. ID sera, serum sample number.](image-url)

| Country (serum sample source) | OIE-ELISA | pA104R-ELISA | p54-ELISA | pK205R-ELISA | pB602L-ELISA |
|-----------------------------|-----------|--------------|-----------|--------------|--------------|
|                             | Se | Sp  | Se  | Sp   | Se  | Sp   | Se  | Sp   | Se  | Sp   |
| Uganda (pigs)               | 3/3 | 248/251 | 2/3 | 247/251 | 2/3 | 247/251 | 2/3 | 247/251 | 2/3 | 248/251 |
| Mozambique (pigs)           | 4/4 | 85/91  | 1/4 | 88/91  | 3/4 | 90/91  | 3/4 | 90/91  | 3/4 | 90/91  |
| Nigeria (pigs)              | 4/4 | 2/2   | 4/4 | 2/2   | 4/4 | 2/2   | 4/4 | 2/2   | 4/4 | 2/2   |
| Burkina Faso (pigs)         | 5/5 | 15/15 | 5/5 | 15/15 | 5/5 | 15/15 | 5/5 | 15/15 |
| Uganda (warthogs)           | 17/26 | 7/26  | 23/26 | 2/26 | 26/26 | 2/26 |

TABLE 2. Performance of recombinant protein (pA104R, p54, pB602L, and pK205R)-based ELISAs compared to that of the conventional ELISA (OIE-ELISA)

- A total of 375 serum samples obtained from domestic pigs in East Africa (Uganda, n = 254 [2 immunoblotting positive]; Mozambique, n = 95 [4 immunoblotting positive]) and West Africa (Nigeria, n = 6 [4 immunoblotting positive]; Burkina Faso, n = 20 [5 immunoblotting positive]) and 26 field serum samples obtained from warthogs in Uganda (all previously characterized as positive by immunoblotting) were analyzed.
- Se, sensitivity; Sp, specificity. The data represent the number of serum samples positive (Se) or negative (Sp)/total number of serum samples tested.
two of three, two of three, and two of three of the serum samples from Uganda, respectively, were positive and that one of four, three of four, three of four, and three of four of the positive serum samples from Mozambique, respectively, were positive [Table 2]), we may conclude that these recombinant proteins are unlikely to provide acceptable sensitivities for the diagnosis of ASF with sera from East Africa. An explanation for this is not immediately obvious but is an urgent priority for the control of ASF in that part of Africa.

Strikingly, all 26 Ugandan warthogs tested positive by immunoblotting but only 17 of the 26 were positive by the OIE-ELISA. Analysis of these 26 warthog serum samples by the recombinant virus protein-based ELISAs was particularly interesting. A total of 23 of 26 were positive by the pB602L-ELISA and p54-ELISA, a marked improvement over the results of the OIE-ELISA (Table 2). In contrast, fewer positive serum samples (7 of 26) were detected by the pA104R-ELISA. Finally, the pK205R-ELISA was positive with 20 of 26 of the warthog serum samples.

**Kinetics of anti-recombinant protein antibody responses in experimentally infected pig sera.** As low sensitivities were observed when sera from East African were assayed with the recombinant viral proteins, pigs 2U and 3U were experimentally infected with the virulent isolate Uganda ASFV Ug03H (Fig. 5). To provide a positive control, two pigs (pigs 1 and 2) were infected with the attenuated Spanish E75 CV14 isolate (Fig. 5). As expected, the pigs infected with the Spanish ASFV isolate did indeed make detectable antibodies to the recombinant proteins. Specifically, antibodies to the pB602L and p54 proteins were detected as early as 10 dpi in pigs 1 and 2. A similarly early antibody response was also observed when the same sera were tested by the OIE-ELISA against the total semipurified ASFV antigen (data not shown). For pig 1, infected with the attenuated Spanish isolate, a positive, but low antibody response against pK205R was detected on days 15 and 21 postinfection, but antibodies to the pA104R protein were undetectable. The other pig (pig 2), infected with the same attenuated Spanish isolate, responded with antibodies against pK205R and pA104R by day 15 postinfection. Challenge of pig 2 at 30 dpi with homologous virulent Spanish isolate E75 did not appreciably raise the titers of antibodies against the four proteins. Reinoculation on day 45 of the same pig with heterologous virulent Spanish isolate E70 resulted in marginally increased antibody levels.

Of the two pigs infected with virulent East Africa isolate Ug03H, one (pig 2U) responded with anti-pB602L and anti-pK205R antibody levels that were already above the cutoff line at day 8 postinfection, and in agreement with the results observed with pigs 1 and 2, the anti-p54 and anti-pB602L antibody levels were impressively high at 10 dpi. A similarly significant antibody response on day 10 was also observed when the same sera were tested by the OIE-ELISA (data not shown). The anti-pK205R antibody response showed a similar increase at day 12 postinfection. At this early time, as was
observed with the Spanish E75 CV4 virus, no antibody against the pA104R protein was observed. The other pig infected with the Uganda isolate died on day 9 and had no detectable antibodies at day 8 postinfection.

**DISCUSSION**

In this study, we have assessed the use of four recombinant ASFV proteins (pA104R, pB602L, p54, and pK205R) as tools for a diagnostic ELISA for the serological diagnosis of ASF with samples of both European and African origin.

By analyzing European porcine field sera, ELISAs with recombinant proteins p54, pK205R, and pB602L performed well; and the results were in almost perfect agreement with those of the gold standard immunoblotting test for ASFV (κ values, 0.95 [95% CI, 0.90 to 0.99], 0.92 [95% CI, 0.86 to 0.97], and 0.92 [95% CI, 0.86 to 0.97], respectively). Thus, these ASFV recombinant proteins function as well as the OIE system, but without the necessity of working with infectious virus. Although the sensitivity of the pA104R-ELISA was low (79%; 95% CI, 69.8 to 87.7), it had the advantage of detecting only 1 sample with a false-positive result of the 119 control field serum samples tested, and so it may still be of value for the diagnosis of ASF when it is applied at the herd level.

In agreement with previous results with the p30 recombinant protein (22), both the OIE and the recombinant virus protein assays gave similar results with sera from Europe and Western Africa. Sera from Eastern African countries, however, gave variable results. The specificities of the four recombinant protein-based assays were very high and similar to the specificity of the OIE-ELISA. However, with the limited number of positive serum samples from Uganda (n = 3) and Mozambique (n = 4), the recombinant protein ELISAs (particularly the pA104R-ELISA) were less sensitive than the OIE-ELISA.

The decreased sensitivity with East African sera was evident and surprising. Clearly, the testing of more samples is required in order to make firm conclusions. As East African isolates are more variable and genotypically distant (5), the recombinant proteins were investigated for possible variations in their sequences. Bioinformatic analysis, however, showed that their sequences, and hence their immunological epitopes, are highly conserved. In view of the structural conservation of the four proteins that we have studied, an explanation for their unexpectedly low sensitivities with sera from East Africa may reside in the pig rather than the virus; for example, the pigs may have different immunological repertoires, clonal deletions, swine leukocyte antigen types, and balances of innate immune mechanisms. Surprisingly, the pA104R protein, although it is highly conserved in viruses isolated from pigs, warthogs, and ticks from Europe and East and West Africa, was the least sensitive of the four proteins with sera from East Africa.

A similar analysis of sera collected from warthogs was particularly interesting and highly relevant for the control of sporadic outbreaks in domestic pigs in contact with warthogs, raising the possibility of the performance of proactive serosurveillance of both domestic and wild pigs in order to determine the status of ASF in East African countries where the sylvatic cycle plays a crucial part in the epidemiology of the disease. Although the number of warthog serum samples was small, the comparative serology results obtained by the recombinant protein-based ELISAs and the OIE-ELISA strongly suggest that the pB602L- and p54-based ELISAs are more sensitive than the OIE-ELISA or the ELISA with well-known antigenic protein p30 (22).

In addition to the high sensitivities and specificities, antibodies against proteins p54 and pB602L were detectable in sera as early as 10 dpi following experimental infection of pigs with either Ugandan and Spanish isolates (similar results were obtained when whole virus was used as the antigen). The antibody titers were maintained for up to 30 dpi (the last time point at which samples were obtained) after infection with the Spanish E75 CV4 virus isolate. After the boost with the homologous virulent isolate (isolates E75), the antibody response against all the proteins tested was slightly increased 15 days after the boost. Boosting with heterologous virulent isolate E70, however, resulted in a more significant increase in the antibody response.

A particular disadvantage of the conventional ELISA (which uses semipurified virions as the antigen) is the lack of sensitivity and the unacceptable level of false-negative results obtained with poorly preserved samples, hence demanding the routine confirmation of the result by immunoblotting (2, 3, 20). In order to evaluate the stability of the new ELISAs, 39 ASFV-positive field serum samples were incubated for 1 month at 37°C in order to simulate deterioration in the field and were then retested by the ELISA with the recombinant proteins. With these sera, a sensitivity of 95% (95% CI, 87.9 to 100) was obtained with protein p54, whereas a sensitivity of 87% (95% CI, 76.7 to 97.7) was obtained by the OIE-ELISA. Only the pA104R-ELISA gave a major loss in sensitivity, which was reduced to 31% (95% CI, 16.3 to 45.3). Thus, the best candidates for the serodiagnosis of ASF are the p54 and pB602L proteins, although the pB602L protein had a marginally diminished sensitivity with the heat-treated sera. Importantly, taking together the results obtained with three proteins, p54, pB602L, and pK205R, only 1 of the 39 heat-treated serum samples was negative, and so sensitive diagnosis with recombinant viral proteins is possible even with poorly preserved sera.

It is not clear why the same sera should lose reactivity against one recombinant protein but not another. Two possibilities, proteolytic degradation of the recombinant antigen by heated sera and differential representation of porcine IgG classes, have been eliminated (our unpublished work). A possible explanation for this could be that the antibodies against the p54 protein are more stable or display a higher affinity than the other proteins, and this allows them to react even after the long incubation at 37°C.

In conclusion, this work extends the recombinant antigens (2, 9, 19, 22) with the potential for use for the diagnosis of ASFV infections, has confirmed the usefulness of p54, and has identified pB602L as a novel diagnostic tool for use in areas where ASF is endemic. Given that the variability of ASFV isolates in Africa is greater than that in Europe, further studies are needed in African countries in order to adequately validate the ELISAs. Work is in progress to address this issue by using sera collected from East Africa and Eastern European countries where recent outbreaks of ASF have occurred.

**ACKNOWLEDGMENTS**

This work was supported by European Union project ASFRISK grant agreement 211691 and Wellcome Trust project WT075813MA.
Work at INIA was supported by the Community Reference Laboratory for ASF (grant UE-LR PPA/03) and Spanish grants from MEC (grant SFRH/BD/6071/2001). A. L. Reis was a recipient of a fellowship (SFRH/BD/6071/2001) from the Fundação para a Ciência e a Tecnologia.

We thank E. Martin and A. Simon for their valuable technical assistance.

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