Detection of Antibody to Pseudorabies Virus in Swine Sera by Indirect Immunoperoxidase Plaque Staining

KUNIHIKO SATO,1 YOSHIO TANAKA,1 HIROSHI KUROGI,1 SHUICHI TOKUHISA,1 KOICHI NUMBA,1 YUJI INABA,1,4 AND MINORU MATUMOTO2

National Institute of Animal Health, Tsukuba, Ibaraki,1 and Kitasato Institute, Minato-ku, Tokyo,2 Japan

Received 14 May 1987/Accepted 28 September 1987

A new method for the detection of pseudorabies antibody in swine sera was developed by use of the indirect immunoperoxidase plaque staining test. The indirect immunoperoxidase plaque staining test demonstrated serologic responses in pigs that were subcutaneously infected with pseudorabies virus. The test had a sensitivity and a specificity comparable to those of the complement-dependent neutralization test and appeared to be somewhat more sensitive than the neutralization test and the enzyme-linked immunosorbent assay. The test was simple to perform, and the results could be read with the unaided eye under ordinary light, making the test particularly suitable for the routine testing of large numbers of serum samples.

Pseudorabies is a ubiquitous virus disease of pigs, and its causative virus, suid herpesvirus type 1, is a member of the family Herpesviridae. Clinically normal swine can be carriers of pseudorabies virus and can disseminate the virus intermittently, thus becoming the source of epizootics among swine (7). Consequently, a simple, reliable test is needed to detect potential carriers of the virus, particularly among breeding stock. Cutaneous allergic tests have been used (8). Various serologic tests are now available for the detection of antibodies against pseudorabies virus. They include the enzyme-linked immunosorbent assay (ELISA) (2-5, 10), the neutralization (NT) test (4), and the complement-dependent NT (CNT) test (1).

Recently, Pan et al. (6) developed a new method of antibody detection by indirect immunoperoxidase plaque staining (IIPS) for the serodiagnosis of African swine fever. In the present study, we attempted to adapt this method for the detection of antibody against pseudorabies virus in swine sera.

MATERIALS AND METHODS

Cell cultures. The HmLu-1 cell line, derived from hamster lung, and the PK-15 cell line, derived from swine kidney, were grown in Eagle minimum essential medium containing 10% tryptose phosphate broth (Difco Laboratories, Detroit, Mich.), 10% calf serum, 100 U of penicillin per ml, 100 µg of streptomycin per ml, and 10 µg of fungizone per ml. The maintenance medium was minimum essential medium containing 10% tryptose phosphate broth, 0.05% yeast extract, 0.5% sodium glutamate, 0.1% glucose, and antibiotics as described above. Monolayer cultures were prepared in tubes (11 by 100 mm) by seeding the tubes with 1.5 × 107 cells suspended in 0.5 ml of growth medium and incubating them at 37°C for 5 days in a stationary rack. Cell cultures were also prepared in flat-bottom, 24-well plastic plates (Costar, Cambridge, Mass.) by seeding each well with 4 × 106 cells suspended in 1 ml of growth medium and incubating them at 37°C for 5 days in an atmosphere of 5% CO2 in air.

Virus. The Indiana S strain of pseudorabies virus was kindly supplied by P. Gustafson, Purdue University, West Lafayette, Ind. Virus was passaged in PK-15 or HmLu-1 cells before use in these cells.

Infectivity assay. Serial 10-fold dilutions of the virus material were made in maintenance medium and inoculated in 0.1-ml volumes in HmLu-1 or PK-15 cell cultures in tubes; three tubes were used for each dilution. After the addition of 0.5 ml of maintenance medium, the cultures were incubated at 37°C for 4 days and examined for cytopathic effect, and the 50% tissue culture infective dose (TCID50) was calculated by the method described by Kärber. The infectivity assay was also performed by plaque count in cell cultures prepared in plastic plates (Costar). Each virus dilution was inoculated in 0.1-ml volumes; three wells were used for each dilution. After the virus was adsorbed at 37°C for 60 min, the cultures were washed with maintenance medium, 1 ml of 0.8% methylcellulose was added, and the cultures were incubated in a CO2 incubator at 37°C for 3 days. The cultures were fixed with dry methanol at room temperature for 10 min. 4 ml of Giemsa stain (Merck & Co., Inc., Rahway, N.J.) diluted 50-fold with distilled water was added, and the cultures were incubated at room temperature for 30 min. The infectivity was expressed in PFU.

NT test. Serial twofold dilutions of the serum inactivated at 56°C for 30 min were made in maintenance medium, and each dilution was mixed with an equal volume of maintenance medium containing 200 TCID50 per 0.1 ml of virus. After incubation at 37°C for 60 min, the virus-serum mixtures were inoculated in 0.1-ml volumes into tube cultures of HmLu-1 cells; two tubes were used for each serum dilution. After virus was adsorbed at 37°C for 30 min, the cultures were fed 0.5 ml of maintenance medium, incubated in a roller drum at 37°C for 4 days, and examined for any cytopathic effect. The antibody titer was expressed as the reciprocal of the highest serum dilution which showed complete inhibition of cytopathic effect in at least one of the two tubes.

CNT test. Serial twofold dilutions in 0.1-ml amounts of the inactivated serum were made as described above for the NT test, and each dilution was mixed with 0.1 ml of maintenance medium containing 400 TCID50 of virus and incubated at 4°C for 24 h. At the end of the incubation, each tube received 0.1 ml of maintenance medium containing 16 hemolytic units of guinea pig complement, as determined by the method described by Takahashi et al. (9), and incubated at 37°C for 60 min. Each mixture was then inoculated in 0.1-ml volumes into two tube cultures of HmLu-1 cells. After virus was adsorbed at 37°C for 60 min, 0.5 ml of maintenance medium

* Corresponding author.
was added to each tube, and the tubes were incubated at 37°C for 4 days in a roller drum and then examined for any cytopathic effect. The antibody titer was expressed as the reciprocal of the highest serum dilution showing complete inhibition of cytopathic effect in at least one of the two tubes.

ELISA. The virus antigen for ELISA was prepared from PK-15 cells incubated at 37°C for 36 h after virus inoculation. Antigens were prepared from uninfected PK-15 cells as control antigens. Cells suspended in 50 mM Tris hydrochloride buffer (pH 7.4) were solubilized with 0.2% Nonidet P-40 and centrifuged at 80,000 × g for 60 min, and the resulting supernatant was used as the antigen.

Virus antigen was appropriately diluted with 0.05 M bicarbonate-carbonate buffer (pH 9.6) and delivered in 100-μl volumes into wells of 96-well, flat-bottom Microelisa plates (Dynatech Laboratories, Inc., Alexandria, Va.). The plates were allowed to stand at 4°C for 18 h and washed 4 times with a 0.85% NaCl solution containing 0.02% Tween 20, and 100 μl of the test serum diluted 100-fold with phosphate-buffered saline (0.15 M NaCl, 0.02 M phosphate buffer (pH 7.2)) containing 10% bovine serum was added to each well. After incubation at 37°C for 60 min, the plates were washed as described above and 100 μl of the optimal dilution of horseradish peroxidase-conjugated rabbit antibody against swine immunoglobulin G (IgG; Cooper Bio-medical) was added to each well. After incubation at 37°C for 30 min, each well was washed, 100 μl of substrate solution was added, and the plates were incubated at 25°C for 20 min in a dark room. The substrate solution was prepared by dissolving O-phenylenediamine dihydrochloride at a concentration of 0.1 mg/ml in 0.1 M citric acid–0.2 M Na2HPO4 buffer (pH 4.8) and adding 0.2 ml of 30% H2O2 per ml. At the end of the incubation, the reaction was stopped with 3 N H2SO4 and the A492 was measured. The value obtained by subtracting the absorbance of the control antigen from that of the viral antigen was taken as the ELISA value. In each series of tests a standard positive serum sample was included. ELISA values of 0.200 or higher were taken as positive, when the mean ELISA value of the standard serum was taken as 1,000.

IIPS test. For preparation of the antigen for the IIPS test, HmLu-1 cell cultures prepared in plastic plates (Costar) were inoculated with 0.1 ml of maintenance medium containing 100 PFU of pseudorabies virus. After virus was adsorbed at 37°C for 60 min, 1 ml of 0.8% methylcellulose in maintenance medium containing 0.5% bovine serum albumin was added to each culture and incubated at 37°C for 36 h. After methylcellulose was removed, the plates were washed once with chilled phosphate-buffered saline, fixed with methanol at room temperature for 10 min, and dried. The plates were sealed with tape and stored at −70°C until use.

For performance of 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. The test serum, which was diluted 100-fold with a 2% NaCl solution containing 5% bovine serum, was applied in 0.2-ml amounts to the wells of the antigen plates. The plates were left at room temperature for 30 min. After the serum was removed, the wells were rinsed 3 times with a 2% NaCl solution, filled with saline, allowed to stand for 10 min, and decanted. To the wells was added 0.2 ml of horseradish peroxidase-conjugated rabbit antibody against swine IgG (see above), the optimal concentration of which was predetermined, and the plates were incubated at room temperature for 30 min. After the conjugate was removed, the wells were rinsed 3 times with a 2% NaCl solution, filled with saline and left at room temperature for 10 min, and decanted. To the wells was added 0.5 ml of 0.02% 3,3′-diaminobenzidine (1 mg/ml) in 0.01% H2O2 saline solution, and the plates were incubated at room temperature in a dark room for 20 min. The plates were rinsed with tap water and examined for dark brown plaques.

RESULTS

In preliminary experiments, the factors that influenced the plaque formation of pseudorabies virus in HmLu-1 cell cultures in plastic plates (Costar) were investigated, and the procedure described above was established. Furthermore, factors that influenced IIPS were investigated. Swine sera showed nonspecific staining of cell sheets up to a 50-fold dilution. On the other hand, swine antisera against pseudorabies virus clearly stained plaques at a 100-fold dilution; even with antisera with a very low NT titer (1:1) and ELISA value (0.200), plaques were stained at this dilution, even though no staining was observed at a 200-fold dilution. In the following experiments the test serum was diluted 100-fold for the IIPS test.

Important factors in the IIPS test were the diluents used for dilution of the test serum and the conjugate and for washing the cell sheets after incubation with the test serum and the conjugate. We tested various diluents in various combinations and found the results to be best when a 2% NaCl solution containing 5 or 10% bovine serum was used for dilution of the test serum and the conjugate, and when a 2% NaCl solution was used for washing. With the other diluent combinations, nonspecific staining of the cell sheets obscured specific staining of plaques.

In the IIPS test thus developed, dark brown-colored plaques developed rapidly within seconds, when sera with high antibody titers were used (Fig. 1). A swine antiserum with an NT titer of 128 clearly stained plaques up to a 1,600-fold dilution. A goat antiserum with an NT titer of 1,024 against pseudorabies virus, however, did not stain plaques. IIPS tests were negative with swine antiserum with an NT titer of 5,000 against hog cholera virus and another swine antiserum with an NT titer of 2,000 against transmis-

![FIG. 1. Results of the IIPS test with positive and negative sera. The ELISA value and the NT titers of the sera used were, respectively, 0.037 and <1 (A), 0.101 and 1 (B), 0.078 and <1 (C), and 0.047 and <1 (D). Columns 1, 2, 3, 4, 5, and 6 received sera diluted 100-, 100-, 1,000-, 10,000-, 100,000-, and 1,000,000-fold, respectively. Wells in column 1 received no peroxidase-labeled antibody. The results of the plate shown here were read, from columns 1 to 6, respectively, as follows: A, −, −, −, −, −, −; B, −, −, −, +, +, −; C, −, +, +, +, −, −; D, +, −, +, +, −, −.
VOL.

Subcutaneously inoculated against all the tests 9 days, test that where farm possible gastroenteritis virus. Antigen plates ELISA and by the ELISA and the ELISA tests have summarized in by the IIPS, or CNT, and NT tests. The results were compared with that by the IIPS, CNT, and NT tests (Table 2). The degree of agreement by ELISA with that by the other tests was 92%. By the IIPS and CNT tests, 74 of 99 sera were positive, but 8 of these positive sera were negative by ELISA. By the NT test 66 sera were positive, and 4 of these sera were negative by ELISA, whereas of 66 sera positive by ELISA, 4 were negative by the NT test.

**DISCUSSION**

In this study a new method for detecting pseudorabies antibody in swine sera was developed by the use of the IIPS test.

Previously, Pan et al. (6) reported a similar method for the detection of antibody against African swine fever. In the present study we attempted to adapt this method for the detection of pseudorabies antibody in swine sera and encountered difficulties in preventing background staining. Various factors influencing the specific staining of plaques and the background staining were investigated, and a standard method was developed.

The IIPS test thus developed demonstrated serologic responses in pigs that were subcutaneously infected with pseudorabies virus, as did the ELISA and the NT and CNT tests. The NT test had a sensitivity and a specificity comparable to those of the CNT test and appeared to be somewhat more sensitive than the NT test and ELISA.

Antigen plates for the IIPS test could be stored at -70°C for 12 months or longer. The test was simple to perform, and the results could be read with the unaided eye under ordinary light, making the test particularly suitable for the routine testing of large numbers of serum samples. The test developed here could find wide application for the serodiagnosis of pseudorabies in swine.

**LITERATURE CITED**


---

**TABLE 1. Detection of antibody by the IIPS, CNT, and NT tests and by ELISA in testing 99 serum samples**

<table>
<thead>
<tr>
<th>Test and result</th>
<th>No. of results by IIPS</th>
<th>% Agreement between two tests*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Positive (n = 74)</td>
<td>Negative (n = 25)</td>
</tr>
<tr>
<td>CNT</td>
<td>Positive 74</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Negative 0</td>
<td>25</td>
</tr>
<tr>
<td>NT</td>
<td>Positive 66</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Negative 8</td>
<td>25</td>
</tr>
<tr>
<td>ELISA</td>
<td>Positive 66</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Negative 8</td>
<td>25</td>
</tr>
</tbody>
</table>

* Percent agreement is the total number of sera tested/number of sera positive or negative by both tests. Probabilities, as determined by the chi-square test, are given in parentheses.

---

**TABLE 2. Detection of antibody by ELISA and by the IIPS, CNT, and NT tests in testing 99 serum samples**

<table>
<thead>
<tr>
<th>Test and result</th>
<th>No. of results by ELISA</th>
<th>% Agreement between two tests*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Positive (n = 66)</td>
<td>Negative (n = 33)</td>
</tr>
<tr>
<td>IIPS</td>
<td>Positive 66</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>Negative 0</td>
<td>25</td>
</tr>
<tr>
<td>CNT</td>
<td>Positive 66</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>Negative 0</td>
<td>25</td>
</tr>
<tr>
<td>NT</td>
<td>Positive 62</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>Negative 4</td>
<td>29</td>
</tr>
</tbody>
</table>

* See footnote a to Table 1.