Development and Evaluation of an Indirect Enzyme Immunoassay for Detection of Porcine Antibodies to Pseudorabies Virus

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ABSTRACT

An indirect enzyme immunoassay is described for detection of porcine serum antibody to pseudorabies virus. The analytical sensitivity of the enzyme immunoassay was found to be approximately 4.5 log 4 x 10 (5120 times) greater than the serum neutralization test, based on parallel end point titrations. The diagnostic sensitivity of the enzyme immunoassay was comparable or superior to that of the serum neutralization, based on the earliest detectable antibody after infection of swine with pseudorabies virus by intranasal or intrauterine routes or by contact with infected pigs. The enzyme immunoassay, at a screening dilution of 1:20, gave 100% agreement with ELISA results provided with a U.S. Department of Agriculture-Animal and Plant Health Inspection Service proficiency panel of 40 sera. One serum having demonstrable antibody by the enzyme immunoassay was seronegative by the serum neutralization test.

Key words: Enzyme immunoassay, pseudorabies virus, Aujeszky's disease, porcine, serum neutralization.

RÉSUMÉ

Les autres rapportent la mise au point d'une épreuve immuno-enzymatique indirecte, pour la détection des anticorps sériques porcins contre le virus de la pseudo-rage. Des titrages parallèles révélèrent que la sensibilité analytique de cette épreuve s'avérait environ 4,5 log 4 x 10, i.e. 5120 fois, supérieure à celle de l'épreuve de la séroneutralisation. Par ailleurs, sa sensibilité diagnostique se révéla équivalente ou supérieure à celle de l'épreuve de la séroneutralisation, parce qu'elle permit de déceler plus tôt des anticorps sériques, chez des porcs soumis à une infection intranasale, intrautérine ou horizontale. L'épreuve sérologique de 40 échantillons de référence du ministère de l'agriculture des États-Unis, à la dilution de dépistage 1:20, à l'aide de cette épreuve immuno-enzymatique indirecte, donna des résultats identiques à ceux de l'épreuve ELISA. Un seul des échantillons de référence s'avéra positif par l'épreuve immuno-enzymatique indirecte, mais négatif par celle de la séroneutralisation.

Mots clés: épreuve immuno-enzymatique, virus de la pseudo-rage, maladie d'Aujeszky, porcs, séroneutralisation.

INTRODUCTION

Since 1977 pseudorabies (PR) in swine has been designated by Agriculture Canada as a reportable disease in Canada. Consequently, the demand for serological surveillance has increased steadily. Historically, the serum neutralization (SN) test has been and remains the accepted method for detection of porcine antibodies to pseudorabies virus (PRV) (1). However, the inherent complexity of this test and increasing operating costs have stimulated efforts to develop alternative assays (2,3). Enzyme immunoassay (EIA) techniques have proven to be highly sensitive and versatile tools in viral serology and such techniques have been applied to the detection of porcine antibody to PRV (4-9) and in PR control programs in the United Kingdom (10) and some parts of the United States (Carbrey EA, USDA-APHIS, Ames, Iowa, personal communication).

The increase in the prevalence of PR in the United States (11,12) has increased the potential risk of an introduction of PR in otherwise disease-free Canadian swine herds (1). Therefore, the development and evaluation of an indirect EIA for intensified serological surveillance was undertaken.

This communication describes an EIA protocol for the detection of porcine antibody to PRV and compares its performance to that of the SN test currently in use.

MATERIALS AND METHODS

VIRUS AND CELL CULTURES

Monolayer cultures of Vero (African green monkey kidney) cells in plastic roller bottles, of 490 cm^2 surface area, were grown in Eagle's minimum essential medium (MEM), pH 7.2 containing penicillin (200 unit/mL), streptomycin (200 μg/mL), 1% nonessential amino acids (Gibco Laboratories, Grand Island, New York 14072, U.S.A.) and 10% fetal bovine serum (FBS). Following a wash with 0.05 M phosphate buffered saline (PBS), pH 7.4, each bottle was inoculated with 10 mL of PRV suspension diluted 1:10 in PBS to contain $10^{6.5}$ $TCID_{50}$/mL, giving a multiplicity of infection (MOI) of

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approximately 3. The virus, isolated originally as a field strain, was obtained from the Central Veterinary Laboratory (CVL), Weybridge, England. After an adsorption period of 60 min at 37°C, the cultures were maintained in Earle's MEM without FBS, incubated at 37°C and rolled at 0.5 rpm. When an 80 to 90% cytopathic effect (CPE) developed, approximately 36 hours after inoculation, the cultures were harvested by twice freezing at -70°C and thawing at 37°C.

PRV ANTIGEN PREPARATION

The harvested virus suspension was centrifuged at 1800 *g* for 30 min at 4°C, and the supernatant fluid was concentrated 15-fold with an Amicon filter (Amicon Corporation, Lexington, Massachusetts 02173, U.S.A.) using a 50,000 Dalton retention membrane. The cell pellet was resuspended in 10 mL of concentrated supernate, sonicated with three bursts of 30 second duration with an ultrasonic disrupter (Branson Ultrasonic Corporation Canada, 705 Progress Ave., Scarborough, Ontario M1H 2X1) and then pooled with the remaining concentrated virus suspension. An equal volume of 0.005 M Tris, 0.01 M glycine buffer, pH 8.9 containing 1% Triton X 100 (Sigma Chemical Co., P.O. Box 14508, Saint-Louis, Missouri 63178, U.S.A.) was added to the virus suspension which was stirred continuously for 24 hours at 4°C. This solubilized virus preparation was centrifuged at 100,000 *g* for 120 min at 4°C in an IEC ultracentrifuge (International Equipment Co., 1234 Soldiers Field Road, Boston, Massachusetts, U.S.A.; fixed-angle rotor A-320). Three separate layers resulted; the top and bottom layers contained lipoprotein and cellular debris, respectively and were discarded. The middle layer which contained most of the PRV antigen, as demonstrated by gel immunodiffusion (1) was collected, dispensed in aliquots and stored at -70°C. This stock PRV antigen (15.3 mg total protein/mL) and a stock of control antigen (16.0 mg total protein/mL) which was prepared in the same way from uninfected Vero cells were used throughout this study.

SERUM SAMPLES

Sera from serial bleedings of two groups of pigs experimentally infected with PRV (ATCC strain VR-135, American Type Culture, 12301 Parklawn Drive, Rockville, Maryland 20852, U.S.A.) were studied. In the first group, two pigs (P34 and P36) each had received 1000 $TCID_{50}$ of PRV intranasally and a third pig (P2) was kept in contact with them. The details of this infection experiment, together with the SN antibody results, have been described elsewhere (13). In the second group, three sows (P133, P134, P138) were infected intrauterinely by implanting pig embryos which had been exposed *in vitro* to PRV stock derived from the CLV strain. Another two sows (P131, P135) were maintained as noncontact controls, all five animals were part of an embryo transfer experiment (F.C. Thomas, unpublished data). However, no preinoculation sera were available from the above five animals. Forty porcine sera comprising a proficiency panel (set No. 14) for PRV-ELISA standardization in the United States, kindly provided by Dr. Carbrey of the USDA-APHIS, Ames, Iowa were also included in this study.

EIA PROCEDURE

The EIA was based on a previously described method (14). Flat bottom, 96 well, polystyrene microtiter plates (Linbro, Flow Laboratories, 1760 Meyerside Drive, Mississauga, Ontario L5T 1A3) were optimally coated by passive adsorption of 100 μL/well of PRV stock antigen diluted 1:500 in 0.05 M carbonate buffer, pH 9.6, for 18 hours at 4°C. The wells were emptied and washed three times with PBS, pH 7.2 containing 0.05% Tween 20 (PBST). Inactivated sera (56°C/30 minutes) were tested at a single dilution of 1:20 for screening or serially diluted (fourfold) from a starting lowest dilution of 1:40 for end point titrations. All dilutions were made in PBST and 100 μL samples were applied in duplicate in a diagonal sample placement pattern (14,15) and incubated for 90 minutes at 25°C. The wells were again emptied and washed and the wells were filled with 100 μL of a 1:2000 dilution of horseradish peroxidase-labelled rabbit antiswine IgG (H+L) (Lot #S-186, Miles Laboratory Ltd., 77 Belfield Road, Rexdale, Ontario M9W 1G6) in PBST. After incubation for 60 minutes at 25°C, the conjugate solution was discarded and the plates were washed with PBS without Tween. The wells were filled with 100 μL of substrate solution, 1.0 mM ABTS [2,2-azino-di(3-ethylbenzothiazoline-6-sulfonic acid] - 4 mM H_2O_2 in 50 mM citrate buffer, pH 5.0 and the plates then were shaken continuously at 25°C for precisely 20 minutes. The optical density (OD) values of the enzymatic reaction products were then recorded at 414 nm, using Titertek Multiskan MC microtiter plate reader (Flow Laboratories, 1760 Meyerside Drive, Mississauga, Ontario L5T 1A3), interfaced with an Apple II plus microcomputer. By means of a custom computer program OD values were expressed as the mean OD value resulting from the reaction with PRV antigen minus the mean OD value resulting from the reaction with the control antigen for each individual plate. Samples were considered negative if their OD values, as calculated above, were less than that of a 1:20 dilution of negative control serum (P50), obtained from a specific pathogen free (SPF) pig at ten weeks of age. Enzyme immunoassay titers were expressed as that dilution of sera which resulted in an OD value equal to or less than that given by a 1:40 dilution of the negative control serum (P50).

SERUM NEUTRALIZATION TEST

The SN tests were performed as previously described (13).

RESULTS

The profiles of EIA and SN antibody titers in the sera from the two intranasally infected pigs (P34 and P36) and the single contact pig (P2) are shown in Figure 1. No PRV antibody was found in sera collected prior to infection. Antibody was demonstrated by both the EIA and SN tests in the sera from the intranasally infected pigs at one week postinfection. Both EIA and SN titers rose exponentially during the first two to four weeks postinfection before becoming relatively stable at elevated levels for the duration of the 40 week experiment. In serum from the contact pig, antibody was demonstrable by

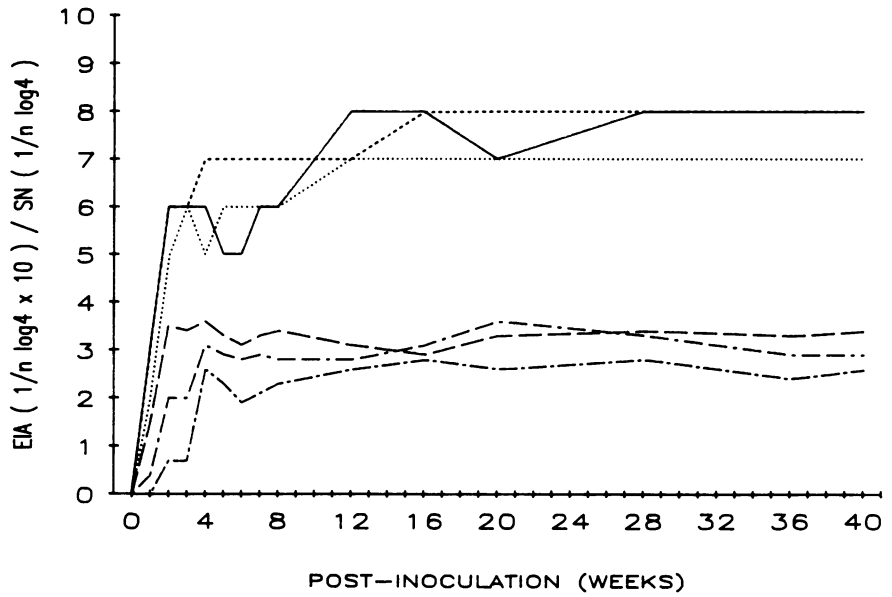

Fig. 1. Comparative development of PRV antibody titers measured by EIA (_____ P34, — — — — P36, ... P2) and SN (___ ___ P34, . ___ ___ P36, ___ . ___ P2) in sera of pigs intranasally infected (P34, P36) and exposed to infected pigs (P2).

EIA but not by SN at one week postexposure; however, at two weeks postexposure antibody was demonstrable by both methods. In general, antibody in the sera of all three pigs was demonstrated with greater sensitivity and increased in titer more rapidly when assayed by EIA rather than by SN. The EIA titers were approximately 4.5 log 4 x 10 (5120 times) greater than SN titers once peak antibody activity was observed.

The profiles of EIA and SN antibody titers in the sera from the three intrauterinely infected sows are shown in Fig. 2. At ten days postinfection, sera from two sows (P133 and P134) had substantial antibody activity by EIA. Two were negative by SN, and the third sow (P138) had only a weak positive SN reaction (1:2). Similar to the previous experiment, EIA titers rose to levels approximately 4.5 log 4 x 10 (5120 times) greater than SN titers. Sera from the two control sows remained negative by both EIA and SN tests throughout the 60 day experimental period.

The EIA, at a screening dilution of 1:20 gave 100% agreement with the positive/negative ELISA results provided with the 40 sera in the USDA-APHIS proficiency panel. Furthermore, regression of EIA titers (expressed as 100% end points) on SN titers (expressed as 50% end points for 39 of these 40 sera indicated a general positive correlation ($R = 0.884$) between these tests (Fig. 3). Serum #40 of the panel had no detectable neutralizing antibody (SN $< 1:2$) yet had an EIA titer of 1:2560 and was not included in the regression analysis.

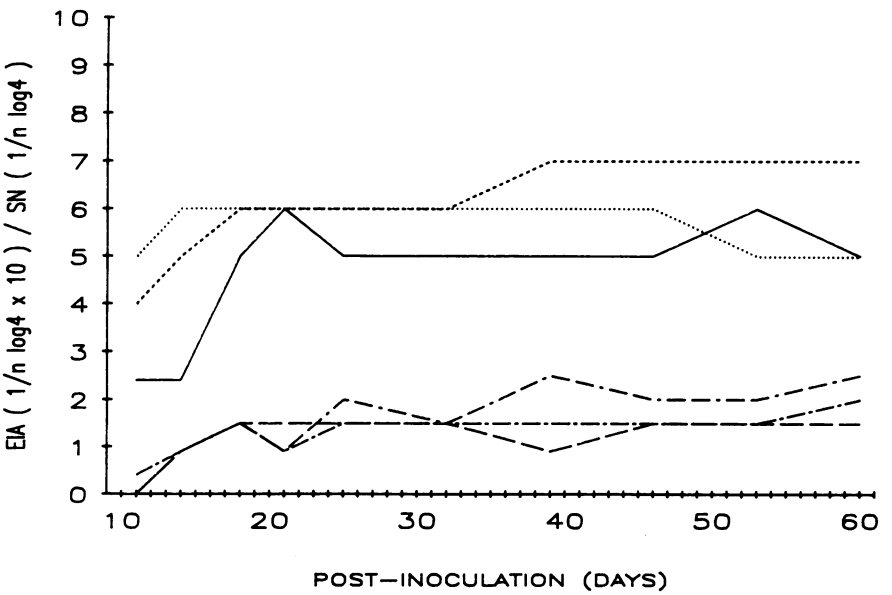

Fig. 2. Comparative development of PRV antibody titers measured by the EIA (_____ P133, — — — — P134, ... P138) and SN (___ ___ P133, . ___ ___ P134, ___ . ___ P138) in sera of sows infected by the intrauterine routes.

DISCUSSION

The SN test, despite its widespread application for detection of antibody to PRV, has a number of inherent disadvantages. It requires the maintenance and use of live viruses and tissue culture cell lines. The test must be performed under aseptic conditions and requires strict biocontainment procedures in Canada where PR is absent (1). Some sera cannot be assayed due to their cytotoxic effect. The test requires at least three to five days for completion and does not lend itself to large scale application or automation. The indirect EIA is free of these disadvantages.

Both intact PRV particles (5-7,9-16) and PRV glycoprotein antigens (16,17) have been incorporated into indirect EIA protocols. However, the relative efficiencies of these various antigen preparations have yet to be compared. In the present study, a Vero cell line, rather than a porcine line, was chosen for PR viral propagation and FBS was eliminated from the maintenance medium. This was done to reduce the potential risk of reactions

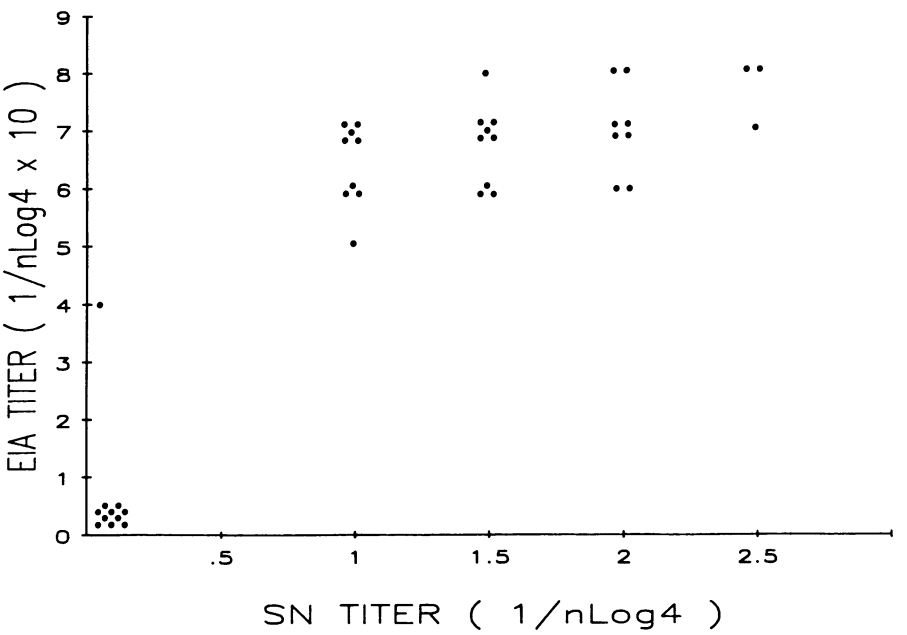

Fig. 3. Comparison of EIA and SN titers for 40 reference sera from USDA-APHIS.

occurring in the EIA due to the presence of porcine or cross-reacting bovine antigens in the PRV glycoprotein antigen preparation. As a further precaution mean OD values were corrected for nonspecific background activity by subtracting the mean OD value of the control antigen reaction from the mean OD value of the viral antigen reaction for each serum tested.

A commercial horseradish peroxidase conjugated rabbit antiporcine IgG was chosen for this study. Previous studies (14) have demonstrated that rabbit antisera, prepared against intact IgG, will contain a substantial amount of both antiheavy chain and antilight chain activity. As IgG may not be the predominant antibody class present early in infection, an antiglobulin with antilight chain may be beneficial as it will also allow for the detection of other classes such as IgM or IgA.

In previous studies (6,9,18) antibodies were demonstrable by EIA but not by SN in the sera of pigs, five to seven days after intranasal infection with PRV. In other experiments (8,19) antibody was not detected by EIA until ten days postintranasal infection. Our study indicates that antibody can be detected by both EIA and by SN in the sera of pigs seven days after intranasal infection. However, antibody was detectable by EIA alone in sera from two of three sows, ten days after receiving PRV-infected embryos by intrauterine route. As well, serum antibody was detectable by EIA alone within one week (five days) of exposure of a pig to others infected by intranasal inoculation. Differences between these results could be related to a number of factors such as assay conditions, individual animal responses or infection protocols. In general, all studies would suggest that the analytical sensitivity of the EIA is comparable, if not superior, to that of the SN test.

Peak antibody activity has been observed between 10 and 35 days postexperimental infection with a number of PRV strains and elevated titers have been observed to remain relatively stable for long periods of time (18). We have observed similar responses, irrespective of the route of infection and antibody titers remained elevated for the entire ten month duration of the intranasal infection experiment. Although SN antibody profiles were essentially similar, EIA titers were consistently 2.5 log 4 x 10 higher than SN titers once peak activity had been obtained. The higher relative analytical sensitivity of the EIA has been previously demonstrated in comparisons of OD values at a single test serum dilution with SN results expressed as end points (9,18). However, given the accepted method of expression of EIA and SN end point titers, as presented here and elsewhere (8), the superior analytical sensitivity of the EIA is readily evident.

Serum screening dilutions ranging from 1:10 to 1:30 have been reported in the literature (5,6,9,19). We have found that a dilution of 1:20 suitably minimizes the nonspecific background activity of antibody negative sera in the EIA. This dilution also serves as a convenient base for serial twofold dilutions in end point titrations. At this dilution, one of the 40 proficiency panel sera from USDA-APHIS was EIA positive while being SN negative at a dilution of 1:2. When titrated by EIA, this particular serum demonstrated a weak to moderate end point (1:2560). This would suggest that the antibody in question was not of a neutralizing isotype.

Before the EIA can be accepted into routine use, it must first be field tested on a larger number of samples to determine its diagnostic specificity and sensitivity. In a PR-free country, such as Canada, diagnostic specificity is a very critical factor in surveillance. On the other hand, in countries where eradication programs are required, enhanced diagnostic sensitivity is often more important. One of the major advantages of the EIA is that the appropriate seropositive/negative threshold can be chosen to optimize the diagnostic requirements of the individual surveillance, control or eradication program. An extensive field survey has been undertaken to evaluate the diagnostic sensitivity and specificity performance of the EIA protocol.

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