DEVELOPMENT OF AN INDIRECT AND A DOT ELISA TO DETECT ANTIBODIES TO PORCINE PSEUDORABIES VIRUS IN ARGENTINA

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Abstract

DEVELOPMENT OF AN INDIRECT AND A DOT ELISA TO DETECT ANTIBODIES TO PORCINE PSEUDORABIES VIRUS IN ARGENTINA.

Indirect and dot enzyme linked immunosorbent assays (ELISAs) were developed to detect antibodies to pseudorabies virus (PRV) in serum samples of 321 pigs. The Argentine CL 15 PRV strain was used as the source of virus. Nonidet P-40-solubilized infected and mock infected cell lysates were used as antigens. The results of these tests were compared with those of a blocking ELISA and virus neutralization (VN) test. There was a 100% agreement between the indirect and dot-ELISAs. Using VN as standard, the blocking ELISA and the indirect ELISA showed 97.6% and 100% relative sensitivity and 96.9% and 99% relative specificity respectively. The sensitivity of the blocking ELISA was lower than that of the other tests.

1. INTRODUCTION

Pseudorabies (PRV), also known as Aujeszky's disease, is a disease of pigs caused by a herpesvirus (suid herpes virus type 1). The disease causes economic losses to the swine industry and, as with other herpesviruses, the pseudorabies virus (PRV) frequently establishes latent infections, and thus, when a herd becomes infected, the virus remains among the adult pigs. A control programme for PRV requires the identification and removal of infected animals within a herd. For this purpose, several serodiagnostic tests are available. The virus neutralization (VN) test is the most frequently used test, although the enzyme linked immunosorbent assay (ELISA) is now being used more frequently for the serological diagnosis of PRV.

In Argentina, several outbreaks of pseudorabies have been described and confirmed by virus isolation [1-4]. Using the VN test and the blocking ELISA (B-ELISA) kit provided by the Joint FAO/IAEA Division, a large serological survey was carried out [6] and a comparison of the results was made with an indirect (I) and a and a dot ELISA and to compare the results with the VN and B-ELISA.

2. MATERIALS AND METHODS

2.1. Pseudorabies virus antigen

Madin-Darby Bovine Kidney (MDBK) cells were grown in Eagle's MEM supplemented with 10% foetal calf serum, 10% tryptose phosphate broth (Difco, Detroit, USA) and antibiotics. The PRV CL 15

strain isolated in Argentina was used to infect the cells. After virus infection, the cells were maintained in the same medium without serum. Nonidet P-40 (NP-40) at a concentration of 1% in TEN buffer was used to solubilized infected and mock infected cells. These lysates were used as antigen in the I-ELISA and dot ELISA [5].

2.2. Sera

Three hundred and twenty one sera collected from pig herds were inactivated at 56°C. These sera which had been previously tested by VN (6) were used in all three ELISAs. Positive, weak positive and negative reference sera included in the FAO/IAEA B-ELISA kit were used as controls.

2.3. Indirect ELISA (I-ELISA)

ELISA 96-well plates (Nunc-Maxisorb, Denmark) were coated with 100 μl antigen per well diluted in coating buffer (50 mM Na_2C0_3, 50mM $NaHC0_3$, pH 9.6). The working dilution was 1:600 as calculated from chessboard titration. This represented approximately 0.008 mg protein per well. The protein concentration was calculated reading the samples at 280 nm in a UV spectrophotometer, and by reference to BSA standards under the same conditions. After incubation in a humid chamber at 4°C for 24 hrs, the antigen solution was discarded and replaced by 100 μl blocking solution (0.2% bovine serum albumin in PBS) and incubated for 1 hour at 37°C and then the wells washed 3 times with PBS containing 0.05% Tween 20 (washing solution, WS).

The sera to be tested in duplicate (50 μl/well) were diluted 1:30 in dilution buffer (PBS with 0.5% Tween 20 and 0.5% skimmed milk powder, DB), and incubated overnight at 4°C. The working dilution was calculated using 6 known sera, 3 positive and 3 negative. Following 3 consecutive washes, 50 μl of a 1:3000 dilution (as calculated from chess board titration) of HRP-labelled rabbit anti-swine IgG (Cappel, USA) in DB were added per well, and plates were incubated for 60 minutes at room temperature. Finally, after 5 washes, 100 μl of substrate solution (0.1 M citric acid, 0.2 M PO_4HNa_2, 0.3 mM ABTS, 0.01% H_20_2) was added and incubated in the dark for 30 minutes at room temperature.

The optical density (OD) values were then recorded at 405 nm using a Titertek Multiskan Microtiter Plate reader (Flow Laboratories, UK). Sera that reacted equally to or higher than the weak positive standard serum supplied with the FAO/IAEA B-ELISA kit were considered as positive. The results were calculated with reference to the standard negative serum of the B-ELISA kit. The positive samples were compared with the results obtained using the mock control antigen. A sample was considered positive if the ratio of absorbance with PRV and mock-infected cell lysates antigens was greater than a OD of 1.8 [7-9].

2.4. Dot ELISA

The antigens, buffers and solutions used in this test were the same as for the I-ELISA. Nitrocellulose (NC) paper (Bio-Rad, USA) was cut into individual strips of about 0.5 x 2cm, labelled using a suitable marker, washed in distilled water and then air dried before use. In preliminary experiments, strips

were placed in individual wells of a microtiter plate previously filled with a 1:300 dilution of either PRV or mock antigen. After incubation in a humid chamber for 24 hrs at 4°C, the antigen solution was removed using a pipette, blocking solution was added and incubated at 37°C for 1 hour. The strips were then washed 3 times and 50 μl of a 1:30 dilution of serum was added. Plates were then incubated overnight at 4°C in a humid chamber. Strips were then removed from the plates and washed 3 times in WS.

Rabbit anti-swine IgG-HRP conjugate diluted 1:5000 was added to cover the strips and incubated at room temperature for 1 hour. After 2 additional washes in PBS and a final one in TBS (20mM tris, 0.5M NaCl, HCl, pH 7.5), the strips were immersed in a freshly prepared solution of 3.3 Diamino-benzidine tethrahydroxychloride (DAB) (0.009g of DAB to 30ml TBS containing 0.009ml H_2O_2), and then incubated for approximately 3 minutes at room temperature until colour became visible. The enzymatic reaction was stopped with distilled water [11]. A variation of this method was also performed as described by Bennett and Yeoman [10]. The NC paper was cut to fit the top of a 96 well plate and immersed in the antigen solutions. After 24 hours incubation at 4°C, papers were blocked, washed and then air dried. Serum samples were placed in duplicate in a 96-well plates. The plate was covered with the antigen coated NC paper, then with parafilm, and finally with 3 sheets of filter paper. The lid of the plate was clamped down using 4 paper clamps. The plate was inverted, so that the sample came in contact with the coated NC paper, and placed at 4°C overnight. The assay was then developed as described above. The strip assay was assessed using 96 sera including control antisera supplied with the kit. The dot ELISA was evaluated using the 321 sera described before.

2.5. Virus neutralisation (VN) test and blocking (B) ELISA

The VN test has been described elsewhere [6]. The B-ELISA was performed as described in the manual supplied with the FAO/IAEA ELISA kit. The ELISA kit used in the present study, had been stored at the customs for a prolonged period.

3. RESULTS AND DISCUSSION

The VN test and B-ELISA were used for detection of PRV antibodies in swine sera. We also developed an indirect and a dot ELISA using a PRV strain isolated in Argentina as antigen. The procedures followed for the antigen preparation and the ELISAs were basically those described by Toma and Eloit and Bennett and Yeoman respectively [5,10]. A total of 321 porcine sera (field sampled) were tested by the methods described. In our hands the B-ELISA performed with the kit showed a relative sensitivity of 97.6% and relative specificity of 96.9% (Table I). The I-ELISA showed a relative sensitivity of 100% and a relative specificity of 99% (Table II).

In the I-ELISA, the test sera were diluted 1:30. A maximum colour was obtained at this dilution. At further dilutions, a gradual decrease in colour was observed. To examine negative serum populations and for establishing OD limits, we used 40 negative and 40 positive sera in duplicate. There were small difference between duplicates of the same sample. The mean OD value of negative population was 0.4, and

TABLE I. SENSITIVITY AND SPECIFICITY OF B-ELISA COMPARED WITH THOSE OF THE VN TEST

	VN test		
	reactive	non-reactive	total
Reactive	123	6	129
Non-reactive	3	189	192
Total	126	195	321

TABLE II. SENSITIVITY AND SPECIFICITY OF I-ELISA COMPARED WITH THOSE OF THE VN TEST

	VN test		
	reactive	non-reactive	total
Reactive	126	2	128
Non-reactive	0	193	193
Total	126	195	321

the same value was obtained for the sera collected from pigs herds, the mean OD value was 0.7. The weak positive OD value from the kit was 0.75. The field sera showed similar OD values to the strong reference positive sera (1.2).

The sensitivity of B-ELISA was lower than the other tests performed. The B-ELISA kit was inadvertently stored for 3 months in the customs and this may have affected its performance, although the components were lyophilized. The PRV and mock antigens were used in parallel in the I-ELISA. Seven sera which showed similar OD values on both positive and negative antigens were considered as negative. These were also found negative by the other methods. In order to fully verify these results it will be necessary to check all positive sera using the mock antigen. The seven sera which gave high OD values on both negative and positive antigens in the I-ELISA showed no colour in the dot ELISA, with either antigens. This cannot be easily explained. The background for negative sera was very low and no false positives were detected using mock antigen. There were some practical problems in performing the dot ELISA using NC strips since they were difficult to manipulate. However, a limited number of results were obtained and they are shown in Fig. 1. The results were read visually and sera were considered positive by comparing the

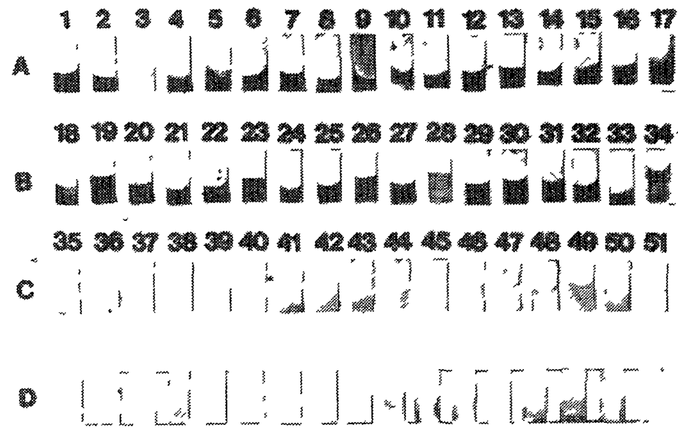

Fig. 1. Dot-Immunoassay for PRV antibodies detection on Nitrocelullose paper using individual strips. Rows A, B and C: PRV antigen coated to paper. Row D: mock-infected cell lysates antigen coated to paper. Strips 1, 2 and 3: positive, weak positive and negative reference sera respectively. Positive reaction (strips 4 through 34); negative reaction, row C.

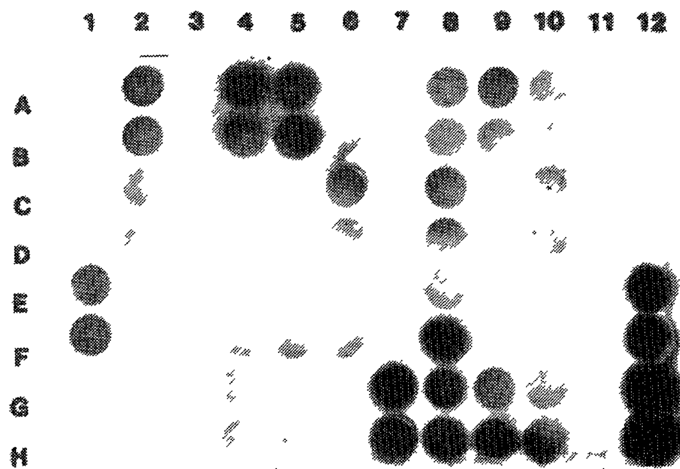

Fig. 2. Dot-Immunoassay for PRV antibodies detection on Nitrocelullose paper using whole sheet. PRV antigen coated to paper.

intensity of colour development in strips to that obtained with the weak positive control sera. Strips showing a more intense coloration than that obtained with the weak positive control sera were considered positive. Fig. 2 shows the results of a dot ELISA. The advantages of dot ELISA of lower background, the provision of a permanent record and its availability to field laboratories is offset by the difficulties in manipulation of the strips themselves and, at present, full validation of this assay.

Further studies using the I-ELISA with larger number of serum samples are in progress.

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