Detection of African Swine Fever Virus Antibodies by Immunoblotting Assay

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ABSTRACT

An immunoblotting assay has been adapted to detect antibodies against African swine fever virus. The electrophoretic transfer of proteins and the immunoreaction conditions were optimized, using 4mA/cm² of current intensity and 10 μg of soluble cytoplasmic antigen of infected cells per strip. Filters of polyvinylidene difluoride showed the highest capacity for protein absorption, but nitrocellulose filters showed lower backgrounds. The specificity and the pattern of the proteins induced by African swine fever virus that react with the antisera were determined in immunoblotting assay, IP30 being the most reactive protein.

RÉSUMÉ

Cette expérience consistait à mettre au point une épreuve d'immunoempreinte, pour détecter les anticorps contre le virus de la peste porcine africaine. Les auteurs optimisèrent à cette fin le transfert électrophorétique des protéines et les conditions d'immunoréaction, en se servant d'un courant électrique de 4 mA/cm² de 10 μg d'antigène cytoplasmique soluble des cellules infectées, pour chaque bande. Les filtres de difluorure de polyvinylidène se révélèrent les meilleurs pour l'absorption des protéines, mais ceuz de nitrocellulose donnèrent moins d'artéfacts. L'épreuve d'immunoempreinte servit à déterminer la spécificité et le profil des protéines produites par le virus de la peste porcine africaine et qui réagissent avec les antisérums spécifiques; la protéine IP30 s'avéra celle qui réagissait le plus.

The eradication of African swine fever (ASF) requires rapid and specific diagnosis, since an effective vaccine does not exist. More than 90% of infected pigs can be detected by the demonstration of specific antibodies against ASFV(1). Therefore, tests based on antibody detection are currently the most frequently used. However, some authors recommend the use of confirmatory tests when large populations are screened in order to avoid false positives produced by problems inherent to the techniques employed (1).

Most types of biochemical and immunochemical analyses make use of sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis to identify and characterize proteins by their relative molecular weights. On the other hand, it is possible to achieve essentially complete and quantitative transfer of most proteins from SDS-gels to filters, while preserving the antigenic properties of the proteins under denatured conditions (2,3).

In the present work we have adapted an immunoblotting assay for ASFV-antibody detection. This technique offers an alternative method to antibody-immunofluorescence (4) which also confirms positive results obtained in routine screening techniques.

The soluble cytoplasmic ASFV antigen used in this technique was obtained from MS cells (monkey kidney cell line) infected at a multiplicity of infection of ten with Spain 70 ASFV strain after 48 passages on MS cells (5). As positive and negative controls, a pool of ten sera from pigs experimentally inoculated with Spain 70 strain (6), showing an antibody ELISA-VP73 titer (7) higher than 1/2000 and a pool of ten sera from noninfected pigs, negative for ELISA-VP73 were used.

Soluble ASFV cytoplasmic proteins resolved in 17% acrylamide-N,N'diallyltartardiamide (DATD) gels (8), were transferred as described (2), with a constant current intensity of 4 mA/cm² for 90 min at 4°C. The transfer buffer was 20% methanol in 0.02 M tris, 0.169 M glycine. After the transfer, the 16 x 20 cm filters were dried and cut into 0.5 cm wide strips, each containing about 10 μg of ASFV antigen. They were then stored at room temperature.

Three different filters were tested: nitrocellulose (Biodine, Pall) (Fig. 1, panel 1.A), nylon (Hybond-N, Amersham) (Fig. 1, panel 1.B) and polyvinylidene difluoride (PVD) (Immobilon, Millipore) (Fig. 1, panel 1.C). The PVD filter showed the best transfer of high and low molecular weight proteins. However, both PVD and nylon showed higher backgrounds than nitrocellulose filters in repetitive immunoreactions (data not shown).

The immunoreaction with the antisera was carried out using three different blocking solutions. They were tested to saturate the remaining protein binding sites: 2% gelatine, 1% bovine serum albumin and 2% nonfat dry milk, all in phosphate buffered

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Submitted December 22, 1987.

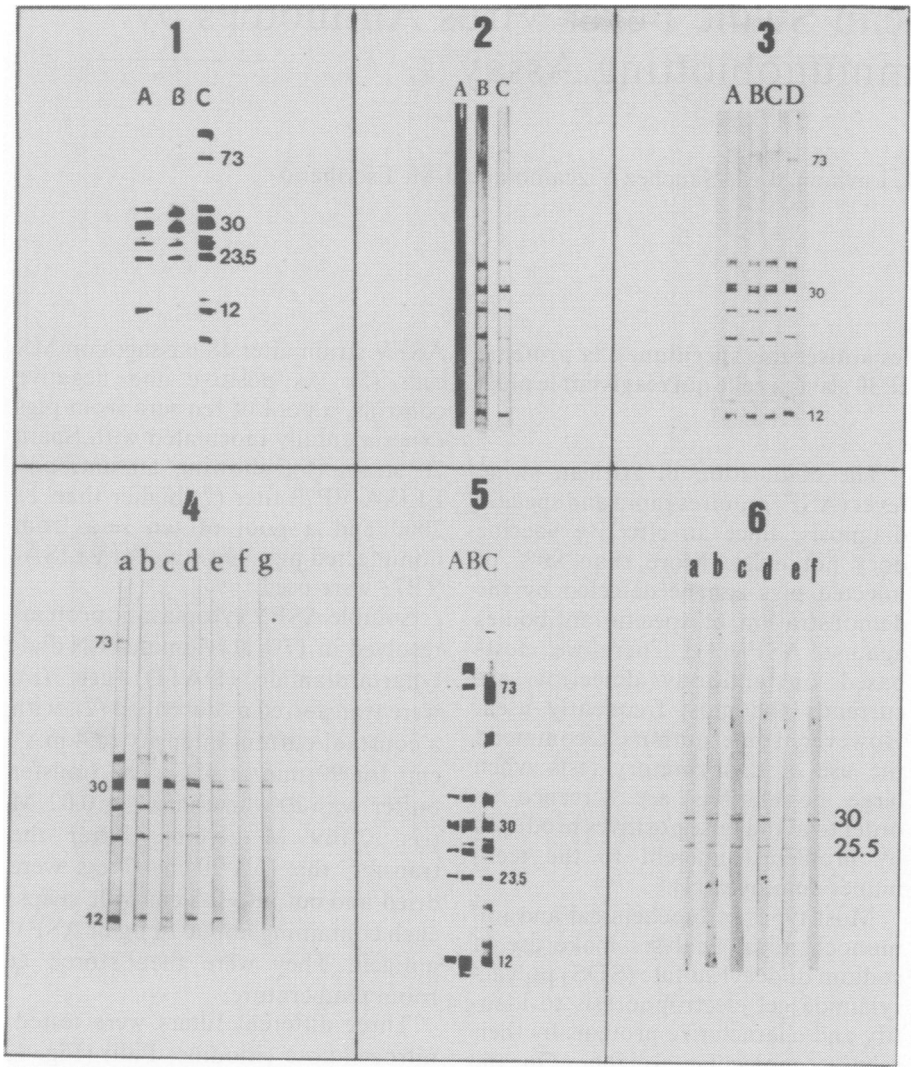

Fig. 1. Immunoblotting assay conditions for detection of specific antibodies against African swine fever virus. Ten μg of soluble cytoplasmic ASFV antigen were transferred from acrylamide gels to each filter strip and reacted with a pool of ten ASF positive sera diluted to 1:30. Different conditions were assayed and compared in separate experiments. With the exception of conditions studied in each case, the standard conditions employed in all cases were: nitrocellulose filters; 2% nonfat dry milk as blocking solution; 15 min blocking incubation, 30 min serum incubation and 45 min conjugate incubation; detection of immunocomplexes by protein A-peroxidase. Results from one representative experiment of each of the conditions studied are shown as follows:

Panel 1) Type of filter: nitrocellulose (A), nylon (B) and polyvinylidene difluoride (C).

Panel 2) Blocking solution: 2% gelatin (A), 1% bovine serum albumin (B) and nonfat dry milk (C).

Panel 3) Time of incubation for reactions

	Blocking	Serum	Conjugate
A	60 min	60 min	60 min
B	15 min	60 min	60 min
C	15 min	30 min	60 min
D	15 min	30 min	45 min

Panel 4) Remaining reactivity of the proteins after serial dilutions of the control positive serum, starting at 1/30 (A).

Panel 5) Alternative methods to detect specific antibodies using protein A-peroxidase (A), rabbit antiswine IgG biotinylated plus avidin-peroxidase (B), and rabbit antiswine IgG plus protein A-peroxidase (C).

Panel 6) Immunereactivity of six sera weakly positive by ELISA-VP73, but not confirmed as positive by antibody immunofluorescence.

saline (PBS). Nonfat dry milk gave the best results as shown by other authors (9) (Fig. 1, panel 2).

The incubation times of the different processes were reduced without loss in protein intensity. In the present assay the filter blocking time was reduced to 15 min, the incubation of the filter strips with the serum was reduced to 30 min and the incubation with the protein A-peroxidase conjugate was reduced to 45 min. This reduction of the processing time, saving about 1.5 h from conventional procedures described for ASF diagnosis by ELISA, did not reduce protein intensity nor increase backgrounds (Fig. 1, panel 3).

The proteins best detected by the hyperimmune sera at a working dilution of 1/30 were IP12, IP23.5, IP25.5, IP30 and IP34, of which IP34, IP30 and IP12 are early proteins which induce high antibody responses in infected pigs and are antigenically constant in the different viral isolates studied (8).

To detect specific antibodies against the proteins transferred to the filters a second rabbit antibody against pig IgG was added to increase the sensitivity of the assay compared with the direct use of protein A-peroxidase (Fig. 1, panel 5.A). Two different methods to reveal the immune complexes were assayed. In the first method, the rabbit antibody was conjugated with biotin (10) and revealed with avidin-peroxidase (Fig. 1, panel 5.B). In the second, protein A-peroxidase was added to the rabbit anti-IgG complexes (Fig. 1, panel 5.C). The rabbit antibody was used at a dilution predetermined by titration to be in excess. The washing process after the addition of first and second antibodies was very important in order to reduce backgrounds. A negative pool of sera used as control in both methods did not react with any protein. Increasing the sensitivity of the immunoblotting, the capacity of antibody detection compared with ELISA-VP73 was very similar, and confirmed all the positive reactions obtained by this technique. "False negatives" in ELISA-VP73 have not been described. However, since immunoblotting was not more sensitive than ELISA-VP73 in antibody detection, this technique will not avoid

possible false negatives not detected by ELISA-VP73.

The semipurified VP73 protein, which is used commonly as an antigen in ELISA (7) cannot be employed efficiently in immunoblotting assays because proteins with molecular weights higher than 40K are not well transferred to the filters under the conditions described above. This protein was best transferred and detected with more efficient filters (PVD) (Fig. 1, panel 1.C), as well as with a rabbit antiswine IgG (Fig. 1, panel 5.B and C).

Two hundred field pig sera positive by ELISA-VP73 were confirmed by antibody-immunofluorescence and immunoblotting, with at least the proteins IP12, IP23.5, IP25.5, IP30 and IP34 reacting in the latter assay. However, six sera weakly positive by ELISA-VP73 were not confirmed as positive by antibody immunofluorescence, but gave positive reactions in immunoblotting against proteins IP30 and IP25.5 (Fig. 1, panel 6).

The stability of the filter strips throughout storage and transportation at room temperature in a dry atmosphere was tested during six months, with no observable loss in the antigenicity of the proteins transferred. For transportation to other laboratories the filter strips have advantages over both soluble antigens and slides containing fixed infected cells. In addition, there is no risk of infective material in the strips.

We conclude that immunoblotting is a very powerful technique for diagnosis of ASF. It is highly specific, sensitive and easy to interpret, and provides an alternative method to antibody immunofluorescence by which it is possible to confirm positive ELISA reactions.

ACKNOWLEDGMENTS

We are grateful to Dr. F. Alonso for his useful discussion, and to L. Gomez Marti for technical assistance.

This work was supported by a grant from Instituto Nacional de Investigaciones Agrarias, Madrid, Spain.

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