Comparison of a Modified Counterimmunoelectrophoresis Test and a Microimmunodiffusion Test for Detection of Pseudorabies Virus Antibodies in Porcine Sera

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ABSTRACT

The correlation of a modified counterimmunoelectrophoresis (CIE) test and a microimmunodiffusion test for detecting pseudorabies virus antibodies in porcine sera was investigated, using as reference a standard virus neutralization test. The counterimmunoelectrophoresis test exhibited a sensitivity comparable to the microimmunodiffusion test but was not as sensitive as the virus neutralization test. The best feature of the modified counterimmunoelectrophoresis test is that it is a rapid test. It provides an alternative to currently used diagnostic tests for detection of pseudorabies virus antibodies in sera from field reared and experimentally reared swine exposed to pseudorabies virus.

Key words: counterimmunoelectrophoresis, microimmunodiffusion, virus neutralization, pseudorabies virus, pig, porcine serum, pseudorabies virus antibodies.

RÉSUMÉ

Cette expérience consistait à déterminer la corrélation entre une épreuve modifiée d'électrosynérèse et une microtechnique de précipitation en milieu gélifié, comme moyen de déceler les anticorps contre le virus de la pseudo-rage, dans le sérum de porcs, en se basant sur une épreuve standard de séroneutralisation. La première épreuve se révéla d'une sensibilité comparable à celle de la deuxième, sans toutefois atteindre celle de l'épreuve de séroneutralisation. Le principal avantage de l'épreuve modifiée d'électrosynérèse découle de sa rapidité d'exécution. Elle fournit une alternative aux épreuves de diagnostic utilisées couramment pour la détection des anticorps contre le virus de la pseudo-rage, dans le sérum de porcs élevés de façon conventionnelle ou spéciale et exposés au virus de la pseudo-rage.

Mots clés: électrosynérèse, microtechnique de précipitation en milieu gélifié, neutralisation du virus, virus de la pseudo-rage, porc, sérum porcin, anticorps contre le virus de la pseudo-rage.

INTRODUCTION

The high incidence of pseudorabies virus (PRV) in animals (1), especially swine (3,4,9), has prompted intensive efforts to develop serological techniques for detection of specific viral antibodies. A variety of these techniques, including the indirect solid-phase radio-immune test (5), immunodiffusion tests (1,3,8,10), the enzyme-linked immunosorbent assay (ELISA) (11,14), the immunoelectrophoretic test (16), the counterimmunoelectrophoresis (CIE) test (6,12) and the virus (serum) neutralization (VN) test (3,5,10,12) have been reported. Detection of antibody by any of these methods is taken as proof of exposure to PRV and may also indicate an asymptomatic animal with latent infection.

Comparative studies have shown that each of the microimmunodiffusion (MID), VN and ELISA tests are adequately sensitive, specific and repeatable for detecting PRV antibodies in sera collected from pig herds (3,7,10,12,13).

This investigation was undertaken to evaluate the sensitivity and specificity of the CIE test (7) with modifications and to compare the modified CIE test with the VN and MID tests for detection of PRV antibodies in sera of field reared and experimentally reared swine exposed to PRV.

MATERIALS AND METHODS

PREPARATION OF ANTIGENS

Strains of PRV tested were an avirulent live-virus vaccine (ALVV) strain (Norden Laboratories, Lincoln, Nebraska) and three virulent PRV strains (Indiana, P-2208; Iowa, S62/26; and Shope). Seed virus of each strain was grown on pig kidney cell monolayers and was concentrated about 100 times as original titers with ammonium sulfate according to the methods previously described (3). In addition, PRV was harvested as above, and then pooled and concentrated (about fiftyfold) in dialysis tubing by applying polyethylene glycol (mol wt 20 000) to the exterior of the tubing at 4° C. The concentrated virus preparations were then isoelectrically focused on granulated dextran gels (15). Control non-PRV infected porcine kidney cells were also processed by the above procedures.

ANIMALS

Thirty pigs, 3 to 3.5 months of age and seronegative to PRV (by VN test), were infected and challenged according to the schedule shown in Table I.

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These pigs were bled on days 1, 14, 28, 57 and 119 for serum samples.

Field samples of porcine serum were obtained from the Veterinary Diagnostics Laboratory, Veterinary Medical Research Institute at Iowa State University and from the National Veterinary Services Laboratories, Ames, Iowa. Each of the serum samples were filtered through a 0.45 μ filter, an aliquot was taken and coded and its identity was concealed before testing by CIE, VN and MID procedures.

VIRUS NEUTRALIZATION TEST

The microtitration VN test was performed as proposed by the Pseudorabies Diagnostic Standardization Committee of the American Association of Veterinary Laboratory Diagnosticians (5). Each series of VN tests included PRV-positive and PRV-negative (control) sera and back titration of virus. The VN titers were expressed as reciprocals of the highest final dilutions of serums that completely neutralized the test virus.

MICROIMMUNODIFFUSION TEST

The MID test, with reference antiserum, was performed as previously described (1,3). Semisolid medium contained 0.69% agarose in 0.05 M Tris buffer, pH 7.2, with 0.025% sodium azide. Wells of 4 mm in diameter and 2.5 mm apart were prepared in the semisolid medium.

COUNTERIMMUNOELECTROPHORESIS TEST

The CIE test (7) was modified as follows. Equipment used included a Millipore immunoelectrophoresis unit (Millipore Biomedica, Acton, Massachusetts) and an LKB-2117 multiphor system (LKB-Producter AB, Stockholm, Sweden). Plastic plates (83 x 94 mm; LKB-Producter AB, Brommel, Sweden) were each coated on their hydrophilic surfaces with 10 mL of 0.8% agarose (Seakem agarose, Marine Colloids, Rockland, Maine or Litex agarose, Glostrup, Denmark) in barbital-calcium lactate buffer. The buffer contained (in grams per liter) sodium barbital (1.64), barbital (0.26), calcium lactate (0.40) and sodium azide (0.40). All ingredients were dissolved in triple distilled water and adjusted to pH 8.6, at an ionic strength of 0.017.

Agarose gels on plastic plates were stored overnight at 4°C in a humid atmosphere and used between 24 and 96 hours after preparation. Rows of wells (Fig. 1), 2.5 mm in diameter, were punched out of the agarose gels at a row distance of 3.0 mm (edge to edge) from each other. Each well was filled with 4.0 μL of antigen or antibody reactant. The wells containing antigen were located on the cathodic side and wells containing antiserum were located on the anodic side of the electrophoresis chamber. The gels were attached to the buffer reservoirs of the electrophoretic units by paper wicks (Whatman No. 1) and were subjected to a constant voltage of 2.5 V/cm for 20 to 60 min at room temperature or 15°C. The gels were examined for immunoprecipitin bands (against a dark background in oblique transillumination) immediately after electrophoresis and after cooling to 4°C. Thereafter, the gels were pressed and dried and immunoprecipitin lines were stained with Coomassie brilliant

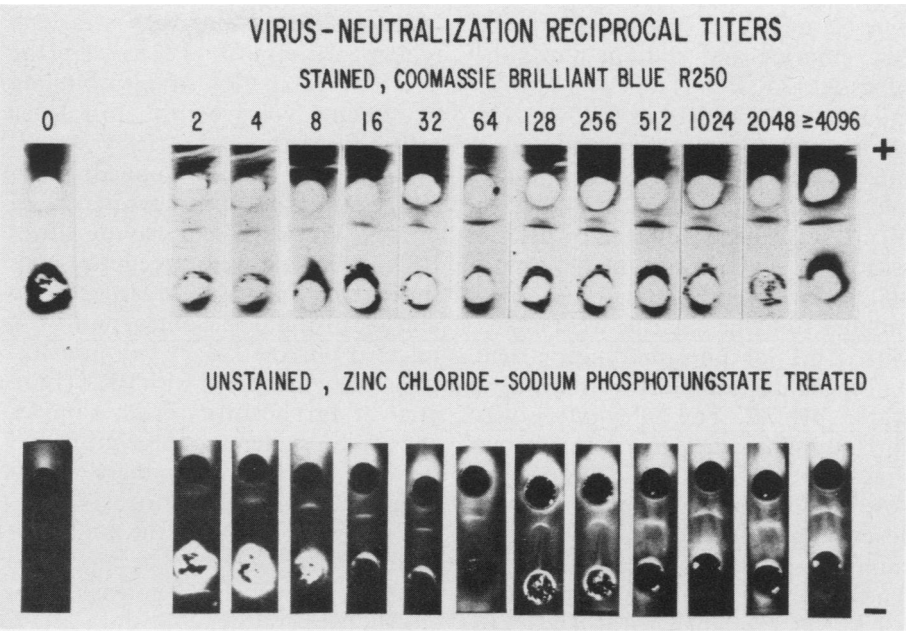

Fig. 1. Composite photograph illustrating the determination of type specificity of pseudorabies viral antigens exposed to various sources of porcine serum antibody. Viral antigens (strains: Shope or ALVV = avirulent live-virus vaccine) were placed in the lower wells and antiserum was placed in the upper wells as indicated. + = positive charge; – = negative charge.

TABLE I. Pseudorabies Virus (PRV) Vaccination and Challenge Treatment Schedule of Animals from the National Animal Disease Center Swine Herd[a]

Group	Number of animals	Route of vaccination	Day interval vaccination given	Type of PRV vaccine used[b]	Day pigs were intranasally challenged with virulent PRV[c]
1	10	Intramuscular	1 and 24	Avirulent live	Not challenged
2	10	Intramuscular	1 and 24	Avirulent live	57
3	10	Intramuscular	1 and 24	Killed	57
Control	10	Not vaccinated	—	—	Not challenged

[a]Animals prior to the above treatments were 3 to 3.5 months of age and seronegative to PRV by the VN test
[b]Avirulent live: PRV strain (Norden Laboratories, Lincoln, Nebraska); 1.0 mL/animal/vaccination
Killed: PRV strain (Salsbury Laboratories, Charles City, Iowa); 2.0 mL/animal/vaccination
[c]Indiana strain: 10^4 40% tissue culture infective doses in 1.0 mL/animal

blue (2) or reacted with a mixture of zinc chloride and sodium phosphotungstate (ZP; Fig. 1) solution. The ZP mixture was prepared as follows: zinc chloride (40.0 g) was dissolved in distilled water and made up to 100 mL and phosphotungstic acid (4.0 g in 50 mL water) was adjusted to pH 7.6 with 1 N NaOH and made up to 100 mL with additional water. Two milliliters of zinc chloride were mixed with 2 mL of phosphotungstic acid and made up to 100 mL with distilled water, pH 7.2. The ZP mixture was filtered (Whatman No. 1) to remove precipitates and was used to flood agarose gels. The ZP solution usually reacted and enhanced visualization of immunoprecipitin bands within 5 min. Thereafter, the gels were washed with 0.1 M NaCl and then distilled water to remove the excess ZP solution. Immunoprecipitin bands appeared as distinct opaque lines between antigen-oriented and antibody-oriented wells as shown in Figure 1.

Test sera were applied undiluted. Antigens were applied after checkerboard titrations to determine their optimum dilution for MID and CIE testings. Total antigen binding was determined by incubating equal volumes of the optimal antigen dilution and the test serum dilution for 60 min at 37°C, then dispensing the antigen-serum mixtures into cathodic wells of the gel and subjecting them to electrophoresis for 45 min as above. A PRV-hyperimmune porcine serum was then added to the corresponding anodic wells of the gel and subjected to electrophoresis for 60 min as above. All serum samples were tested against PRV antigen and noninfected porcine kidney cell antigen.

RESULTS

The viral antigens (ALVV, Indiana, Iowa and Shope), regardless of method of concentration, reacted equally with serum PRV antibodies. When titrated and tested with MID and CIE procedures, the PRV antigens diluted 1:16 reacted optimally with 1:8 dilutions of serum containing PRV antibodies. When PRV antigen and positive PRV sera (by VN test) were tested by CIE in a common cathodic well, then reacted with anodically applied positive PRV sera with reciprocal titers of 1: $\leqslant$ 8 (based on the VN test), total PRV antigen binding occurred. No precipitation lines ocurred with positive PRV serum samples (cathodically applied) at an antigen titer of 1:128 or greater based on the VN test. Sera having lower PRV titers showed precipitin lines indicating incomplete antigen to antibody reactions. Control non-PRV infected porcine kidney cell preparations when incubated with PRV (Indiana strain) positive serum samples showed no evidence of precipitin lines by MID or CIE tests. In these experiments, both electrophoresis units worked well. However, the multiphor system was preferred because gels could be subjected to a constant controlled temperature.

A composite of serum samples repeatedly tested by VN and CIE procedures is presented in Figure 1. Samples that were positive by the VN test (reciprocal titers ranging from 2 to $\geqslant$ 4096) were proven to react by CIE. The intensity of the opaque precipitin bands, appearing between wells of the positive antigen and positive sera, was greater with the higher titered PRV antibodies ($\geqslant$ 32 by VN). However, the CIE test proved capable, though inconsistently, of detecting a VN reciprocal titer of 2. Control sera that were negative by the VN test were negative by the CIE test.

The Coomassie brilliant blue R250 and the ZP procedures for enhancing the detection of precipitin bands were capable of detecting positive samples with reciprocal VN titers ranging from 2 to $\geqslant$ 4096 with the CIE test. Some of these samples showed an additional precipitin band at about 0.5 mm distance from the antibody well. Similarly, when these samples were run by MID procedures, additional precipitin bands were also observed (3). By either CIE or MID procedures, test serum samples incubated with non-PRV material in cell culture preparations were negative. No precipitin bands were observed.

Serum samples submitted for PRV antibody determinations with identities concealed were tested by VN, CIE and MID procedures (Table II). There was 100% agreement between precipitin reactions as tested by CIE and MID procedures. Based on the VN test, there were false-negative reactions disclosed by the CIE and MID tests in sera with lower VN titers: 19 of 32 (1:4), 3 of 6 (1:8) and 1 of 6 (1:16). The VN test appears more sensitive than either the CIE or the MID test, for the VN test detected more PRV-positive serum samples at the 1:4 dilution on both field and vaccinated animals. In samples with VN titers of 1:32 and higher, there was 100% agreement between the VN, CIE and MID tests.

Serum samples from swine exposed in the field or experimentally to PRV with known processing and storage records were analyzed by VN, CIE, and MID tests (Table III). In most cases, there was agreement between the tests except for samples with VN titers of 1: $\leqslant$ 4, for which false negatives occurred in field and PRV-vaccinated swine samples. In consideration of all samples (Tables II and III and 266 other samples [14]) repeatedly tested, correlation coefficients were calculated as 0.93 for VN and CIE tests and 0.87 for VN and MID test results. No precipitin bands were obtained in CIE and MID tests with control negative serums and PRV

TABLE II. Correlations of Virus Neutralization (VN), Counterimmunoelectrophoresis (CIE) and Microimmunodiffusion (MID) Tests for Detection of PRV Antibodies in Selected Sera of Swine

Reciprocal VN titer	Total number of serum samples tested	Results[a]	
		CIE+/MID+	CIE-/MID-
negative	10	0	10
4	32	13	19
8	6	3	3
16	6	5	1
32	4	4	0
64	6	6	0
128	6	6	0

[a]CIE+/MID+, positive reactions indicating the presence of PRV antibodies
CIE-/MID-, negative reactions indicating the absence of PRV antibodies

TABLE III. Correlations of VN, CIE and MID Tests for Detection of Serum PRV Antibodies in Field and Experimental Swine[a]

Range of reciprocal VN titers	Field		PRV-vaccinated[b]		PRV-vaccinated and challenged[c]	
	CIE+/MID+	CIE-/MID-	CIE+/MID+	CIE-/MID-	CIE+/MID+	CIE-/MID-
⩽4	32	28	7	10	0	0
8-16	18	0	4	0	0	0
32-64	11	0	4	0	0	0
128-256	6	0	1	0	2	0
512-2048	14	0	0	0	14	0
⩾4,096	4	0	0	0	4	0

[a]CIE+/MID+, positive reaction indicating presence of PRV antibodies; CIE-MID-, negative reaction indicating absence of PRV antibodies. All samples were tested by the VN test to determine the ranges of reciprocal titers

[b,c]Pigs were PRV seronegative on day 1; other values of samples taken on days 14, 28, 57 and 119 were run with identities concealed. Serum samples were run in duplicate over two storage periods with two PRV antigens. Duplicate samples were in agreement

antigen. All serum samples found negative by the VN test were also negative by CIE and MID tests (specificity 100%).

DISCUSSION

The CIE test was modified for the serodiagnosis of PRV in swine. The precipitation reaction was specific inasmuch as a line of identity appeared between positive PRV antigen and a serum sample containing PRV antibodies with a reciprocal VN titer ranging from 2 to ⩾ 4096. In samples with VN titers of 1:128 or higher, an additional precipitin band occurred similar to that observed by the MID test and as previously described (3). However, this apparently is not a problem, for all tests VN, CIE and MID were in agreement that the serums contained PRV antibodies. The extraneous precipitin band was not avoided by propagating the PRV strains for antigen production in a porcine kidney cell system supplemented with porcine serum as had been suggested (3). The band is not likely due to cross-reacting antibodies to antigens of other viruses, based on evidence with cytomegalovirus serum (7), but may be due to antibody to additional antigens or PRV itself. In any case, it does not affect the value of the CIE test.

In comparing the CIE and MID tests to the VN test, sensitivities were comparable, but false negatives were a problem in sera with low VN titers. However, factors that adversely influence the VN test, such as contamination or cytotoxicity, do not affect the results of the CIE nor the MID tests (3,7). Results are independent of the presence of living virus, which means that CIE, like the MID test, can be used with specimens inactivated by acetylethyleneimine (3). In positive samples, precipitation reactions occur sooner (1 h or less for the CIE test; 12 to 34 h for the MID test) than the development of cytopathic effects in cell culture (VN test).

The CIE test, as reported, and the MID test, as previously described (3) and tested, do not allow for the identification of nonspecific precipitation lines that may develop. Our findings support previous suggestions (7) that the CIE test can be used for screening large numbers of sera suspected to have PRV antibodies. The CIE test combines specificity, sensitivity and simplicity comparable to the MID test for surveillance of PRV in pigs. However, it is not as sensitive, qualitatively, as the VN test. Also, it is not useful as a quantitating test for detecting PRV antibody in serum samples, unlike the VN and ELISA tests (14). The positive feature of the CIE is time. It is rapid and equivalent to the MID test in sensitivity. It provides an alternative to currently used diagnostic tests for detection of PRV antibodies in sera of swine.

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REFERENCES

1. **CRANDALL RA, GUTEKUNST DE, KANITZ CL, McADARAGH JP, SEAWRIGHT GL, SNYDER ML, SOLORZANO RF, STEWART WC, HILL HT.** Standard procedures for microimmunodiffusion test for detecting pseudorabies viral antibody. Am Assoc Vet Lab Diagnost 1978; 21: 53-56.
2. **CROWLE AJ, CLINE LJ.** An improved stain for immunodiffusion tests. J Immunol Methods 1977; 17: 379-381.
3. **GUTEKUNST DE, PIRTLE EC, MENGELING WL.** Development and evaluation of a microimmunodiffusion test for detection of antibodies to pseudorabies virus in swine serum. Am J Vet Res 1978; 39: 207-210.
4. **GUSTAFSON DP.** Pseudorabies. In: Diseases of swine, 5th ed. Ames: Iowa State University Press, 1981: 209-223.
5. **HILL HT, CRANDELL RA, KANITZ CL, McADARAGH JP, SEAWRIGHT GL, SOLORZANO RF, STEWART WC.** Recommended minimum standards for diagnostic tests employed in the diagnosis of pseudorabies (Aujesky's disease). Am Assoc Vet Lab Diagnost 1977; 20: 375-390.
6. **KELLING CL, STAUDINGER WL, RHODES MB.** Indirect solid-phase microradioimmunoassay for detection of pseudorabies virus antibody in swine sera. Am J Vet Res 1978; 39: 1955-1957.
7. **PAPP-VID G, DULAC GC.** Pseudorabies: Adaptation of the countercurrent immunoelectrophoresis for the detection of antibodies in porcine serum. Can J Comp Med 1979; 43: 119-124.
8. **PFEIFFER NE, SCHIPPER IA.** An immunodiffusion test for detection of antibody to pseudorabies virus. Am Assoc Vet Lab Diagnost 1977; 20: 33-46.
9. **PIRTLE EC.** Pseudorabies virus antibodies in swine slaughtered in Iowa. Can J Comp Med 1982; 46: 128-129.
10. **SMITH PC, STEWART WC.** Agar gel immunodiffusion assay for pseudorabies virus antibody. J Clin Microbiol 1978; 7: 423-425.
11. **SNYDER ML, STEWART WC.** Application of an enzyme labeled antibody test in pseudorabies. Am Assoc Vet Lab Diagnost 1977; 20: 17-32.
12. **STEWART WC, SWANSON MR, SNYDER ML, KRESSE JI.** A comparison of three serologic techniques for the detection of pseudorabies antibodies. Am Assoc Vet Lab Diagnost 1978; 21: 43-52.
13. **WILLIAMS PP, CORIA MF, PIRTLE**

EC. Counterimmunoelectrophoresis in the diagnosis of pseudorabies in pigs. Abst Ann Meet Am Soc Microbiol 1981; 81: 290.

14. **WILLIAMS PP, CUE DR, PIRTLE EC.** Detection of pseudorabies virus antibodies in porcine colostrum and serum by the enzyme-linked immunosorbent assay (ELISA). Proc 3rd Intern Symp Vet Lab Diagnost 1983; 1: 249-254.
15. **WILLIAMS PP, PIRTLE EC, CORIA MF.** Isoelectric focusing of infectious particles of porcine pseudorabies virus strains in granulated dextran gels. Can J Comp Med 1982; 46: 65-69.
16. **YUSAPOV RK.** Primenenie immunoosmofereza diya laboratornoi diagnostiki bolenzni Aujeskzky. Uch Zap Kazan Vet Inst 1972; 112: 151-153.