

Detection of African Swine Fever Virus-Specific Antibodies Using the Recombinant Viral Proteins p32 and p54 Expressed in Insect Cells

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Abstract

African swine fever (ASF) is one of the most important infectious diseases of wild and domestic pigs. To develop safe and efficient diagnostic tools for ASF, we amplified the CP204L and E183L genes, which encode the major antigenic proteins p32 and p54, respectively, of the ASF virus Lisbon'60BM89BC1 strain by polymerase chain reaction and expressed them in recombinant baculoviruses. Each recombinant protein was recognized as a single band in baculovirus-infected insect cells by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Specific reaction was observed between these recombinant proteins and test sera from pigs infected with several strains of ASF virus in Western blot analysis and indirect enzyme-linked immunosorbent assay (ELISA). In Western blot analyses, p54 showed relatively higher sensitivity than p32 in antibody detection in the early stage of infection. On the other hand, p32 exhibited better reactivity in indirect ELISA to discriminate between positive and negative reactions. These data indicate that the recombinant p32 and p54 are useful as diagnostic reagents for serological tests of ASF. In order to diagnose ASF accurately, both recombinant proteins should be used in Western blot analysis and indirect ELISA.

Discipline: Animal health

Additional key words: baculovirus expression, diagnostic assay

Introduction

African swine fever (ASF) virus is an enveloped, large DNA virus with icosahedral symmetry. It is the only member of the family *Asfarviridae*. The virus shares several structural features with Iridoviruses but has a genomic organization similar to that of Poxviruses. The double-stranded DNA genome is 170 to 190 kbp long and contains 151 open reading frames (ORFs) in both DNA strands^{5,23}. About 40 proteins synthesized in infected pig cells, mainly macrophages and monocytes, are incorporated into the virion^{4,6,23}. Recently, 14 serological immunodeterminants were identified by screening of a representative lambda phage cDNA expression library of the Ba71V strain¹⁰. These include five structural proteins (A104R, p10, p32, p54 and p72),

three non-structural proteins (RNA reductase, DNA ligase and thymidine kinase) and six proteins with unassigned functions. Together with previous immunological investigations^{3,7-9,24}, it is suggested that some of these proteins (such as p32 and p54) play an important role in protection against virus infection, irrespective of the virus strain, and may be useful for the development of diagnostic and preventive methods for ASF.

ASF virus causes various forms of disease from rapidly lethal to subclinical in domestic pigs²². Currently, no effective vaccine is available. The virus infects not only pigs and wild boars but also soft ticks of the *Ornithodoros* genus, which makes control and eradication of ASF difficult in the affected countries, mainly in Africa. Furthermore, since an infectious virus can survive for several months in fresh and salted dried-meat products²², ASF is recognized as one of the most important diseases

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in the worldwide trade of animals and animal products. Countries free from ASF must prepare safe, sensitive and reliable diagnostic tools without use of the dangerous live virus, in order to prevent incursion of the disease, especially through unapparent carriers. Serological examinations may be the best way to detect pigs infected with ASF virus. In this study, we produced two of the antigenic viral proteins p32 and p54 of the Lisbon'60BM89BC1 strain by using a baculovirus expression system and performing assays to detect antibodies specific to ASF virus in affected pigs. In this report we discuss the utility of these recombinant proteins as diagnostic reagents.

Materials and methods

All experiments were carried out in the bio-containment facility of the Department of Exotic Diseases, National Institute of Animal Health, in Tokyo.

1. Virus and antisera

An avirulent strain of ASF virus, Lisbon'60BM89BC1¹², and five sera obtained from pigs infected with different strains of ASF virus were kindly supplied by Dr. Eugene V. Genovesi, USDA-ARS, Plum Island Animal Disease Center (PIADC), USA. Details of the antisera are as follows: ET034 was collected from a pig infected with highly virulent strain Lisbon'60¹¹. ET343 and ET6106 were collected from pigs that recovered from infection with the moderately virulent strain DR-2 by intranasal/oral inoculation, and by tick transmission challenge, respectively. ET7007 was collected from a pig multiply inoculated with ASF virus strains (Iberian and African isolates). ET9891 was collected from a pig inoculated with the Brazilian strain.

Two conventionally raised pigs (approximately 10–15 kg in weight) were inoculated intramuscularly with a 10⁷ 50% tissue culture infective dose of the Lisbon'60BM89BC1 strain. After inoculation, these pigs were observed daily for 4 weeks. Sera of these pigs were collected periodically (at 0, 7, 12, 14, 16, 20, 24 and 28 days post-inoculation) throughout the experiment.

2. Construction of baculovirus transfer vectors containing CP204L and E183L genes of ASF virus and production of recombinant viruses

Two primer pairs, p32F/p32R (5'-AGCGGATCCTTGAATGGATTTTATTTTA-3' / 5'-TAAGGATCCC TAAAACATTAATGTAG-3') and p54F/p54R (5'-CGCGGATCCTTAAATGGATTCTGAATTTTTTCAA CC-3' / 5'-CGCGGATCCTTACAAGGAGTTTTCTA GGTC-3'), containing *BamH* I sites and covering the

whole ORFs of the CP204 and E183L genes which encode p32 and p54, respectively, were synthesized according to the sequence data previously presented^{19,20,23}. The template DNA for polymerase chain reaction (PCR) was extracted from alveolar macrophages infected with the Lisbon'60BM89BC1 strain using the High PureTM viral nucleic acid kit (Roche Diagnostics, Indianapolis, Ind., USA) according to the manufacturer's instructions. The CP204 and E183L genes were amplified using these primer sets, template DNA and *Pfu* DNA polymerase (Stratagene, La Jolla, CA, USA) by the following protocol: 30 cycles of denaturation (94°C for 1 min), annealing (55°C for 1 min) and extension (72°C for 1 min) and then a terminal extension step at 72°C for 10 min. The PCR products were gel-purified and blunt-end ligated into the *Srf*I site of the pCR-script SK(+) (Stratagene), then excised by digestion with *BamH* I and cloned into the *BamH* I site of the pAcYM1 transfer vector. The derived recombinant transfer vectors were characterized by restriction enzymes and by sequencing across the insertion site. Sf21 cells were transfected with a mixture of the recombinant transfer vector (pAcYM1/ASFVp32 or pAcYM1/ASFVp54) and *Bsu*36 I -digested BacPAK6 viral DNA containing the beta-galactosidase gene under the control of the polyhedrin promoter (Clontech, Palo Alto, CA, USA). Putative recombinant baculoviruses were selected by their *lacZ*-negative (white plaque) phenotypes following exposure to X-gal and purified by three rounds of plaque assays. The derived recombinant viruses expressing p32 and p54 were designated BacASFVp32 and BacASFVp54, respectively.

3. Western immunoblot assay

Western blot analysis was performed using ECL Plus Western blotting detection reagents (Amersham Biosciences, Piscataway, NJ, USA) according to the manufacturer's protocol. The recombinant proteins expressed in Sf21 insect cells infected with BacASFVp32 and BacASFVp54 were resolved by 12.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and electroblotted onto a Hybond-P PVDF membrane (Amersham Biosciences). Strips of the membrane were soaked in blocking buffer (5% non-fat dried milk, 0.1% Tween 20 in phosphate-buffered saline (PBS)) at 4°C overnight, and then each strip was transferred to PBS-T (PBS with 0.1% Tween 20) containing a 1:2,000 dilution of the pig serum from PIADC or from the experimental infection described above and incubated for 1 h at room temperature on an orbital shaker. After four washes in PBS-T, each strip was incubated for 1 h in PBS-T containing a 1:40,000 dilution of horseradish peroxidase (HRP)-conjugated rabbit affinity purified antibody to

swine IgG (Cappel/ICN, Aurora, OH, USA) and rinsed four times with PBS-T. The immune complexes were detected by chemiluminescent reaction of luminogen PS-3 with HRP.

4. Indirect enzyme-linked immunosorbent assay (ELISA) using recombinant proteins

Sf21 or High Five insect cells were infected with the recombinant virus at approximately 5 multiplicity of infection and harvested at 4 days post-infection. Each recombinant protein was extracted from the infected cells (approximately 1×10^8) by three cycles of freezing and thawing in 40 mL of PBS and diluted with coating buffer (0.05 M carbonate/bicarbonate buffer, pH 9.6) just before use as an antigen. Each well of a 96-well microtitre plate was coated with 50 μ L of diluted recombinant antigen at 4°C overnight. After absorption of the antigen, the wells were washed four times in PBS-T and incubated with blocking buffer for 1 h at 37°C. Next, serially diluted pig sera from PIADC (1:50 to 1:6,400) or appropriately diluted sera collected from experimentally infected pigs at 0–28 days post-inoculation were added to the wells and incubated for 1 h at 37°C. After four washes, the wells were incubated with a 1:2,000 dilution of HRP-conjugated rabbit affinity purified antibody to swine IgG for 1 h at 37°C. Following washing as before, the plates were developed with *o*-phenylenediamine (OPD). The absor-

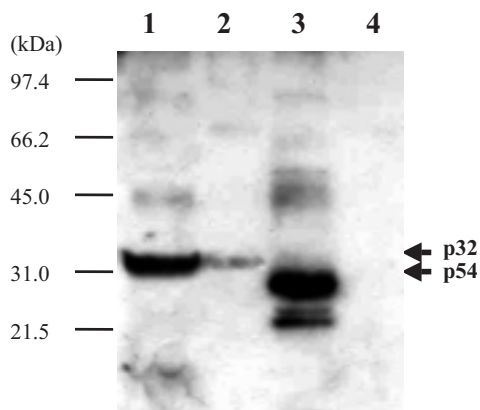


Fig. 1. Characterization of the proteins p32 and p54 expressed by the recombinant baculoviruses

BacASFVp32- and BacASFVp54-infected cells and their supernatants were harvested at 4 days post-infection and were separated by 12.5% SDS-PAGE. The recombinant p32 and p54 proteins were detected by Western immunoblot analysis using an anti-ASF virus pig serum, ET034.

Lane 1: BacASFVp32-infected cells, lane 2: culture medium of cells infected with BacASFVp32, lane 3: BacASFVp54-infected cells, lane 4: culture medium of cells infected with BacASFVp54.

bance at 492 nm was measured using a multichannel spectrophotometer.

Results

The amplified CP204L and E183L genes were individually inserted into the transfer vector pAcYM1, and the recombinant baculoviruses expressing p32 and p54 (BacASFVp32 and BacASFVp54), respectively, were obtained by transfection and subsequent plaque purification. The recombinant proteins, p32 and p54, were detected by SDS-PAGE and Western blot analysis using one of the anti-ASF virus sera provided by PIADC (ET034) (Fig. 1). The recombinant p32 was expressed as a single band of 32 kDa and recognized not only in infected Sf21 cells but also in extracellular medium as described previously¹. Although the recombinant p54 was also expressed as a single band of 25 kDa in infected cells, no secretion of the protein was observed. These

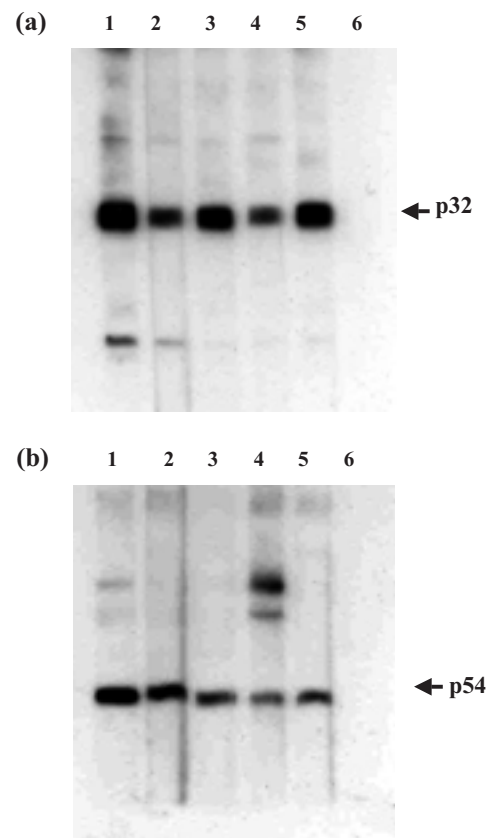


Fig. 2. Western immunoblot analyses of the recombinant proteins (a) p32 and (b) p54 with antisera against several strains of ASF virus obtained from Plum Island Animal Disease Center, USA

Lanes 1–5: Antisera; 1: ET034, 2: ET343, 3: ET6106, 4: ET7007, 5: ET9891. Lane 6: Normal pig serum.

recombinant proteins equivalent to native p32 and p54 appeared in Sf21 cells from 12 h post-infection (data not shown). Like an antiserum ET034, the other four antisera from PIADC showed positive reactions with both recombinant proteins p32 and p54 in Western blot analysis (Fig. 2). No signal was recognized between a normal pig serum and these recombinant proteins. The corresponding proteins were not present in cells infected with wild-type baculovirus AcNPV, BacPAK6 or mock-infected cells.

We performed the time course of antibody detection by Western blot analysis using the recombinant proteins and sera from pigs experimentally infected with the Lisbon'60BM89BC1 strain. Antibodies against p32 were efficiently detected from 12 days post-inoculation,

whereas antibodies against p54 were detected as early as 7–12 days post-inoculation (Fig. 3).

To apply the expressed p32 and p54 to indirect ELISA, we determined the optimal dilution of these recombinant antigens by box titration using antiserum ET034. Since p32 was secreted into extracellular medium (Fig. 1), the supernatant fluid of BacASFVp32-infected cells was also used as an antigen. As shown in Fig. 4, both proteins exhibited strong reactivities with anti-ASF virus pig sera from PIADC. OD values of these antisera were significantly higher than those of the control, an uninfected normal pig serum. The supernatant fluid of BacASFVp32-infected cells (a 1:100 dilution) and extract of BacASFVp54-infected cells (a 1:200 dilution) presented a similar tendency of the reaction patterns with antisera. On the other hand, a 1:1,000 dilution of the extract of BacASFVp32-infected cells showed relatively higher OD values than those of the former two antigens. This cell-extracted recombinant p32 also exhibited the lowest OD value with a normal serum. The ELISA titers of sera of experimentally infected pigs rose day by day, as presented in Fig. 5. When the cell-extracted p32 was used as an antigen, the titers rose markedly between 7 and 12 days post-inoculation and remained high (e. g. > 1.1 OD at 1:100 dilutions of sera from pigs nos. 1 and 2) until 28 days post-inoculation. The amount of the recombinant p32 and p54 extracted from 1×10^8 cells was sufficient for coating almost 8,000 and 1,600 ELISA plates (40 samples/plate for screening), respectively.

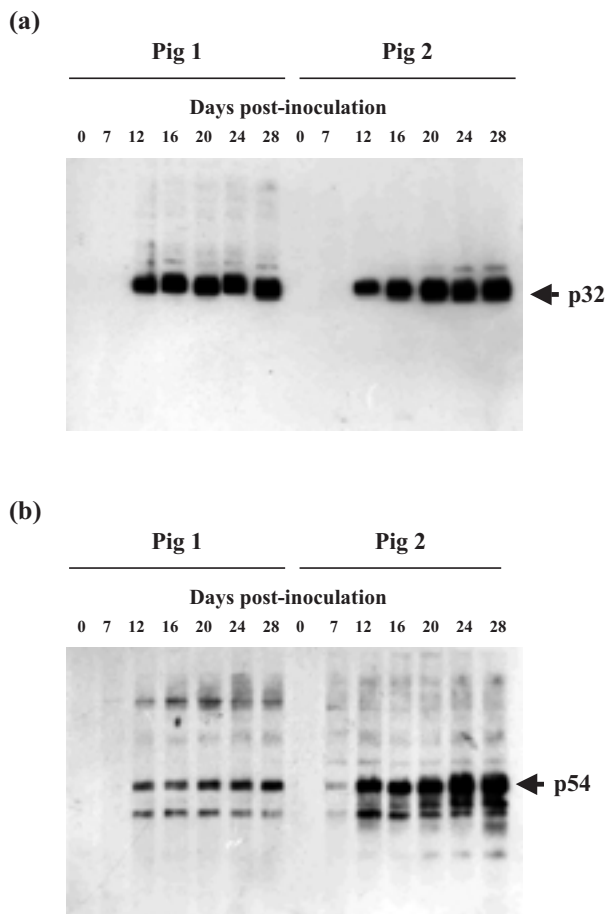


Fig. 3. Time course of antibody detection by Western immunoblot analyses using sera from pigs experimentally infected with the Lisbon'60BM89BC1 strain

The recombinant proteins (a) p32 and (b) p54 were resolved by SDS-PAGE and blotted onto Hybond-P membranes, then reacted with two pig sera collected periodically throughout the experiment.

Discussion

Recombinant proteins have been used frequently to develop novel diagnostic systems for detecting antibodies against important veterinary viruses. It is especially convenient for serological diagnosis of the Office International des Epizooties list A diseases to use recombinant proteins instead of the virus itself. In the case of ASF, one of the 15 list A infectious diseases, the recombinant proteins derived from a Spanish strain, E70, have already been prepared for this purpose^{2,14}. Gene expression systems using bacteria, yeast, mammalian cells, baculovirus and vaccinia virus are powerful tools for producing antigens. In particular, the baculovirus expression system is considered to be ideal for the production of large quantities of the recombinant proteins that exhibit antigenic properties similar to those of their native counterparts¹³. We, therefore, expressed p32 and p54 of ASF virus Lisbon'60BM89BC1 strain by recombinant baculoviruses to investigate their potential diagnostic use.

The expressed p32 and p54 reacted with all five antisera from PIADC in Western immunoblot assay and

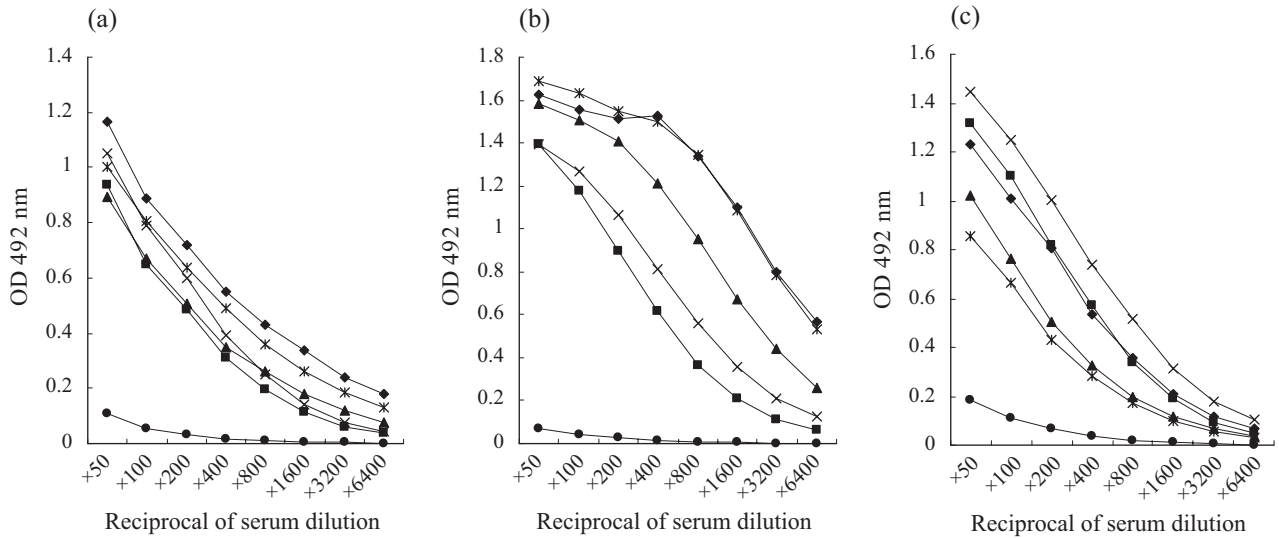


Fig. 4. Indirect ELISA tests using (a) the supernatant of BacASFVp32-infected insect cells (1:100 dilution) and the extracts of (b) BacASFVp32- and (c) BacASFVp54-infected cells (1:1,000 and 1:200 dilutions, respectively) as antigens
 OD₄₉₂ values were obtained from serial dilutions of anti-ASF virus pig sera from Plum Island Animal Disease Center and a normal pig serum. —◆—: ET034, —■—: ET343, —▲—: ET6106, —×—: ET7007, —*—: ET9891, —●—: Negative control.

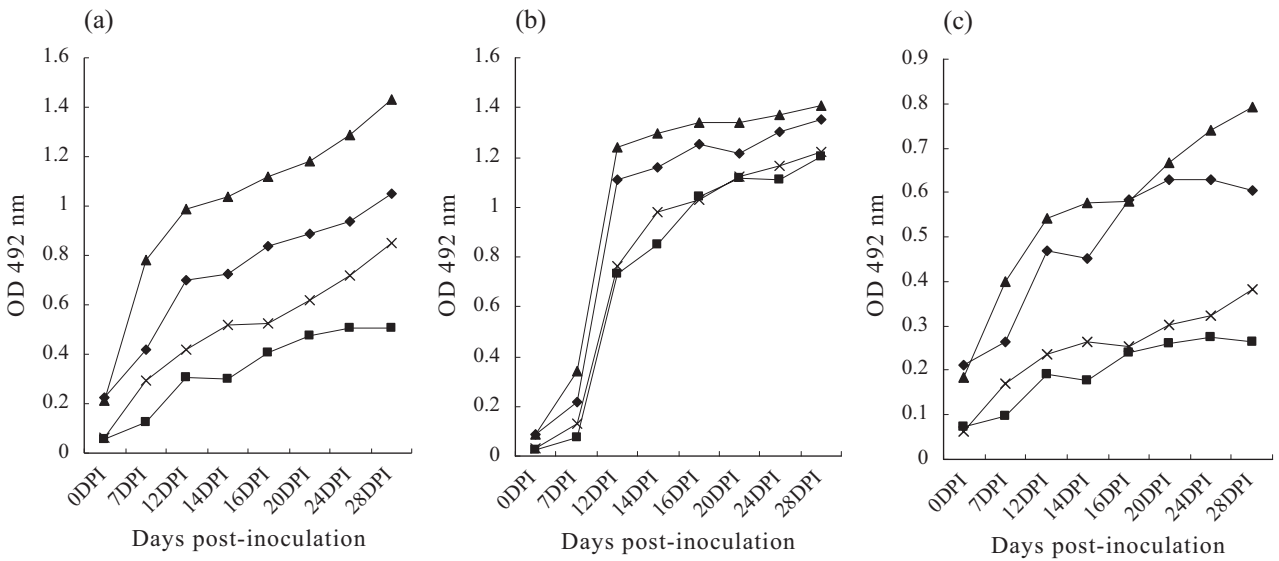


Fig. 5. Time course of antibody detection by indirect ELISA tests using (a) the supernatant of BacASFVp32-infected insect cells (1:100 dilution) and the extracts of (b) BacASFVp32- and (c) BacASFVp54-infected cells (1:1,000 and 1:200 dilutions, respectively) as antigens

Pig sera experimentally infected with the Lisbon'60BM89BC1 strain were collected at 0–28 days post-inoculation. OD₄₉₂ values of 1:100 and 1:400 dilutions of these sera were obtained.
 —◆—: Pig no. 1 (1:100), —■—: Pig no. 1 (1:400), —▲—: Pig no. 2 (1:100), —×—: Pig no. 2 (1:400).

indirect ELISA, suggesting that these proteins can be used to detect antibodies against various strains of ASF virus. In Western blot analysis, antibodies to p32 and p54 were detected simultaneously from 12 days post-inoculation in pig no. 1 experimentally infected with the Lisbon'60BM89BC1 strain. However, in pig no. 2, antibody to p54 was detected earlier than that to p32. The time course experiments of antibody detection indicate that the

recombinant p54 has relatively higher sensitivity than p32 and should be used mainly in Western blot analysis to find pigs in the early stage of ASF virus infection. In contrast, in indirect ELISA, the recombinant p32 is more efficient than p54 for antibody detection. Since the extract of BacASFVp32-infected cells can be diluted more than that of the Lisbon'60BM89BC1-infected cells, it is possible to lower non-specific (false positive) reac-

tion and to prepare plenty of 96-well ELISA plates for screening and/or titration. These data demonstrated that the cell-extracted p32 is most suitable for an ELISA antigen to facilitate discrimination between positive and negative sera. The supernatant fluid of BacASFVp32-infected cells is also useful because it is simple to prepare as an antigen. These results suggest that serological diagnosis of ASF for detecting unapparent carriers could be carried out by screening with ELISA subsequent to a Western blot confirmatory test using both recombinant proteins without any need to propagate ASF virus and without special safety precautions.

In general, pigs infected with ASF virus develop antibodies that can be demonstrated from 7 days post-infection by various serological methods^{15–18,21}. Although the data obtained from this study revealed that the antibody detection system using the recombinant proteins p32 and p54 represents an interesting alternative to the conventional and hazardous method, it is still necessary to determine the appropriate cutoff value by using field sera of ASF virus-infected pigs in cooperation with animal health laboratories in foreign countries, in order to evaluate the utility of our ELISA tests.

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