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Evaluation of a blocking ELISA using a urease conjugate for the detection of antibodies to pseudorabies virus

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Abstract. A blocking enzyme-linked immunosorbent assay (ELISA) using a urease conjugate (U-B-ELISA) was evaluated for screening sera for antibodies to pseudorabies virus under field conditions. A total of 764 serum samples were analyzed by U-B-ELISA. Of these, 264 were evaluated by both virus neutralization and U-B-ELISA, and the results were compared. U-B-ELISA showed 98.5% and 98.9% sensitivity and specificity, respectively. This test combines the sensitivity and specificity of the blocking ELISA format while allowing visual assessment of results.

Pseudorabies (Aujeszky's disease) virus (PRV) is a very important pathogen for the swine industry worldwide, and eradication programs for PRV are in progress in many countries. Most if not all of PRV eradication programs are based on the use of differential vaccines and appropriate serodiagnostic techniques. In Argentina, the National Animal Health Service established in 1996 a PRV control program based on serologic detection of infected animals with interdiction of vaccine usage. In accordance with the National PRV control program, the seropositive animals are segregated and/or sent to slaughter. Two serologic techniques are accepted as official by Argentina's animal health authorities: the latex agglutination test[4] and the nondifferential enzyme-linked immunosorbent assay (ELISA).[8] The kits for these 2 different tests are imported from other countries, which results in a significant additional surcharge for the performance of PRV serology by diagnostic laboratories in Argentina. Likewise, there are few laboratories in the country that can run the commercially available PRV ELISAs because ELISA plate readers are not readily available. Therefore, the development of serodiagnostic techniques that are reliable, easy to perform, and require minimal equipment are of fundamental importance for the control and eradication of PRV in developing countries such as Argentina. The ELISA is probably the most widely used test for screening of PRV exposure in pig populations. In 1977, indirect ELISAs (I-ELISAs) for detection of antibodies to PRV were developed in the USA.[8] Since then, a number of reports on the application of ELISA as a serodiagnostic test for PRV have been published.[1,2,10] Most of the ELISA methods use alkaline phosphatase (AP) or horseradish peroxidase (HRPO) antibody conjugates. The evaluation of the results of these tests usually requires the use of an ELISA plate reader. If a urease conjugate is used instead, results can be obtained visually without using specialized equipment. The urea-containing substrate causes a pH shift, which in turn is visualized by the use of bromocresol purple as a pH indicator. In the present study, a blocking ELISA using a urease conjugate (U-B-ELISA) was evaluated for screening sera for antibodies to PRV under field conditions. This test combines the sensitivity and specificity of the blocking ELISA format while allowing visual assessment of results.

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Two groups of serum samples were used throughout this study. Five hundred porcine serum samples were obtained from an epizootic that occurred in the province of Buenos Aires in 1990 and subsequently were stored at −20 C until use.[5] These samples were used for the HRPO blocking ELISA (HRPO-B-ELISA) and the U-B-ELISA but were not suitable for use in the virus neutralization (VN) test. Two hundred sixty-four serum samples were obtained from different breeding farms at various locations in Santa Fe and Buenos Aires provinces between 1995 and 1997 and were analyzed by VN test and U-B-ELISA. Reference negative and positive anti-PRV sera were used in each case.

Rabbit anti-PRV hyperimmune gamma globulin was prepared by immunizing rabbits with potassium tartrate-purified PRV ($10^{6.5}$ $TCID_{50}$) which had been inactivated by ultraviolet light and mixed with Freund's complete adjuvant. The immunization schedule consisted of 4 subcutaneous applications at intervals of 2–3 weeks. Six days after the last immunization, the rabbits were euthanized and serum was collected and precipitated with saturated ammonium sulfate. The precipitate was resuspended in distilled water and extensively dialyzed against phosphate-buffered saline.

For the VN test, all serum samples were heat treated at 56 C for 30 minutes before titration. Antibodies to PRV were detected by adding 100 $TCID_{50}$/0.025 ml of virus to 2-fold dilutions of serum in microtiter plates. Each sample was tested in duplicate. Following incubation for 1 hour at 37 C, RK13 cells were added to each well. The neutralizing titer was expressed as the reciprocal value of the highest serum dilution that neutralized 100 $TCID_{50}$ of virus.[2] An endpoint titer of $\geq$1:4 was considered positive.

PK15 cells were grown in Eagle's minimum essential medium[a] supplemented with 10% fetal calf serum, 0.3 mg/ml glutamine, 200 IU/ml penicillin, and 0.5 mg/ml streptomycin. The local PRV CL15 strain[5] was propagated in the PK15 cell cultures. The virus culture was harvested when 90% of the cells exhibited cytopathic effect, and the culture was clarified by centrifugation. Nonidet P-40[b] at a concentration of 1% in Tris ethylenediaminetetraacetic acid (EDTA)-saline buffer was used to lyse and solubilize the pellet of PRV-infected cells. These lysates were used as the source of antigen for the U-B-ELISA.[6]

To standardize the U-B-ELISA, 0.1 ml of the predetermined antigen dilution (1:1,000) in carbonate/bicarbonate buffer (0.05 M, pH 9.6) was used in all microtiter wells. After incubation at 4 C overnight in a humid chamber, 4-

fold dilutions of pig sera to be tested were added to the antigen-coated wells in duplicate and incubated overnight at room temperature. Subsequently, rabbit anti-PRV serum diluted 1:1,000 was employed as secondary antibody to detect antigen sites that had not been blocked by the primary sera through incubation for 30 minutes at 37 C. Anti-rabbit urease conjugate[b] diluted 1:1,000 was used as a third antibody and incubated for 60 minutes at 37 C. In all cases, phosphate buffer containing 0.05% Tween 20 and 0.1% bovine serum albumin (BSA) was used as the diluent. The washing solution (WS) was similar to the diluent but did not include BSA. After each step of incubation, the plates were washed 3 times with WS and 3 additional times with distilled water before the addition of substrate. The substrate containing 0.1% urea, 0.008% bromocresol purple,[b] and 0.2 mM EDTA was added to each well. The plates were sealed and incubated at room temperature for 30–60 minutes until the negative control serum developed a purple color without noticeable change in the positive control, which remained yellow.

As expected for the blocking test format, absence of antibodies in the pig serum samples resulted in the binding of the secondary antibody (rabbit anti-PRV serum). The absorbance values were measured in a microplate spectrophotometer.[c] Optimal dilutions of the components of the system were determined by checkerboard titration, to identify the concentration of each reagent that would provide the best discrimination between the positive and negative reference sera. Additionally, various blocking substances (to adsorb nonspecific antibodies from the rabbit anti-PRV serum), i.e., skimmed milk, BSA, and egg albumin, were tested as dilution buffers. In each plate, 2 wells (to which only anti-rabbit conjugate was added) were used for control antigen, 2 wells were positive controls, 2 wells were negative controls, and 2 wells were controls for the overall detection system (antigen + rabbit anti-PRV + anti-rabbit conjugate). The reproducibility of the results obtained by this test was determined by using 2 negative and 2 positive sera and by agreement of the positive and negative control sera in 10 different repetitions. To perform the HRPO-B-ELISA, an ELISA kit was used.[d] The steps are similar to those described for U-B-ELISA except for the use of peroxidase as the enzyme with H_2O_2 as substrate and 3′3′5′5′-tetramethyl benzidine as chromogen. The reaction was stopped by adding 1 M H_2SO_4, and the results were read on a microplate spectrophotometer.[c]

A total of 764 serum samples were analyzed by U-B-ELISA (Table 1). Of these, 264 sera were studied by both VN and U-B-ELISA, and the results of the 2 tests were compared. Using the chi-square method, the agreement between the serologic techniques was significant: $\chi^2 = 249$, $P < 0.001$. U-B-ELISA showed 98.5% sensitivity and 98.9% specificity (Table 1). Absorbance values for the urease reaction were determined by spectrophotometer using a 450-nm filter. Samples were considered positive if the OD value was at least 3 SD above the mean of 70 negative serum samples for both VN and U-B-ELISA (0.593). The choice of the optimal concentration of antigen used for coating onto the wells was based on obtaining maximal absorbance readings for PRV-positive control samples and minimal background for PRV-negative control samples. Rabbit anti-PRV antibodies are added to bind to the specific sites on the antigen free from pig's antibodies. Weak positive sera develop a greenish color. Development of a purple color indicates unequivocally negative sera. Using repeated measures analysis of variance, the reproducibility of the test was acceptable ($F = 2.38$) for the sera analyzed on 10 occasions.[7]

Table 1. Comparison of results of urease blocking ELISA (U-B-ELISA), virus neutralization (VN), and peroxidase blocking ELISA (HRPO-B-ELISA) for pseudorabies virus obtained with 764 field serum samples.

Test	VN		HRPO-B-ELISA	
	+	−	+	−
U-B-ELISA +	68	2	112	13*
U-B-ELISA −	1	193	0	375
Total	69	195	112	388
Relative sensitivity (%)	98.5		100	
Relative specificity (%)	98.9		96	

* Sera tested by ELISAs but unfit for VN testing.

This study demonstrates the efficacy of an alternative urease conjugate that permits the visual evaluation of the endpoint of the PRV blocking ELISA. The use of the regular peroxidase enzyme for ELISAs has some disadvantages, e.g., HRPO conjugates can lose activity if they become contaminated with microorganisms. This problem cannot be overcome by the use of bacteriostatic agents because HRPO activity is very sensitive to antibacterial agents such as methanol and sodium azide.[11] In addition, the substances used as substrates are frequently unstable. The use of an ELISA plate reader is always necessary. Further, both HRPO and AP are present in many mammalian tissues, which limits their application in assays in which whole cells are used as substrate.[3] These problems can be overcome by using urease as the enzyme label. The urease substrate is stable in aqueous solution at ambient temperature, and the enzymatic reaction can be stopped by the addition of a small quantity of thiomersal. Incubation at room temperature for 24 hours was preferred because evaporation at 37 C causes drying, especially at the wells along the edge of the plate. A visual format using urease as the enzyme marker was adapted for this blocking-ELISA; the nonreactive sera developed a definite purple color. When the U-B-ELISA was compared with the HRPO-B-ELISA, there was no evidence that 1 enzyme was superior to the other for increasing the sensitivity or the speed of the assay.[6] The endpoint as determined by the urease conjugates may appear more clear-cut than that obtained with other enzyme conjugates.[3] Experience does indicate that the urease conjugates provides more clear-cut endpoints than do other enzymes. HRPO and AP conjugates give a gradual color development, which makes accurate assessment of titration endpoint difficult. This difficulty does not occur with the urease conjugate.

The use of urease conjugates in ELISAs offers the advantages of convenience and safety. However, use of urease conjugates may result in false-negative reactions upon prolonged incubation when wells containing substrate solution absorb ammonia liberated from an adjacent reactive well. Also, the urease test system is extremely sensitive to pH changes and thus demands special care during the perfor-

mance of the test. This system was highly sensitive when the substrate solution was used at pH 4–4.2. A close correlation between the results obtained with the blocking ELISA and with the VN test has been reported.[9] In this comparison, close overall correlations between VN and the U-B-ELISA (261/264 samples = 98.1%) and between the HRPO-B-ELISA and the U-B-ELISA (487/500 samples = 97.4%) were obtained.

The HRPO-B-ELISA has been used in this laboratory for several years with satisfactory results. However, because of the implementation of a national plan for control of pseudorabies in Argentina, it has become necessary to transfer the testing to less well-equipped laboratories located throughout the swine-producing region of the country. The overall unavailability of ELISA plate readers in those small-scale laboratories precludes the use of the conventional HRPO-B-ELISA. The use of the U-B-ELISA offers a solution to this problem. The U-B-ELISA was sensitive, specific, precise, rapid, and easily automated. The test can be easily transferred for use in routine serodiagnosis in those conditions where access to an ELISA plate reader is limited, as it often occurs in developing countries.

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Sources and manufacturers

a. Nissui, Tokyo, Japan.
b. Sigma Chemical Co., St. Louis, MO.
c. Titertek Multiskan Plus, EFLAB, Finland.
d. Blocking ELISA for detection of antibodies against pseudorabies virus, the Joint FAO/International Atomic Energy Agency Division, Seibersdorf, Austria.

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