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New Method of Antibody Detection by Indirect Immunoperoxidase Plaque Staining for Serodiagnosis of African Swine Fever

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An indirect immunoperoxidase plaque-staining method was developed for detecting antibody to African swine fever virus infection. In both sensitivity and specificity, the test was comparable to indirect immunofluorescence. Because it has all of the desirable features of the indirect immunofluorescence test and may also be readily used for testing large numbers of sera, the indirect immunoperoxidase plaque-staining method can be used as a single and final serodiagnostic test in a large-scale survey of African swine fever. The indirect immunoperoxidase plaque-staining method should be applicable to other viruses that can be adapted to and grown in cell cultures.

African swine fever (ASF) (4), as it usually occurs in Africa and as it was first encountered in the Iberian peninsula, is an acute, systemic virus infection of high mortality that may usually be quickly and accurately diagnosed with the direct immunofluorescence test by demonstrating viral antigens in macrophages of the spleen, lymph nodes, liver, bone marrow, lung, kidney, etc. However, in the recent outbreaks of ASF in Europe and countries of the Western hemisphere, clinically mild forms with low mortality have been common (5). In these cases, quick diagnoses based on the demonstration of viral antigens in the tissues are often not possible, due perhaps to the blockage of antigenic determinants by the rapidly rising antibody levels in the infected animals. Under these conditions, the demonstration of the presence of specific antibody has become the accepted means of quickly detecting the disease. Because there is no satisfactory vaccine, eradication is pursued by the "stamping-out" method, in which all infected and exposed swine are eliminated. The detection of infection in a single animal in a herd is enough to require slaughter and sanitary disposal of the entire herd. This places tremendous responsibility on the laboratory and its serodiagnostic procedures. In Spain, where ASF has been endemic for 20 years, the current serodiagnostic strategy is to use the immunoelectroosmophoresis (IEOP) test (7) as a screening test and to apply the indirect immunofluorescence (IIF) test (1, 2) to confirm any positive reactions. With the application of this combination of tests, a contaminated herd will not escape detection (12).

In the Dominican Republic, a new situation arose. The decision was made to eradicate the disease by eliminating the entire swine population. This was accompanied by a serum survey to determine the extent and distribution of the disease. The enzyme-linked immunosorbent assay (ELISA) test (3) was applied because it was considered to be the most sensitive method available for testing large numbers of sera. However, the test yielded an inordinate number of false-positive reactions, and again the IIF test had to be applied for confirmation (3).

The IIF test is the most accurate and reliable of the methods of detecting viral antibodies because it has two test indicators. The test is run on cell cultures in which only 10 to 20% of the cells are infected with the virus. In a positive reaction, the sparsely distributed infected cells fluoresce and display intracellular antigen typical for that particular virus. Furthermore, if the test serum contains antibodies to the cells per se, the uninfected cells will also fluoresce. Thus, it is possible to easily distinguish positive from false-positive reactions, and much confusion can be avoided in interpreting the result. However, IIF is not readily adaptable to large-scale surveys. We have developed an immunoperoxidase plaque-staining (IIPS) method that has all of the desirable features of IIF, but the results can be read with the unaided eye under ordinary light, making it possible for a single technician to conduct 400 tests in a day. Thus far, we have evaluated the method for the ability to detect antibodies to ASF virus by comparing it with the IIF, ELISA, and IEOP tests.

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MATERIALS AND METHODS

Test sera. A total of 1,176 assorted sera, including pre-inoculation sera, were collected from swine at various stages of experimental infection with five ASF virus isolates, i.e., Lisbon '60 (7), Brazil (5), Dominican Republic (5), Haiti, and Italy (7), and with two cell-attenuated viruses, Lisbon '60 BM_{89} (8) and the Vero cell-attenuated virus (12). A total of 136 field sera collected from the Dominican Republic and 6 sera from Brazil were also included in this study. The six sera from Brazil were known to give false-positive reactions in the IEOP test, in which the conventional IEOP antigen was used; they were used as false-positive controls in developing a new virus antigen for the ELISA test. All 1,318 sera were tested in IIPS, IIF, and IEOP tests, and 1,089 of these sera were also tested by ELISA in a manner described later in this section.

IIF test. For the IIF test, cover slip cultures of Vero cells infected with Lisbon '60 virus were prepared as previously described (1, 2, 12). The cover slips, on which approximately 10 to 20% of the cells were infected, were dried, fixed with methanol for 10 min at room temperature, again dried, and stored in a freezer at −70°C until used. The ovine anti-swine immunoglobulin G (IgG) (OASIg) antibody precipitated from antiserum by one-third saturation with ammonium sulfate and conjugated to fluorescein isothiocyanate (13) was used for staining. Tests displaying cells that had green fluorescence of cytoplasmic inclusion bodies and fine stripplings (11) were regarded as positive. Sera were usually tested undiluted, but some were diluted 1:10 with 0.14 M NaCl in 0.01 M phosphate buffer (PBS; pH 7.3) if the volume was insufficient for testing undiluted or the background fluorescence was too strong.

IEOP test. The conventional IEOP antigen (12) prepared from Lisbon '60 virus adapted to Vero cells was used for the IEOP test (7).

Preparation of HP conjugate. OASIg antibody for making the appropriate conjugates for the IIPS and ELISA tests was prepared as follows. Swine IgG was purified by chromatography on a DEAE-cellulose column equilibrated and eluted with 0.04 M Tris-phosphate buffer solution at pH 8.6 (9). The purity was assessed by immunoelectrophoresis and Ouchterlony double-agar diffusion. Each sheep received a total of 10 mg of swine IgG in complete Freund adjuvant subcutaneously in four sites. A booster injection with the same dosage of IgG in incomplete Freund adjuvant was given 7 days later. Sheep were exsanguinated 14 days after the booster injection. The OASIg antibody was purified from antiserum by affinity chromatography, with swine IgG conjugated to Sepharose-4B-CNBr (Pharmacia Fine Chemicals, Uppsala, Sweden) as an immunoadsorbent. The immunoadsorbent was suspended in ovine antiserum and agitated with a rocking motion on a shaker at 33°C for 2 h. After a thorough washing with PBS until the final wash solution had an optical density of 0 at 280 nm in a spectrophotometer, the antibody was eluted by suspending the immunoadsorbent-antibody complex in a solution of 0.5 M NaCl in 0.1 M glycine-hydrochloride buffer (GBS; pH 3.0). After mechanical shaking for 30 min at 33°C, the entire suspension was poured into a glass column equipped with a sintered glass plate at the bottom. The eluate was collected in fractions, and the column was further washed with GBS until the effluent no longer absorbed at 280 nm. The protein solution was neutralized with 1 N NaOH solution, precipitated by the addition of an equal volume of a saturated aqueous solution of ammonium sulfate, and pelleted by centrifugation. The protein pellet was dissolved in a minimum volume of distilled water and dialyzed against running tap water. Precipitates were removed by centrifugation at 800 × g. The supernatant fluid was dialyzed against 1,000 volumes of PBS and frozen at −20°C until used. The protein concentration was determined by spectrophotometry, assuming that a 10-mg/ml immunoglobulin solution has an absorbance at 280 nm of 14.0. The purified OASIg antibody was conjugated to horseradish peroxidase (HP) (type VI; Sigma Chemical Co., St. Louis, Mo.) by the method of Nakane and Kawaoi (6), and the HP conjugate was used in the IIPS and ELISA tests.

Preparation of antigen plates for IIPS test. Confluent monolayers of Vero cells grown in Costar cell culture dishes with 24- or 96-well clusters and infected with the Vero cell-adapted Lisbon '60 isolate of ASF virus (12) served as antigen plates for the IIPS test. The virus inoculum (0.3 ml/well for a 24-well plate or 0.1 ml/well for a 96-well plate) was adjusted so that with 1 h of adsorption each well contained approximately 150 or 60 plaques, respectively, under an agarose overlay (0.7%), after 5 days of incubation in a humidified 5% CO_2 atmosphere at 37°C (10). After the agarose overlay was carefully removed with a spatula (24-well plate) or by vacuum suction (96-well plate) and the cell sheet was dried with a stream of warm air, the cell sheet was fixed with dry methanol for 10 min and dried. Finally, the plates were sealed with tape and stored at −70°C until used. The antigenicity after 12 months of storage (the longest period tested) equaled that of freshly prepared antigen plates.

IIPS test. For performing the IIPS test, antigen plates taken from the freezer were warmed and dried before the tape was removed to prevent the cell sheets from detaching from the plastic wall during washing. A 0.3-ml (24-well plate) or 0.05-ml (96-well plate) amount of undiluted test serum was applied to the respective wells of the antigen plates. The plates were left at room temperature for 30 min. The serum was removed from the plates, and the residual serum was removed by pressing the well mouths firmly with clean paper towels. Wells were rinsed twice with 2% saline solution, filled with saline and left at room temperature for 10 min, and decanted. A 0.3- or a 0.05-ml amount of HP conjugate, the optimal concentration of which was predetermined, was placed into each well, and the plates were kept at room temperature for 30 min. Wells were washed, and 0.5 or 0.1 ml of 3,3′-diaminobenzidine (1 mg/ml) in 0.01% H_2O_2 saline solution (5°C) was placed into each well. The reaction was stopped after 30 min by rinsing the plates once with tap water and drying them. Wells containing dark brown-colored plaques were recorded as positive (see Fig. 1). When the HP conjugate prepared with the purified antibody was used, the background (uninfected area) was colorless during the 30-min reaction period even when extensively hemolyzed sera were tested.

New method for preparing ASF virus antigen. In our experience, when IEOP antigen (12) prepared by the conventional method was used in the ELISA test (16), substantial numbers of false-positive reactions occurred. Therefore, a new method of preparing antigen was developed. The viral particles were precipitated from viral culture fluid, approximately $10^{8.0}$ PFU/ml, by adding 6% polyethylene glycol (molecular weight, 6,000) to the culture fluid and allowing it to stand overnight at 4°C. The precipitate was pelleted by centrifugation at $800 \times g$ for 30 min at 4°C. The pellet was suspended in PBS (30 ml of PBS per liter of viral culture fluid), dialyzed overnight at 4°C against 100 volumes of PBS, and sonicated for 2 min. The sonic extract was clarified by centrifugation at $85,000 \times g$ at 4°C. Sodium azide was added (0.1%) to the supernatant fluid. The antigen was divided into small volumes and stored at −70°C until used.

The quality of the new antigen was assessed by the IEOP test. The IEOP test was applied to 451 sera that were known to be free of antibodies against ASF virus. Among them were six field serum samples from Brazil that had previously given false-positive reactions when the conventional IEOP antigen was used. The test results with all of these sera were negative when the new antigen was used, and sharp precipitin lines were consistently obtained with positive control sera.

ELISA test. We performed the final version of the ELISA test as follows, using a modification of the method of Voller et al. (16). Disposable, 96-well microtiter polystyrene plates (Dynatech Laboratories, Inc., Alexandria, Va.) were coated with 0.1 ml of ASF virus antigen in 0.1 M bicarbonate-carbonate buffer (pH 9.0) for 18 h at 4°C. The appropriate dilution of antigen for use in the ELISA was determined by serial dilution; the highest dilution that gave the same absorbance value at 420 nm as the next lowest dilution was used (1:50). In the preliminary trials, undiluted sera invariably gave false-positive reactions; therefore, the minimum dilution of serum that did not give a false reaction was determined by testing sera from 436 U.S. swine known to be free of ASF virus infection. When sera were serially diluted and tested, none of the sera diluted at 1:50 gave false reactions with the new antigen. Subsequently, all test sera were diluted 1:50 for testing. At the time of testing, the antigen plates were rinsed twice with PBS containing 0.05% Tween 20, and 0.1 ml of serum diluted 1:50 in PBS–0.05% Tween 20 was added to the wells. The plates were incubated for 1 h at room temperature and then rinsed twice with PBS, and then the wells were filled with PBS for 10 min. After the wells were emptied, a 0.1-ml volume of HP conjugate (1:50) was added, and the plates were incubated at room temperature for 1 h. The plates were again washed, and 0.1 ml of freshly prepared 0.1% H_2O_2 in 1 M citrate buffer solution (pH 4.5) containing 0.1% *O*-phenylenediamine was added. Because the substrate solution in the negative control well remained colorless for 2 h, the results, positive or negative, could be read by eye after 30 min at room temperature.

RESULTS

General appearance of positive IIPS test. In the IIPS test, dark brown-colored plaques developed rapidly within seconds when sera of high antibody titer were tested (Fig. 1A). A strong background coloration was apt to develop with badly hemolyzed sera when a crude immunoglobulin fraction of OASIg serum was used for preparing HP conjugate, and the discrimination of specifically stained plaques from background was difficult (Fig. 1B). This problem was solved by using the HP conjugate of purified antibody; the background remained colorless during the 30-min reaction period even when extensively hemolyzed sera were tested.

Selection of a virus isolate for preparing antigen plates. A total of 686 sera, known to contain antibody against ASF virus by other conventional tests, were tested by the IIPS with the antigen plates prepared from the Lisbon '60, Dominican Republic, Brazil, and Haiti virus isolates. The results indicated that all viral antigens reacted with test sera well within the variation of less than 3% (Table 1). Since the antigen plates prepared from the Lisbon '60 isolate reacted with all of the sera tested (Table 1), the antigen plates (IIPS) or antigen (IIF, IEOP, ELISA) prepared from Lisbon '60 virus were used throughout the experiment.

Sensitivity of the IIPS test in relation to the IIF test. To compare the sensitivity of the IIPS test with that of the IIF test, serial twofold dilutions of two positive sera were made, and the final dilutions which gave a positive result in either test were obtained. With the IIPS test, the final dilutions in which a well contained dark-staining plaques were 1:16,384 (Fig. 1A) and 1:65,536; comparable titers (1:8,192 and 1:8,192) were obtained with the IIF test. Thus, both tests had similar sensitivity.

Agreement of test results among IIPS, IIF, and IEOP tests. The data in the table are arranged so that the degree of agreement in the detection of positive and negative sera by two tests is expressed as a percentage of the total sera examined. The IIPS and IIF tests had 98% agreement, and either test had about 1% more positives than the other test (Table 2). Thus, both tests had comparable sensitivities and specificities; the results of both tests did not differ from each other as tested by the chi-square test (chi square = 0.0137, degree of freedom = 1, P = 0.907). The IIPS and IEOP tests had 88% agreement (Table 2). Although the sensitivity of the IEOP test was only 77% of that of the IIPS test, the IEOP test did not react falsely with negative sera.

Agreement of the ELISA with three other tests. Because the ELISA test is relatively new and has never been properly evaluated by testing swine sera of experimental ASF, sensitivities and specificities were evaluated in relation to the other three tests in this study. Since not all sera were tested in the ELISA test, the data obtained from sera tested by all four tests were treated separately (Table 3). The results indicated that

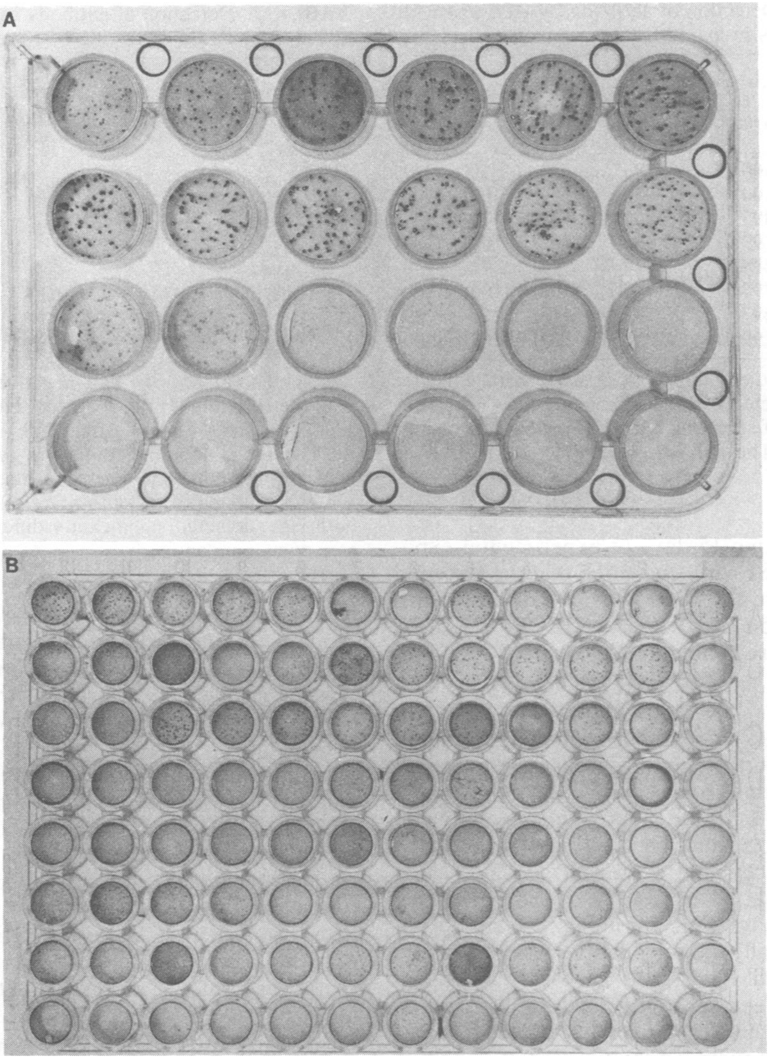

FIG. 1. Results of the IIPS test with HP conjugate of purified OASIg antibody in a 24-well antigen plate (A). Serial twofold dilutions of a positive serum were made, and the undiluted serum was placed in the upper left well; each additional dilution of serum was placed sequentially to the right in the first row and so on for the rest of the rows. The final dilution which produced dark-staining plaques was 1:16,384 (third row, third well from the left); a comparable titer (1:8,192) was obtained with the IIF test. As is seen here, higher serum dilutions gave stronger staining of plaques than did the lower dilutions, including undiluted serum. This occurred only with serum with a high antibody content. For sera with a low antibody content, the undiluted serum gave the strongest staining of plaques. When a crude immunoglobulin fraction of OASIg serum was conjugated to HP, the strong background coloration that developed with intensely hemolyzed sera was apt to hinder the discrimination of plaques from the background, as seen in two sera in the seventh row of the 96-well antigen plate (B). This problem was solved by using the HP conjugate of purified antibody.

the degree of agreement by the ELISA test with the other tests was relatively low, about 87% with the IIPS test, about 86% with the IIF, and 91% with the IEOP test; the ELISA test detected 6% more positive results than did the IEOP test. Nine sera positive in the ELISA test were negative in three other tests; thus, these sera were regarded as false positive. Furthermore, six field sera from Brazil that were known to give false-positive results in the IEOP test with the conventional antigen, but which were negative with the new antigen, consistently gave false-positive results in the ELISA test.

Detection of antibody in sera collected from early phase of infection. The results of antibody detection from 206 sera which were derived

TABLE 1. Detection of antibody by IIPS test with antigen plates prepared from various virus isolates

Virus isolates for antigen prepn	Lisbon '60 (500)[a]	Dominican Republic (78)[a]	Brazil (49)[a]	Haiti (10)[a]	Rome (49)[a]
Lisbon '60	500[b] (100)	78 (100)	49 (100)	10 (100)	49 (100)
Dominican Republic	495 (99.0)	76 (97.4)	49 (100)	10 (100)	49 (100)
Brazil	488 (97.6)	77 (78.7)	49 (100)	10 (100)	49 (100)
Haiti	497 (99.4)	78 (100)	49 (100)	10 (100)	49 (100)

[a] Indicates the number of positive sera collected from pigs infected with each isolate.
[b] Number of sera which showed a positive reaction in each test. The percentage of the total is given in parentheses.

from pigs within 15 days of experimental exposure to the ASF virus indicated that the IIPS and IIF tests were far more sensitive in early antibody detection than were the IEOP or ELISA tests used here (Table 4).

DISCUSSION

A test that is to be applied on a large scale to detect antibodies for viral diseases in livestock should be specific and sensitive, as well as rapid and affordable. Well-defined antigens and proper test controls must be used in such a test to ensure an acceptable degree of specificity and sensitivity. This is especially true when testing field samples, for which preinfection serum samples are usually not available. When a crude antigen is used, it is usually necessary to strike a balance between sensitivity and specificity that will enable the researcher to detect specific antibodies yet yield a minimum of false-positive readings. At best, tests in which crude antigens are used are only suitable for screening, and a more critical test must be used to confirm the positive reactions.

The IIPS test described here had sensitivity and specificity comparable to those of the IIF test. The IIPS test also has the built-in control, as in the IIF test, consisting of uninfected cells, for differentiating a true-positive result from a false one. As to the efficiency of the IIPS test, a trained technician is able to process up to 400 sera a day without difficulty, and the completed test plates may be stored as permanent records.

Since there is evidence indicating that antigenic differences exist among isolates from various geographic locations, the obvious question is whether a virus isolate from each geographic location must be used in preparing antigen for that particular location or whether a single representative isolate can be used throughout. The results in Table 1 indicate that the antigen preparation from the Lisbon '60 isolate was satisfactory in all cases. This conclusion seems to be reasonable and is in accord with the view that all other isolates tested originated from the Lisbon '60 isolate.

Despite the fact that an improved ASF virus antigen and an HP conjugate prepared from purified OASIg antibody were used, the ELISA test as performed in this study was not sufficiently specific or sensitive to be acceptable as a routine diagnostic test for ASF. In addition to being less sensitive than the IIPS and IIF tests, it gave 2% false-positive results. The lack of sensitivity was no doubt partially due to the fact that a 1:50 dilution of the sera was necessary to avoid the false-positive reactions. It appears that a

TABLE 2. Detection of antibody by IIPS, IIF, and IEOP in testing 1,318 sera

Test	Results with IIPS: Positive (688)[a]	Results with IIPS: Negative (630)[a]	% Agreement between two tests
IIF			
Positive	675	16	98[b]
Negative	13	614	($P = 0.91$)[c]
IEOP			
Positive	527	0	88
Negative	161	630	($P < 0.01$)[d]

[a] The value in parentheses indicates the number of sera.
[b] Number of positive and negative sera on which both the IIPS and IIF tests agreed/the total number of sera examined = (675 + 614)/1,318 = 0.977.
[c] The probability by chi-square test that the two tests are providing equivalent results.
[d] The probability by chi-square test indicated that both tests detected significantly different numbers of sera containing antibody.

TABLE 3. Detection of antibody by ELISA, IIPS, IIF, and IEOP in testing 1,089 sera

Test	Results with ELISA: Positive (468)[a]	Results with ELISA: Negative (621)[a]	% Agreement between two tests
IIPS			
Positive	459	135	87
Negative	9[b]	486	($P < 0.01$)[c]
IIF			
Positive	459	142	86
Negative	9[b]	479	($P < 0.01$)[c]
IEOP			
Positive	401	34	91
Negative	67[b]	587	($P = 0.15$)[c]

[a] The value in parentheses indicates the number of sera.
[b] Nine sera were regarded as false positive by ELISA.
[c] Probability by chi-square test.

TABLE 4. Detection of antibody on 206 sera collected from pigs within 15 days after exposure to ASF virus

Test	No. positive	No. negative
IIPS	95	111
IIF	96	110
IEOP	51	155
ELISA	37[a]	169

[a] Two sera were negative in three other tests and were regarded as false positive.

more reliable solid-phase carrier and a purified antigen must be used if one is to obtain acceptable results with the ELISA test in ASF diagnosis (3, 14, 17, 18). At the same time, the cost of the test must not preclude its use in large-scale surveys.

In this regard, a recent report by Tabares et al. (15) described the preparation of a semipurified ASF viral protein (VP73) that may serve as an antigen that greatly increases the reliability of the ELISA test. Although the need for such a purified antigen in the ELISA test is obvious, it remains to be seen whether its use in large-scale surveys is economically feasible.

The conventional IEOP antigen used in the IEOP test was prepared from the PBS extract of Vero cells infected with ASF virus (12). We had experienced that certain batches of antigen or antigen exposed to refrigerator or room temperature for a prolonged period of time is apt to cause false reactions in the IEOP test. However, the false line, if it is present, is usually removed within 10 min by soaking test agarose plates in a 2% saline solution at room temperature. Therefore, the use of the conventional antigen in performing the IEOP test does not pose much of a problem. However, the same antigen was virtually useless in the ELISA test as performed here due to the occurrence of frequent false-positive reactions. Thus, we developed a new method of preparing antigen which improved the specificity, but not the sensitivity, of the ELISA test. In the IEOP test, this improved antigen has never shown false lines, which call for soaking in saline, and the precipitin lines appearing with positive sera were much sharper and stronger than those obtained with the conventional antigen. This improved antigen should be tested further for use in the IEOP test.

Although ASF virus and antisera were used in developing the IIPS test, as stated earlier, the test can no doubt be utilized for other viruses which can be adapted to and grown in cell cultures, regardless of the cytopathic or noncytopathic nature of the viruses.

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