Agar-Gel Immunodiffusion Assay for Pseudorabies Virus Antibody

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Soluble antigen prepared from pseudorabies virus-infected embryonic pig kidney cells was used to demonstrate the presence of pseudorabies virus antibodies in sera taken from naturally and experimentally infected swine. Antibody could be detected by agar-gel immunodiffusion as early as 14 days postexposure and was demonstrable at least 3 months after experimental infection (the longest period tested). The reliability of the agar-gel immunodiffusion test was comparable to that of the microtitration serum-virus neutralization test commonly used in diagnostic laboratories. One advantage of the agar-gel immunodiffusion test was that severely hemolyzed and cytotoxic serum samples could be tested with confidence. The test is simple, rapid, and inexpensive and could be easily adopted by diagnostic laboratories that receive requests for pseudorabies virus antibody determinations in swine sera.

The Ouchterlonly technique (7) for identifying antigen-specific precipitin antibodies in gel has been used to demonstrate herpesvirus antibodies in sera from human patients after herpes simplex (6, 11) and varicella-zoster (10) virus infections. Estela (3) infected rabbits with infectious bovine rhinotracheitis virus (IBRV) and demonstrated that they developed antibodies that could be detected by the Ouchterlonly technique. Concentrated antigens prepared from embryonic calf kidney cells infected with IBRV were used by Charbon et al. (2) to diagnose IBRV infections in cattle by testing for gel precipitin antibodies in paired serum samples (2). These authors suggested that the test would be valuable in epidemiological investigations in cattle. The technique has been used to study the prevalence of anti-IBRV antibodies in farm animals and humans in Iran (1).

The purpose of our study was to evaluate the agar-gel immunodiffusion (AGID) technique as a diagnostic test for the presence of anti-pseudorabies virus (anti-PRV) antibody in sera of swine previously exposed to PRV.

MATERIALS AND METHODS

Antigen preparation. Soluble PRV antigen was prepared from embryonic pig kidney cells infected 24 h previously with Shope strain PRV. At this time, near-maximal cytopathic changes had developed in the cell monolayers. The infected cells were frozen and thawed twice and sonically treated (Branson Sonifier, model S-75, Branson Instruments Co., Danbury, Conn.) for 20 s to release virus from the cells. The preparation was then centrifuged at 400 x g for 30 min to remove cellular debris. The PRV titer of the supernatant fluid was 10^5.0 plaque-forming units per 1.0 ml. A 70% concentrate was prepared by filtration through an Amicon (Amicon Corp., Lexington, Mass.) X-M 50 filter. The preparation was heated for 3 h at 56°C to inactivate the virus. Antibodies were stored at 4°C or frozen at −60°C for periods up to 6 months without loss of activity.

AGID. The AGID technique was a modification of the method described by Pearson et al. (8) for use in testing horse serum for the presence of antibodies to equine infectious anemia virus. Modifications included the use of borate buffer prepared by dissolving 6.2 g of boric acid, 9.5 g of sodium tetraborate, and 4.4 g of sodium chloride in 1,000 ml of distilled water to prepare a stock buffer (pH 8.6). Five parts of stock buffer were then mixed with 95 parts of 0.85% sodium chloride and used to prepare a 1.0% agarose gel (Biomedical Division of Marine Colloids, Inc., Rockland, Me.). Merthiolate was added (final concentration, 1:20,000) to inhibit microbial growth. Seven milliliters of a 45°C borate-buffered 1% agarose gel was then placed on plastic petri plates (60 x 15 mm; Falcon Plastics, Oxnard, Calif.). Six-mm wells were cut around an 8-mm center well, leaving 4.0 mm of agar between all wells. A 100-μl amount of antigen was placed in the center well, and 100 μl of each test serum, including a known positive control, was placed in the peripheral wells. Plates were then placed in a closed, moisturized container and incubated at room temperature for 24 or 48 h. Precipitin lines were visible after 24 h but became more distinct by 48 h of incubation. Final recording of results was made at 48 h.

Serum-virus neutralization (SVN) test. The microtitration technique described by Snyder et al. (9)
was used to test diagnostic sera for PRV-neutralizing antibodies. The Shope strain of PRV was used as the test virus. Sera were diluted 1:4 in Earle balanced salt solution and mixed with an equal volume of the test virus containing approximately 300 50% tissue culture infective doses per 0.025 ml in standard 96-well microtitration plates (Falcon Plastics, Oxnard, Calif.). After the mixture was incubated at 37°C for 1 h, 0.15 ml of Eagle minimum essential medium containing embryonic pig kidney cells prepared from a 1:3 split of confluent monolayers in 75-cm² flasks was added to each well. The microtitration plates were then placed in a 5% CO₂ atmosphere at 37°C. After 2 to 3 days, the cell monolayers were observed for viral cytopathic effects. Serial twofold dilutions were made of each serum that had inhibited cytopathic effects, and the test was repeated to determine end-point titers. Titers were recorded as the highest serum dilution neutralizing 100% of the virus in duplicate wells.

**Comparison of AGID and SVN.** The AGID test was first independently evaluated by testing pre-PRV-exposure and post-PRV-exposure sera drawn from five specific-pathogen-free pigs at selected intervals after intranasal exposure to Shope strain PRV. These sera, a known positive control, and specific-pathogen-free sera were used for an initial evaluation of the AGID test.

Swine sera that had been submitted to the Animal and Plant Health Inspection Service diagnostic laboratory were first tested by SVN for the presence of PRV antibodies by W.C.S. and subsequently tested with the AGID test by P.C.S. without prior knowledge of the SVN results, therefore fulfilling the requirement of a double-blind test.

**RESULTS**

Known positive serum and post-PRV-exposure serum drawn at 14 and 96 days postexposure gave distinct PRV-specific lines between antigen and serum wells (Fig. 1). No evidence of precipitin lines was observed between preexposure or specific-pathogen-free sera and PRV antigen wells (Table 1).

![Fig. 1. Immunodiffusion of inactivated PRV antigen (Ag; center well) and serum from specific-pathogen-free pig (well 1), pre-PRV-exposure (well 2), and post-PRV-exposure sera taken at 7 (well 3), 14 (well 4), and 96 (well 5) days after experimental infection. Known positive control serum was in well 6.](http://jcm.asm.org/)

The results of comparative tests between the AGID and SVN tests of 30 sera are shown in Table 2. There was 100% agreement between the two test procedures for all 13 sera with an SVN titer of 1:32 or greater. An 86% agreement between the two tests existed for sera with an SVN titer of 1:16. The discrepancy involved one serum with an initial SVN titer of 1:16 that was negative on the AGID tests. When the test was repeated, the AGID result was again negative. Two sera were reported to have a titer of 1:8 by SVN, but the results were suspected to be due to nonspecific virus neutralization; these were also negative by the AGID test and by another experimental serological test (enzyme-labeled antibody test, details to be the subject of another report). All other sera with SVN titers less than 1:8 were AGID-test negative.

Two sera from severely hemolyzed blood samples were positive by the AGID test but were toxic to embryonic pig kidney cells when tested by the SVN test.

**DISCUSSION**

Yosupov and Gizatullin (4, 12) used antigens prepared from spleens of PRV-infected pigs to conduct AGID tests for PRV diagnosis, but re-

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* Postexposure to PRV. –, No precipitin lines; +, precipitin lines formed by 48 h; ND, not determined.

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<th>Table 2. Double-blind comparison of AGID test and microtitration SVN test of selected swine serum submitted for PRV antibody determinations</th>
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* Number of sera tested by SVN test.
* Reciprocal of serum dilution preventing cytopathic effect in cells.
* Number of sera positive by AGID test.
* Toxic: no test.
ported that the test was inferior to the immuno-osmophoresis technique used for rapid diagnosis of PRV infection in pigs. Kaminjolo and Gicho (5) reported comparative results between SVN and AGID tests of sera from IBRV-infected cattle which seem to indicate that AGID tests are more sensitive than SVN tests.

Our results indicate that AGID tests for PRV-antibody determination in swine serum may be equal in sensitivity to microtitration SVN tests commonly used in diagnostic laboratories in the United States. Furthermore, AGID tests may prove to be more effective on sera that are cytotoxic to tissue culture cells or are very severely hemolyzed.

We estimate that the costs of conducting AGID tests will be approximately $0.68 per serum sample when the initial cost of antigen preparation is disregarded. This compares favorably with the estimated cost of $3.00 to $5.00 per sample with the microtitration SVN test.

The AGID test is rapid and simple to conduct; these factors favor its adoption by diagnostic laboratories not equipped for cell culture work.

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LITERATURE CITED