

Optimization and Standardization of an Enzyme-Linked Immunosorbent Assay Protocol for Serodiagnosis of *Actinobacillus pleuropneumoniae* Serotype 5

YVON-LOUIS TROTTIER,¹ PETER F. WRIGHT,² AND SERGE LARIVIÈRE^{1*}

Groupe de Recherches sur les Maladies Infectieuses du Porc, Faculté de Médecine Vétérinaire, Université de Montréal, C.P. 5000, Saint Hyacinthe, Quebec, Canada J2S 7C6,¹ and Animal Production Unit, Agency's Laboratory, Seibersdorf, International Atomic Energy Agency, A-2444 Seibersdorf, Austria²

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An indirect enzyme-linked immunosorbent assay protocol has been optimized with special emphasis given to assay standardization and quality control. Technical aspects such as choice of a microplate, antigen immobilization, buffer composition, optimal screening dilution of sera, and kinetics of the enzymatic reaction were studied and evaluated in order to design a standard protocol offering maximal analytical sensitivity and specificity, as well as to obtain minimal within- and between-plate variability. Among the 27 plates tested, the Nunc 475-094 and 269-620 immunoplates were found to be the best in terms of high positive-to-negative ratio and low variability. No significant differences in antigen immobilization were found by using buffers of various compositions or pHs; however, the presence of magnesium ions (Mg^{2+} ; 0.02 M) resulted in a twofold increase in nonspecific background. An optimal screening dilution of sera was established at 1:200. A 1-h incubation period for test serum was found to be optimal. Maximum enzymatic activity for peroxidase was obtained by adjusting both substrate (H_2O_2) and hydrogen donor [2,2'-azinobis(3-ethylbenz-thiazoline sulfonic acid)] concentrations to 4 and 1 mM, respectively. To control between-plate variability, a timing protocol was adopted. Within-plate variability was also controlled by using a sample placement configuration pattern. Sliding scales were determined by repeated testing of a cross section of samples to set acceptance limits for both within- and between-plate variability. These limits were used in a quality control program to monitor assay performance. The results obtained suggest that this standardized protocol might be useful in the serodiagnosis of *Actinobacillus pleuropneumoniae* serotype 5.

The gram-negative bacterium *Actinobacillus pleuropneumoniae* is the causative agent of swine pleuropneumonia, a contagious disease characterized by a severe, often fatal, hemorrhagic necrotizing pneumonia in acutely infected pigs (21). Although pigs of all ages may be affected, pigs of about 3 months of age are the most vulnerable (31). The disease may be acute, subacute, or chronic (18, 21, 31). Often, pigs that survive acute infection develop chronic lung lesions and become carriers (21, 30, 31). The introduction of a subclinical chronic carrier into a nonimmune herd or stress on chronic carriers may produce an outbreak of the disease (23, 29, 31). Serodetection of these clinically unapparent carriers is therefore crucial. On the basis of capsular antigens, 12 serotypes of *A. pleuropneumoniae* have been recognized so far (25). Serotype 5 is divided into subtypes 5a and 5b (24).

In the interest of controlling the disease, a wide variety of serological tests have been developed to detect antibodies against *A. pleuropneumoniae* in exposed pigs. In many laboratories the complement fixation (CF) test is still used as the main reference test (11, 16, 19). However, the procomplementary and anticomplementary activities of swine serum (20) and the lack of diagnostic sensitivity of the CF test (26) are major drawbacks associated with this method. Also, the use of whole-cell or crude extracts of antigen reduce the specificity, since somatic antigens of different serotypes of *A. pleuropneumoniae* cross-react among themselves and other gram-negative bacteria (6, 7, 11, 17, 27). Finally, the CF test is a cumbersome and time-consuming technique

which cannot be used to process large numbers of diagnostic samples (35).

To circumvent these difficulties, several enzyme-linked immunosorbent assay (ELISA) and a few radioimmunoassay techniques have been developed during the last decade (7, 10, 12, 13, 20, 33, 37). The high sensitivity of the radioimmunoassay results in the detection of antibodies to cross-reacting antigens, unless serum samples are highly diluted (12). The ELISA seems to offer better performance than the radioimmunoassay in the detection of antibodies to *Haemophilus influenzae* type b capsular polysaccharide (15). In the serodiagnosis of *A. pleuropneumoniae* serotype 5, the ELISA also offers a better sensitivity than other serological tests (10, 20, 37). However, analytical specificity in the indirect ELISA is achieved through proper antigen and/or conjugate selection.

Two problem areas are encountered in ELISA for the serodiagnosis of *A. pleuropneumoniae* serotype 5. First, there is some controversy among scientists about the nature and choice of a serotype 5-specific antigen (35). Serotype 5-specific antigens have been reported to be associated with capsular polysaccharides (3, 4, 7, 11, 13), smooth lipopolysaccharides (S-LPS) (1, 7, 27, 28), and outer membrane proteins (28). Second, variability associated with each step of the ELISA protocol is often compounded, resulting in problems associated with assay interpretation. Therefore, particular emphasis should be placed on protocol optimization and standardization in order to minimize technical error which may affect estimates of diagnostic sensitivity and specificity.

The first objective of the present work was to review each

* Corresponding author.

parameter involved in the indirect ELISA protocol used at the Faculté de Médecine Vétérinaire de St-Hyacinthe (10). To achieve maximal assay performance, the protocol was modified in accordance with the technique used in the detection of anti-*Brucella abortus* antibodies, as described previously (22, 38-40). The second objective was to define limits of assay variability in order to design a quality control program to monitor assay performance in the serodiagnosis of *A. pleuropneumoniae* serotype 5.

MATERIALS AND METHODS

CF test. All sera were tested in a modified CF test adapted from methods of the Centers for Disease Control (U.S. Department of Health and Human Services, Atlanta, Ga.) as described previously by J. Nicolet (19). Briefly, the test was performed in microplates. Swine sera were inactivated at 60°C for 30 min, fresh calf serum was added (modifying factor) to inactivated sera to a final concentration of 1%, and a whole-cell suspension from a 6-h culture on enriched pleuropneumoniae-like organism (PPLO) agar was used as the antigen.

Reference control sera. The first serum control, referred to as the target control (TGT), was a pool of six high-titered sera (CF test titer, 1:256) obtained from pigs experimentally and field infected with *A. pleuropneumoniae* serotype 5. This serum pool resulted in an optical density (OD) of approximately 1.00 when assayed repeatedly in the ELISA. Obtained from similar groups of pigs, a pool of 12 sera with a CF test titer of 1:32 was used as an intermediate positive control, referred to as QC1. A pool of pig sera obtained from a herd with minimal disease was used as a negative control, QC2. Finally, the phosphate-buffered saline (PBS)-Tween solution used to dilute the test sera was used as a fourth control, BC (or buffer control). These controls were used to standardize and monitor the performance of the assay, as described previously (22, 38, 39).

Antigen preparation (crude extract). The antigen for ELISA was prepared from *A. pleuropneumoniae* 81-750 (obtained from the Faculté de Médecine Vétérinaire, St-Hyacinthe, Quebec, Canada) serotype 5 (subtype 5b), as described previously (10, 27). To summarize, the strain was grown for 6 h on enriched PPLO agar plates, and cells were harvested in PBS containing 0.5% of a 37% (wt/wt) formaldehyde solution. This cell suspension was allowed to stand overnight at 4°C. The density was adjusted spectrophotometrically, and the suspension was then boiled for 1 h. The supernatant was collected, filtered, and lyophilized or stored at -70°C in aliquots of 250 µl. The lyophilized antigen was reconstituted to its original volume with ASTM (American Society for Testing and Materials) grade I pyrogen-free water before use.

Antigen titration. The optimal antigen dilution was determined by using a checkerboard titration in which twofold dilutions of antigen (100 µl per well) were titrated against twofold dilutions of the four reference control sera. The optimal antigen dilution chosen was that which demonstrated a maximum positive-to-negative (P/N) ratio between positive and negative serum controls.

Antigen immobilization. Optimal binding conditions for antigen absorption were determined by using the following aqueous solutions: A, 0.15 M NaCl; B, 0.02 M MgCl₂; C, 0.15 M NaCl plus 0.01 M MgCl₂; D, 0.02 M PBS (pH 7.2); E, 0.02 M PBS (pH 7.2) with 0.03 M NaCl; F, 0.1 M carbonate buffer (pH 9.6); G, 0.01 M carbonate buffer with 0.15 M NaCl; H, 0.05 M citrate buffer (pH 4.00); and I, 20 mM Tris

TABLE 1. Evaluation of microplates

Company	Type	Catalog no.	P/N (OD ₄₁₄)	% CV ^a	In-index ^b
Nunc	Polysorp	475-094	7.28	3.33	2.19
Nunc	ELISA	269-620	5.70	3.20	1.78
Corning	25860	S-70114-17	1.18	0.87	1.36
Dynatech	Microtiter	14-245-145	6.97	5.47	1.27
Nunc	Immuno II-F	442-404	3.70	3.08	1.20
Falcon	3915	087725A	5.79	5.57	1.04
Nunc	Immuno I-F	439-454	2.51	2.45	1.02
Flow	EIA plates	76-381-04	5.10	5.58	0.91
Flow	Multiwell	76-301-05	5.61	6.37	0.88
Flow	Cell culture	76-231-05	4.90	5.59	0.88
Bio-Rad	Costar	224-0096	3.04	3.75	0.81
Falcon	Mk III	3072	1.17	1.80	0.65
Falcon	Mk III	3070	1.17	2.03	0.58
Flow	New design	76-0-05	1.21	2.11	0.57
Falcon	Mk II	3040	1.21	2.66	0.45
Beckmann	ELISA	373-660	2.30	5.37	0.43
Flow	ELISA	76-331-05	5.16	12.19	0.42
Flow	Cell culture	76-003-05	1.24	3.12	0.38
Nunc	Nunclon	167-008	1.20	3.12	0.38
Dynatech U bottom ^c	0010102201	14-245-71	4.36	13.96	0.31

^a Within-plate percent CV between seropositive quadruplicates.

^b Index was obtained by dividing P/N ratio by percent CV. Plates are classified by using this index.

^c Plate in use at the Faculté de Médecine Vétérinaire de St-Hyacinthe.

HCl buffer (pH 8.5). Plates were incubated for 18 h at 4°C with antigen at its optimal dilution. Twofold serial dilutions of the target (TGT) and negative serum (QC2) controls (from 1:200 to 1:3,200) were assayed in the ELISA for each coating solution. A graph of the P/N OD ratio was plotted versus serum control dilution.

Evaluation of microplates. Most of the 27 flat-bottom, 96-well microplates used in this study are listed in Table 1. These microplates were obtained from the following manufacturers: Nunc, Kamstrup, Denmark; Flow Laboratories, Inc., McLean, Va.; Dynatech Laboratories, Inc., Chantilly, Va.; Corning Glass Works, Corning, N.Y.; Bio-Rad Laboratories, Richmond, Calif.; Beckman Instruments, Inc., Fullerton, Calif.; Becton Dickinson Canada Inc., Mississauga, Ontario. For each type of plate, quadruplicates of the serotype 5 positive (TGT) and negative (QC2) serum controls were assayed three times. The mean P/N ratio and the mean within-plate percent coefficient of variation (CV) were calculated for each type of plate. The plates were compared by using an index obtained by dividing the P/N ratio by the percent CV.

Washing procedures. Between each ELISA step, plates were washed five times with a microplate washer (catalog no. 78-470-03; Flow Laboratories) as described previously (22).

Screening dilution of test samples. Four seropositive and four seronegative field sera were serially diluted twofold from 1:12.5 to 1:25,600. The optimal screening dilution for test samples was determined by plotting mean OD values versus serum dilution. The serum dilution which gave the maximum P/N ratio was selected as the optimal screening dilution. All sera were diluted in 0.02 M PBS (pH 7.30) containing 0.05% Tween 20 (Sigma P1379) and 0.30 M NaCl (PBS-Tween 20) and applied at 100 µl per well. The use of 0.30 M NaCl in the diluent PBS was suggested by Tijssen et al. (34) to achieve higher immunological specificity.

Serum incubation period. Four positive and four negative sera were incubated with antigen for periods of 15, 30, 60,

120, and 180 min. The optimal incubation period was determined by monitoring the P/N ratio.

Conjugate titration. Conjugate titration was performed by adapting the procedure described by Nielsen and Wright (22). A checkerboard titration was performed with Immulon I plates (Dynatech Laboratories). Porcine immunoglobulin G (IgG) (catalog no. I-4381; Sigma) at concentrations of 2,000, 1,000, 500, 250, 125, 62.5, 31.25, and 0 ng/ml in PBS (eight rows) was adsorbed at 100 μ l per well for 18 h at 4°C. The plate was washed, and 16 replicates of horseradish peroxidase-conjugated anti-porcine IgG (heavy and light chain) (lot F-286; ICN ImmunoBiologicals, Lisle, Ill.) in twofold dilutions from 1:100 to 1:3,200 in PBS-Tween 20 were added (100 μ l per well) in two adjacent columns; the plate was incubated for 15 min at room temperature. The plate was washed five times, and substrate solution [4 mM H₂O₂ and 1 mM 2,2'-azinobis(3-ethylbenz-thiazoline sulfonic acid) (ABTS) in 0.05 M citrate buffer, pH 5.00] was added to each of the 96 wells at 200 μ l per well. The plate was immediately transferred to a plate shaker (catalog no. 76-475-00; Flow Laboratories), and after exactly 10 min of constant shaking, the color development was measured (OD at 414 nm) with a Multiskan MCC plate spectrophotometer (catalog no. 76-620-00; Flow Laboratories). The mean ODs of replicates were calculated and plotted for each immunoglobulin concentration on a graph showing the mean OD versus the reciprocal of the conjugate dilution on a log scale. The optimal conjugate dilution was determined as that dilution which gave an OD of 1.00 at the maximum porcine IgG concentration which did not appear to oversaturate the wells. To evaluate the optimal conjugate incubation period, an optimal dilution of conjugate was assayed with positive (TGT) and negative (QC2) serum controls for 15, 30, and 60 min. The P/N ratio was evaluated for each incubation time.

Enzyme kinetics. To achieve maximal enzymatic activity, optimal concentrations of hydrogen peroxide (H₂O₂) and hydrogen donor (ABTS) were determined by using the procedure described by Nielsen and Wright (22). In summary, 12 replicates (one row) of ABTS at concentrations of 0.125 to 16 mM in 0.05 M citrate buffer (pH 5.00) were applied at 100 μ l per well. Sixteen replicates (two columns) of H₂O₂ at concentrations of 4 to 128 mM in citrate buffer were applied at 50 μ l per well. Conjugate, at a dilution of 1:50,000 in PBS-Tween 20, was applied to each of the 96 wells at 50 μ l. The plate was shaken, using a two-plate shaker, and color development at 414 nm was measured every 2 min for 10 min. The rate of reaction, expressed in OD units per minute versus H₂O₂ concentration on a log scale, was plotted for each ABTS concentration. Then, by using the optimal H₂O₂ concentration found on this graph, the rate of reaction was plotted against ABTS concentration to determine its optimal concentration.

To study the effect of plate shaking versus nonshaking on the enzymatic reaction rate, two plates were coated with an optimal antigen dilution. Three controls were tested (the positive serum control [TGT], the negative serum control [QC2], and PBS-Tween 20), using an optimal screening serum dilution (1:200). Following a 1-h incubation, the plates were washed and the conjugate was applied to all wells at its optimal dilution. After a 15-min incubation, the plates were washed and the substrate solution was applied to all wells. OD was recorded every 2 min for 30 min. The rate of reaction, expressed in OD units per minute, was calculated for each control serum and plotted versus time.

Timing protocol. A sample placement configuration described by Stemshorn et al. (32) was adopted. In brief, the

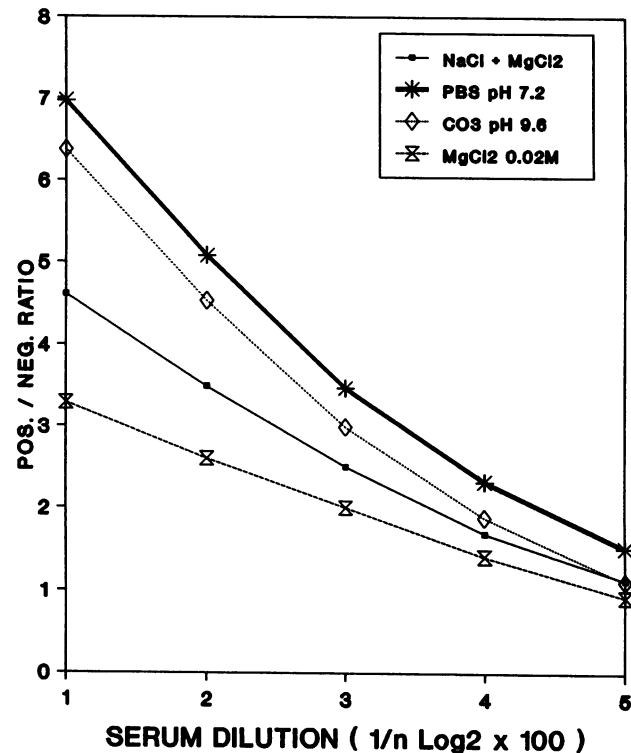


FIG. 1. Effect of magnesium chloride on antigen adsorption.

plate was subdivided horizontally and vertically into four equal quadrants. The four control serum samples were located in each quadrant (quadruplicates). Diagonally opposed quadrants received one of two sample duplicates. A timing protocol developed by Wright et al. (39) was also adopted. The timing protocol is based on the mathematical relationship of the OD value at 4 min of development to a predetermined target OD value of 1.00 for a standard antibody reagent, the target serum (TGT). The enzyme kinetics of horseradish peroxidase is not linear with time; therefore, a correction factor (equation) had to be established. A test plate with the four controls in quadruplicates and duplicates of eight serum samples with reactivities of between 0.100 and 1.000 OD₄₁₄ unit were tested 85 times (8 to 10 plates per day for 10 days). The mathematical relationship to establish the timing protocol was calculated. Mathematical expressions of limits of within- and between-plate variability were evaluated also.

RESULTS

Antigen. The optimal dilution of the two crude antigen preparations, frozen and lyophilized, were found to be different. The maximum P/N ratio of the lyophilized crude antigen was found to be around 1:1,200 compared with 1:50 for the frozen crude antigen. Antigen immobilization was not significantly affected by buffer composition or pH (data not shown). Coating the plates with the optimal antigen dilution in 0.02 M PBS (pH 7.2) gave a slightly higher P/N ratio than coating in 0.1 M carbonate buffer (pH 9.6) (Fig. 1). However, a reduction of the P/N ratio was observed as the MgCl₂ concentration was increased. The P/N ratio was lowered from 7.0 to 4.6 and 3.2 in the presence of 0.01 and 0.02 M MgCl₂, respectively (Fig. 1).

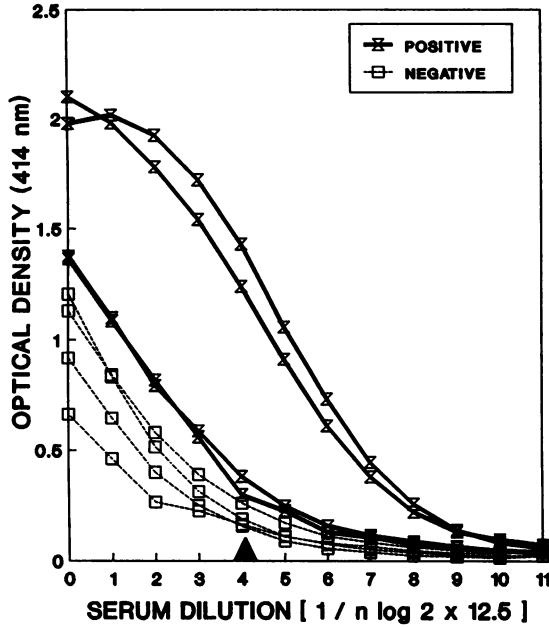


FIG. 2. Determination by ELISA of optimal screening dilution of test samples, using four seropositive (CF) and four seronegative (CF) serum samples.

Microplate. The Nunc Polysorp immunoplate 475-094 was found to be the best plate in terms of low percent CV and high P/N ratio. The P/N ratio of the Nunc 475-094 plate was 67% higher than that observed for the Dynatech U-bottom plates (7.28 and 4.36, respectively). A decrease in the within-plate CV, from 14 to 3.3%, was also observed (Table 1).

Test sera. The ODs observed in the titration of the positive sera reflected a typical dose-response curve over the dilution range, but the background ODs of the negative sera increased sharply at dilutions below 1:200 (Fig. 2). Therefore, a screening dilution of 1:200 for test samples used in the previous protocol (10) was confirmed to be the best dilution. At this dilution, discrimination between positive and negative sera appeared to be reasonable.

A maximum P/N ratio was obtained with four positive serum samples after a 30- to 60-min serum incubation period. Beyond this, a decline in the P/N ratio was associated with an increase in background OD of the negative sera.

Conjugate. The optimal dilution was determined by using the curve representing the maximum porcine IgG adsorbed to the microplate before over-saturation of the wells was apparent. In our case, no significant increase in adsorption was found beyond 1,000 ng of porcine IgG per ml. From this curve, an optimal conjugate dilution of 1:1,600 was chosen relative to achieving an OD of approximately 1.0 after 15 min of incubation (Fig. 3). No significant differences in binding were observed with longer incubation periods (data not shown); therefore, a 15-min conjugate incubation period was chosen.

Enzymatic activity. Maximum enzymatic activity was obtained with 4 mM hydrogen peroxide (Fig. 4). Along with this hydrogen peroxide concentration, 1 mM ABTS was selected as a reasonable concentration for achieving optimal results in a 10-min development period. By selecting this H₂O₂-ABTS combination, an increase of the enzymatic activity of 37.2% ± 0.9% (compared with the previous

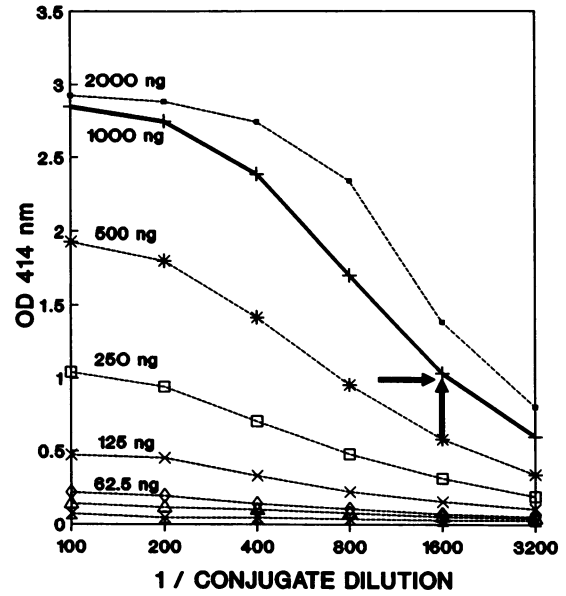


FIG. 3. Conjugate titration. Conjugate dilutions (peroxidase-labeled goat anti-swine IgG [heavy and light chains]) were assayed by using different amounts of swine IgG. The optimal dilution of this conjugate is 1:1,600 (indicated by arrows) for 1,000 ng of IgG.

protocol) was obtained (Fig. 4). Different results were obtained by shaking the plates during enzymatic reaction (Fig. 5). First, erratic variations in the reaction rate observed in the plates without agitation disappeared when agitation was applied. Second, the maximum rate of reaction was reached faster (within seconds) with shaking, and overall, higher rates were observed for the positive control (39.7% faster).

Timing protocol. The mathematical relationship of the OD at 4 min (OD_{4 min}) of development to the predetermined

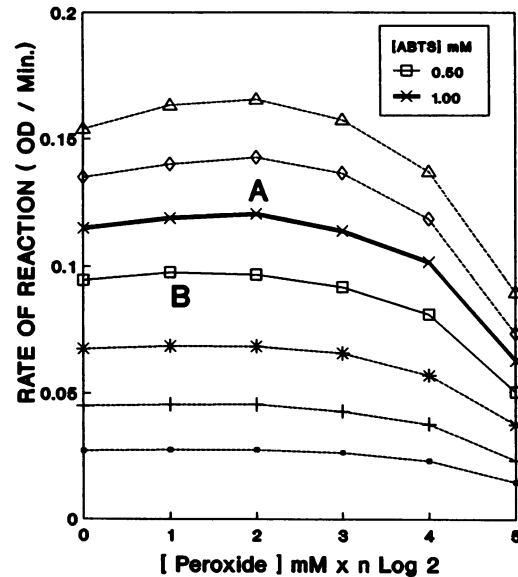


FIG. 4. Optimum hydrogen peroxide concentration for peroxidase activity. (A) Optimal H₂O₂ concentration (4 mM) for ABTS concentration of 1.00 mM. (B) Previous H₂O₂-ABTS concentrations ([H₂O₂], 1 mM; [ABTS], 0.5 mM).

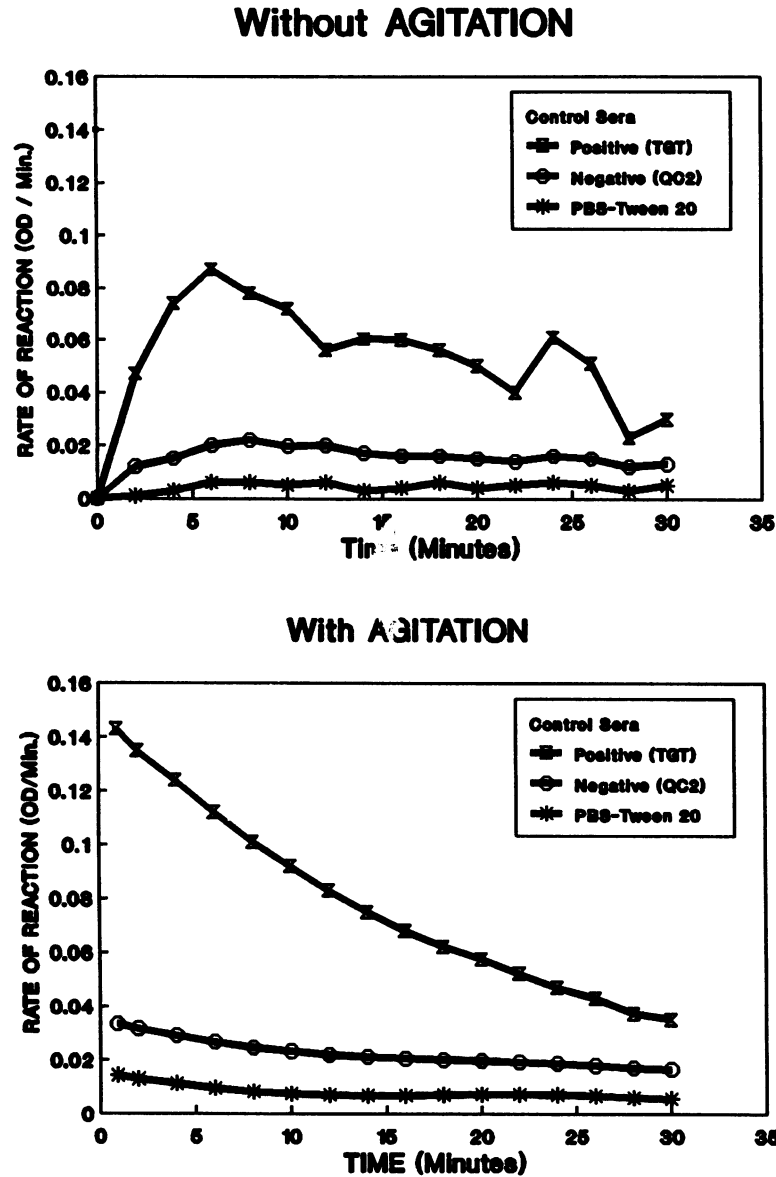


FIG. 5. Effect of plate agitation on enzymatic reaction rate.

target OD of 1.00 (OD_{TGT}) was evaluated in relation to the final development time. The final development time (target time) can be estimated by the following evaluated equation: target time = $(4.666/OD_{4 \text{ min}}) - 0.005374$. The resulting target time of the 85 plates assayed ranged from 6 to 13 min, with a mean of $9.54 \pm 1 \text{ min}$ ($r^2 = 0.9989$) (Fig. 6).

Acceptance limits. The mean percent CV was calculated for each of the eight serum samples from the 85 trials (Table 2). From these data a mathematical sliding scale based on the relationship between OD and percent CV was used to define acceptance limits for duplicate sample variability at 2 standard deviations. To monitor within-plate duplicate variability for ODs higher than 0.150, the mathematical relationship was calculated and expressed as: $\%CV \text{ (limit)} = 11.2358 - (6.6865 \times OD_{414})$, where $r^2 = 0.8047$. To establish assay control, statistical limits of between-plate variability were evaluated with the data from the four reference controls (Table 3). Limits of acceptance were established at 2 stan-

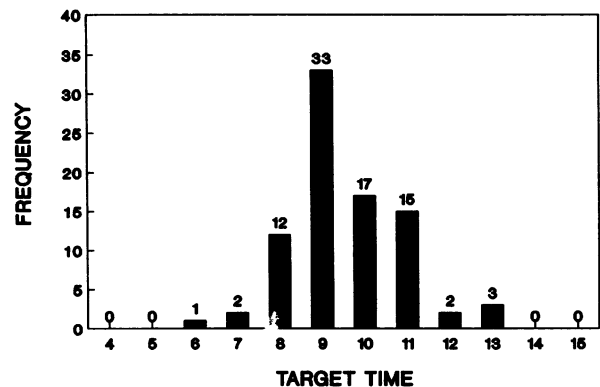


FIG. 6. Target time distribution of 85 plates.

TABLE 2. Statistical limits of variability from a cross section of sera of different activities assayed in ELISA to establish limits of within-plate variability^a

Serum no.	CF test	Mean OD ₄₁₄	%CV (within plate)
PBS-Tween 20	Negative	0.019	54.33
1	Negative	0.128	11.06
2	Negative	0.148	8.93
3	1:128	0.445	8.17
4	1:128	0.487	9.59
5	1:128	0.492	8.49
6	≥1:256	0.628	6.52
7	≥1:256	0.765	4.81
8	≥1:256	1.061	4.54

^a Duplicate assay variability.

standard deviations for the two positive controls (TGT and QC1) and at 3 standard deviations for the negative and buffer controls (QC2 and BC) (Table 4).

DISCUSSION

One of the following explanations may account for the differences in optimal antigen titers for the lyophilized versus the frozen antigen preparations. (i) Lyophilization of *A. pleuropneumoniae* serotype 5 crude antigen could alter the tertiary structure of the antigen, exposing more serotype-specific epitopes. (ii) It has recently been demonstrated that the serotype 5-specific, phenol-extracted antigen of strain 81-750 is associated with the 60-kDa S-LPS (27). Lyophilization may result in a more efficient binding of S-LPS by breaking up S-LPS micelles that may form in an aqueous environment. Similar results were obtained with subsequent lots of antigen preparation.

The high background activity associated with the addition of magnesium chloride (0.02 M MgCl₂) seems to be related to the selective attachment of rough LPS (R-LPS) found in the crude antigen extract (27). Similar results were found by Ito et al. (14) in the coating of R-LPS from *Neisseria gonorrhoeae* and *Escherichia coli* J5. They have reported that the Mg²⁺ ions could act as a bridging molecule between the negative charge of polystyrene (34) and the phosphate ester groups on the R-LPS. The R-LPS of *A. pleuropneumoniae* serotype 5 (strains J45 and 81-750) has been reported to contain no serotype-specific epitopes, but rather species-specific and also cross-reacting epitopes against other gram-negative bacteria (8, 9, 13, 27). To prevent such nonspecific reactions, the use of Dulbecco's PBS or addition of 1 mM EDTA to the coating buffer is advised. That pH and buffer composition did not have a significant effect on antigen immobilization might be related to the hydrophobic nature of this S-LPS antigen.

It has been demonstrated that a great deal of variation in

TABLE 3. Statistical limits of between-plate variability of the four quality controls^a

Control	CF test	Mean OD	SD	% CV
Target sera (TGT)	≥1:256	1.064	0.015	1.41
Positive (QC1)	1.32	0.637	0.039	6.12
Negative (QC2)	Negative	0.120	0.011	9.17
Buffer (BC)	Negative	0.025	0.008	32.00

^a Data were computed from the 85 trials.

TABLE 4. Limits of acceptance of between-plate variability of the four quality controls

Control	Mean OD	Lower limit	Upper limit	Range (SD)
Target sera (TGT)	1.064	1.034	1.094	± 2
Positive (QC1)	0.637	0.559	0.715	± 2
Negative (QC2)	0.120	0.086	0.153	± 3
Buffer (BC)	0.025	0.001	0.049	± 3

test results and antigen immobilization exists among the different types of plates from different manufacturers (22). Therefore, care should be taken when selecting a solid phase for antigen immobilization. The high within-plate variations obtained with the Dynatech 001-010-2201 plate could be associated with light scattering, from the round-bottom shape of the wells, when reading with a spectrophotometer. Also, the site of injection (molding) of polystyrene might be responsible for absorption discrepancies (38). A similar protocol of plate testing was established by Craig et al. (5). We have selected the Nunc ELISA plate 269-620 instead of the Nunc Polysorp 475-094 for economical reasons, considering the large amount of samples submitted to our diagnostic laboratory each year.

The choice of H₂O₂ concentration is one of the most important factors in optimizing enzyme kinetics (34). An excess of H₂O₂ has an inhibitory effect on the reaction rate (Fig. 4). However, in the previous protocol, the H₂O₂ concentration was below optimal enzymatic activity. On the other hand, ABTS concentration is not rate limiting; therefore, a 1 mM concentration was chosen (22) arbitrarily over the 0.5 mM concentration used previously (10).

Another parameter often neglected in enzyme immunoassays is the effect on the enzymatic reaction associated with plate shaking. Kinetics of the enzyme-substrate reaction are altered when the enzyme is immobilized on a solid surface (34). When the plate is left without agitation, accumulation of oxidized products and depletion of substrate in the vicinity of the enzyme result in a sharp decrease of the enzymatic activity after only 6 min (Fig. 5). The impact of this effect, being proportional to the concentration of immobilized enzyme in the well, for the most part will adversely affect the positive serum reactivity. This would explain why agitation has a greater effect on the positive control than on the negative control (Fig. 5). Another benefit associated with shaking is the formation of a uniform meniscus in all wells which diminishes within-plate photometric variation related to the light-scattering effect of menisci (40).

Preliminary studies have shown that the use of a holding buffer (PBS-Tween 20, 0.02 M), added after the removal of unbound conjugate and before substrate application, did reduce the background of some negative serum samples (data not shown). A similar effect was noted when five washes instead of three were used in the initial protocol (data not shown). Beneficial effects on assay specificity related to the appropriate numbers of washings have been reported previously (34).

The first objective of this study was to increase the sensitivity of the assay. The P/N ratio of the controls in a previous assay (10) ranged from approximately 3.0 to 4.5 (1.5 to 1.8 for the positive control; 0.4 to 0.5 for the negative control). By using this new assay, with optimization of most of the parameters involved, these values now range from 3.6 to 12.7. An optimal P/N ratio in ELISA should range

between 5 and, preferably, 10 as reported by Voller et al. (36).

The second objective of this study was to establish limits of assay acceptability. The results obtained for between-plate variability are comparable to those obtained in a similar study on reduced interplate variability after application of a timing protocol for the detection of anti-*B. abortus* antibodies (39), a protocol that also uses an LPS antigen (S-LPS).

Given the limits of variation established here, application of operational quality control will be considered at two levels, as described previously (38). First, intraplate and interassay variability of the target serum (TGT) and the other three controls must fall within specification limits. These limits were established at 2 and 3 standard deviations of the intra- and interassay means of the controls (Table 2 and 4). The interassay variability is monitored by continuous determination of the mean and the standard deviation of the last 40 plates for each of the four controls independently. The target time equation obtained with our results is comparable to the equation obtained previously (39). Second, to determine acceptable duplicate test sample variability, a cross section of samples of different antibody activities has been tested (Table 2). Acceptance limits have been set at 2 standard deviations from a mathematical relationship established between OD and percent CV.

This standardized protocol will be used as a screening assay for the serodiagnosis of *A. pleuropneumoniae* serotype 5. Further studies, including experimental exposure and testing of herds of different serological status, will be conducted to establish the diagnostic threshold, the diagnostic sensitivity and specificity, and also the predictive values of this assay.

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