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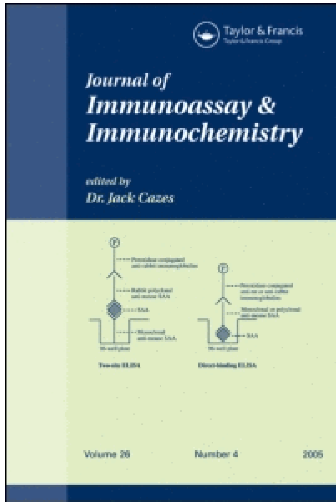

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STANDARDIZATION OF SMOOTH LIPOPOLYSACCHARIDE PREPARATIONS FOR USE IN DIAGNOSTIC SEROLOGICAL TESTS FOR BOVINE ANTIBODY Brucella abortus.

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ABSTRACT

A procedure for standardizing Brucella abortus smooth lipopolysaccharide used in diagnostic tests for brucellosis is proposed. The procedure is based on the reactivity of antigen preparations with a panel of sera with or without antibody to B. abortus using a set of parameters established with 13 antigen preparations. For each serum dilution, a mean and two standard deviations were calculated in the indirect and competitive enzyme immunoassays. If data obtained with an antigen preparation, using the same serum dilutions, falls within the range established using two standard deviations, the antigen would be considered acceptable for diagnostic use.

KEY WORDS

Brucella abortus, lipopolysaccharide, standardization, indirect enzyme immunoassay, competitive enzyme immunoassay.

INTRODUCTION

Preparation of smooth lipopolysaccharide (SLPS) from Brucella abortus was initially described by Redfearn (1960) and Baker and Wilson (1965). The extraction of SLPS was accomplished with a mixture of hot water and hot phenol, followed by various procedures to eliminate contaminants. Unlike SLPS from most other gram negative microorganisms, the SLPS from Brucella sp. is

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hydrophobic and is soluble in the phenol fraction (only free O-polysaccharide is found in the aqueous phase). The phenol phase, in addition to SLPS, contains protein and nucleic acid contaminants. These contaminants may be removed by digestion with enzymes (Cherwonogrodzky et al, 1990) or by precipitation with trichloroacetic acid (Nielsen et al, 1996), resulting in a relatively pure preparation of SLPS.

For use in primary binding assays, especially for assays used for the diagnosis of brucellosis, it is essential that the antigen preparation be standardized in such a way that results are consistent from batch to batch of antigen. The SLPS preparation may be initially standardized on the basis of weight. However, this was found not to be adequate as there were some differences in activity between batches of antigen when used in enzyme immunoassays (ELISA). These differences may be due to the length of the O-polysaccharide side chain in the SLPS molecule which may be a reflection of the conditions of bacterial growth. It became apparent that other means of standardization were required. One such method could be based on the biological activity of the SLPS with a number of reference sera, and comparing the results with those obtained with a large number of batches of SLPS.

The results obtained with 8 batches of SLPS with 12 serum samples in indirect and competitive ELISAs for bovine antibody to Brucella abortus were compared with those obtained with 5 new preparations of SLPS. Some criteria for standardization of the antigen are discussed.

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MATERIALS AND METHODS

Brucella abortus smooth lipopolysaccharide:

Brucella abortus strain 413 and strain 1119.3 were used for preparation of SLPS. Cells were propagated according to Animal Plant Health Directorate Standard Operating Procedure (1993), harvesting cells from tissue culture flasks containing potato infusion agar (for strain 1119.3) or beef infusion agar (for strain 413). The harvested cells were killed by steaming twice for 30 minutes each time. The cells were washed in distilled water and freeze dried.

Smooth lipopolysaccharide was prepared by suspending 5 gm of dried cells in 170 ml of distilled water at 66°C and adding 190 ml of 90% v/v phenol, preheated to 66°C. The mixture was stirred at

66°C for 20 minutes after which it was cooled in an ice bath. All the following manipulations were performed at 4°C. The mixture was centrifuged at 13,000 x g for 15 minutes. The bottom liquid phase, the phenol layer was aspirated with a syringe equipped with a 6 inch 12 gauge cannula and filtered through a Whatman #3 filter to remove any cell debris. Three volumes of methanol containing 1% methanol saturated with sodium acetate were added to the filtrate for 60 to 90 minutes. The precipitate was recovered by centrifugation at 6,000 x g for 15 minutes. To the precipitate was added 80 ml of distilled water. This was left with constant stirring for 18 hours. After centrifugation at 10,000 x g for 15 minutes, the precipitate was resuspended in 80 ml of distilled water for 1 hour with constant stirring. The supernatant solution was kept and the suspension was centrifuged as before. The precipitate was discarded and the two supernatant solutions were pooled. Eight gm of trichloroacetic acid were added to the supernatant solution, incubated for 15 minutes with stirring and centrifuged at 10,000 x g for 15 minutes. The supernatant solution was dialized against distilled water for at least 24 hours, sonicated three times at 6 watts for 30 seconds in an ice bath and freeze dried. A 1.0 mg/ml solution was prepared using distilled water.

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Serum Samples:

Twelve serum samples, consisting of 5 from cattle from which B. abortus was isolated from tissues or milk, 5 samples obtained in the early phases of the antibody response from cattle after routine vaccination with B. abortus strain 19 (early phase sera were selected to provide reactivity in the CELISA) and 2 samples from cattle that had not been exposed to B. abortus were selected. The positive sera for B. abortus represented various levels of antibody. The sera were stored in small volumes to avoid excessive freezing and thawing at -20°C. Additional amounts were freeze dried.

For enzyme immunoassay purposes, four serum controls were included with each assay on each 96 well plate in duplicate. These controls included a strong positive (C++), a weak positive (C+), a negative (C-) and a control in which buffer replaced serum (Cc). The reactivity of these sera determined in each assay determined if the results were acceptable in that each duplicate result was required to fall within a specific range of optical density units.

Enzyme Immunoassays:

Indirect and competitive enzyme immunoassays (I- and CELISA) were performed as described previously (Nielsen et al, 1996) with some modifications. The modifications consisted of the use of three concentrations of SLPS antigen, 0.1, 1.0 and 10.0 ug/ml for passive adsorption of antigen to the polystyrene 96 well plates. Serum dilutions used were 1:100, 1:1,000, 1:10,000, 1:100,000 and 1:1,000,000 for the IELISA (the range was 1:1,000 to 1:1,000,000 for sera from infected cattle; 1:100 to 1:100,000 for sera from vaccinated cattle and 1:100 and 1:1,000 for sera from non-exposed cattle) and 1:10, 1:100, 1:1,000 and 1:10,000 for the CELISA, except for the two negative sera which were only tested at the two lowest dilutions. All samples were tested in duplicate at least twice.

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Briefly, the ELISAs were performed on polystyrene plates (NUNC 2-69620 from GIBCO/BRL, 2270 Industrial St., Burlington, Ontario, Canada L7P 1A1). Plates were coated with 100 ul of the appropriate concentration of SLPS diluted in 0.6M carbonate buffer, pH 9.6 for 18 hours at ambient temperature. The plates were washed four times with 0.01M phosphate buffer, pH 7.2 containing 0.15M NaCl and 0.05% Tween 20 (PBST). For the IELISA, 100 ul of serum, diluted 1:50 in PBS containing 15mM EDTA and 15mM EGTA, pH 6.3 were added to each well and incubated at room temperature for 30 min. After four additional washes with PBST, 100 ul of appropriately diluted mouse monoclonal antibody specific for the heavy chain of bovine IgG1, conjugated with horseradish peroxidase, were added to each well for 60 min. After four further wash cycles with PBST, 100 ul of 4mM 2,2'-azino-bis(3 ethylbenz-thiazoline-6-sulfonic acid) diammonium salt and 1mM hydrogen peroxide dissolved in 0.05M citrate buffer, pH 4.5, were added to each well and incubated for 10 min. With constant shaking. The colour development was evaluated in a spectrophotometer at 414 nm using the substrate/chromogen for blanking. For the CELISA, 50 ul of serum diluted 1:10 in PBS with EDTA and EGTA were added followed immediately by 50 ul of mouse monoclonal antibody specific for the O-polysaccharide of SLPS also diluted in PBS with divalent cations. The plates were shaken for 3-5 min. and left at ambient temperature for 30 min. After four wash cycles, 100 ul of goat

anti-mouse IgG conjugated with horseradish peroxidase (Jackson ImmunoResearch Inc. product from BIO/CAN Scientific, 2170 Dunwin Dr., Unit 5, Mississauga, Ontario, Canada L5L 1C7) were added per well for 30 min. After four wash cycles, colour was developed and assessed as described for the IELISA.

Data:

The acceptability of the data was based on the performance of the control sera. For the IELISA, a range of optical density values for the C++ control had been established based on variability observed in at least 30 different test runs. The range for the C++ was 0.57 - 1.26 optical density units. If the observed optical density values on a given plate were within the range, the % positivity (%P = (sample OD/C++ OD) x 100) for the other controls were calculated using the C++ as 100%. Again, ranges had been established and for data to be acceptable, the %P for the C++, the C+, the C- and the Cc must be within these parameters. The acceptable ranges were: C++: 87 - 113%P; C+: 50 - 80%P; C-: -5 - 31%P and Cc: -4 - 8%P. These ranges were based on using one SLPS at a concentration of 1.0 ug/ml. One control (but not the C++) was allowed to be outside the range and the data would still be acceptable.

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For the CELISA, similar criteria were established. The no serum control, the Cc, was considered to give 0 % inhibition (%I =100 - (sample OD/Cc OD) x 100) and all other controls were calculated based on this value. Ranges established for the Cc were 0.65 - 2.02 optical density units. The %I values for the controls were as follows: C++: 87 - 106%I; C+: 34 - 69%I; C-: -12 - 28%I and the Cc: -14 - 9%I. One control (but not the Cc) was allowed to outside the range and the data was still acceptable. The ranges were determined using one SLPS preparation at 1.0 ug/ml.

For the IELISA, the % P was calculated for each serum dilution based on the reactivity of the strong positive serum control (C++) set at 100%P.

Similarly, for the CELISA, the control to which no serum was added (Cc) was considered to give 0 %I and the %I of all other controls and serum dilutions were calculated based on this value. Results for replicate samples were not averaged.

For each antigen preparation and for each antigen concentration, all data was used to calculate a mean (X) and a standard deviation (SD). Plots were prepared using the means +/- 1SD or 2SD.

RESULTS

Data obtained with the IELISA for the 13 antigen preparations used at 1.0 mg/ml are presented in Figures 1 and 2. Ten-fold dilutions of serum, ranging from 1:100 to 1:1,000,000 were tested in duplicate with each antigen (1:100 to 1:100,000 for sera from vaccinated cattle; 1:1,000 to 1:1,000,000 for infected cattle and 1:100 and 1:1,000 for the sera from non-exposed cattle). The grand mean was calculated for each dilution with 1 SD (Figure 1) or 2 SD (Figure 2). Each panel in the Figures represent data from a positive serum, serum from a vaccinated animal and serum from a non-exposed cow. One SD gives a much narrower range (encompassing approximately 68% of the population) than does 2 SD (encompassing 95% of the population), resulting in 6 and 1 antigen preparations giving results outside the range, respectively (raw data not shown). The data obtained when the antigen concentration was 10 ug/ml was very similar, with the SD values being somewhat less in most cases (data not shown). The criteria for acceptance of the data were met. If antigen was used at 0.1 ug/ml, there was a considerable increase in the SD values and in all cases, the strong positive control serum (C++) did not give sufficiently high optical density values for the data to be acceptable (data not shown).

Data for the CELISA using an antigen concentration of 1.0 ug/ml for the 13 antigen preparations with the 12 test sera are presented in Figures 3 and 4. Figure 3 represents the mean of the %I with +/- 1 SD and Figure 4 is the mean +/- 2 SD. As was the case with the IELISA, 6 of the antigen preparations gave %I values outside 1SD while only 1 was outside 2SD (data not shown). Using an antigen concentration of 0.1 ug/ml, the optical density values for the no serum control (Cc) in the CELISA were below acceptable levels with all antigens. Therefore, these data were rejected. Antigen at 10 ug/ml gave optical density values that did not significantly differ from those obtained with an antigen concentration of 1.0 ug/ml and the interpretation of the data was the same. Therefore, only data for 1.0 ug/ml antigen are shown.

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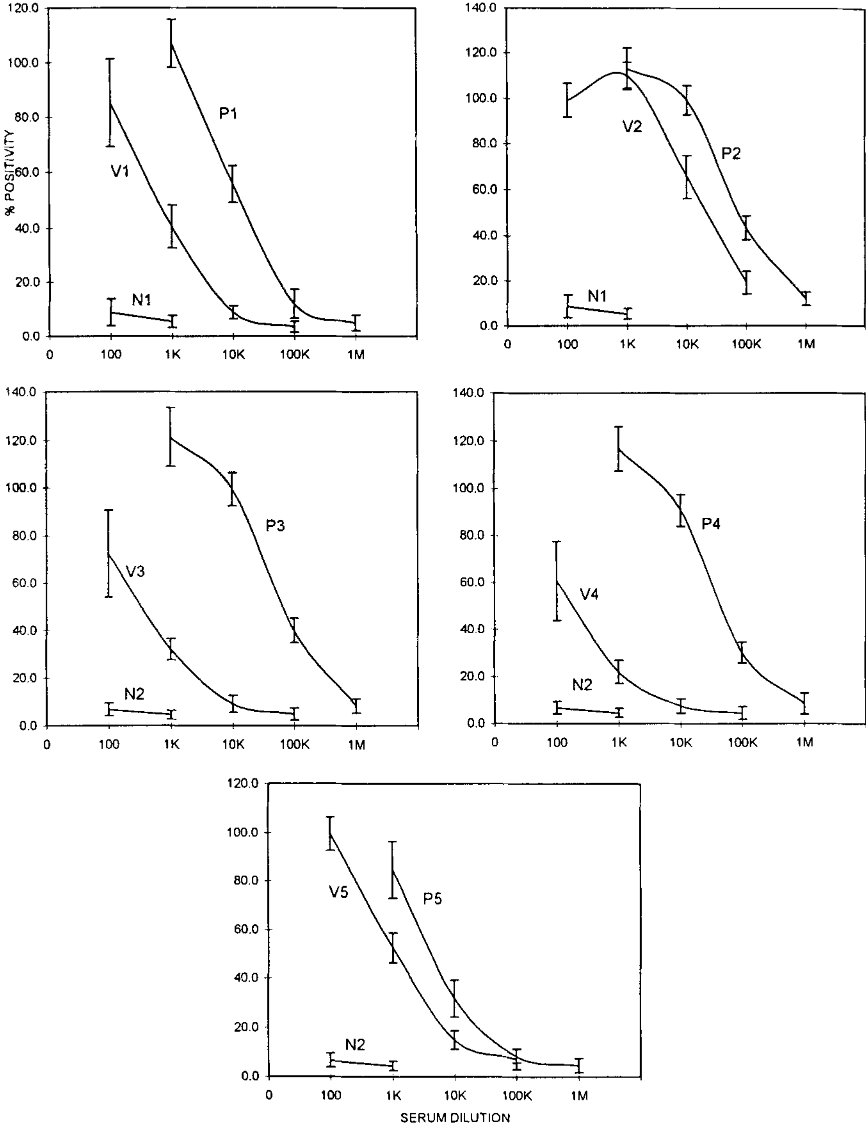

Figure 1

Panels each represent % Positivity values (Y-axis) of an indirect enzyme immunoassay obtained with sera from a cow infected with B. abortus (P); vaccinated with B. abortus strain 19 (V) or not exposed to B. abortus (N). A total of 5 sera from infected, 5 sera from vaccinated and 2 sera from non-exposed cattle were used at various dilutions (X-axis). Bars indicate range of +/- one standard deviation.

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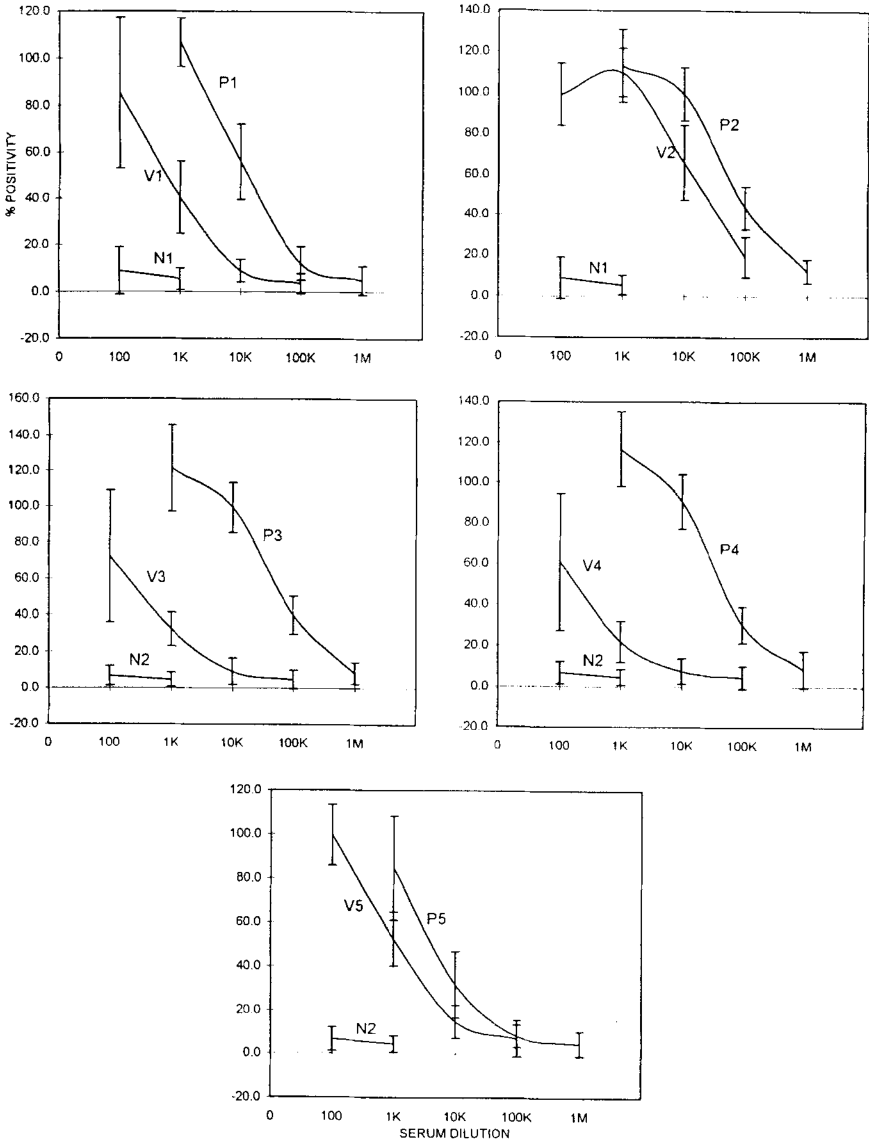

Figure 2

Same legend as Figure 1 but the bars represent +/- two standard deviations.

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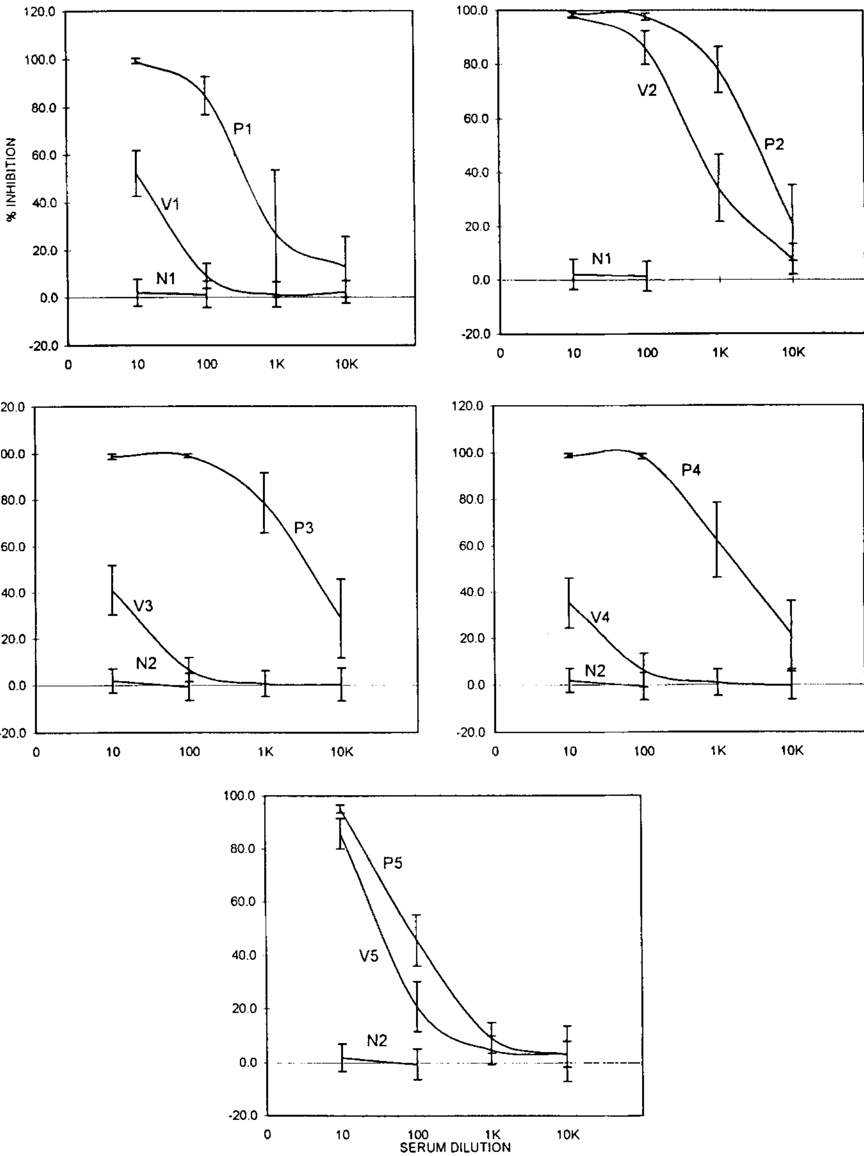

Figure 3

Same legend as Figure 1 but % Inhibition values were obtained with a competitive ELISA.

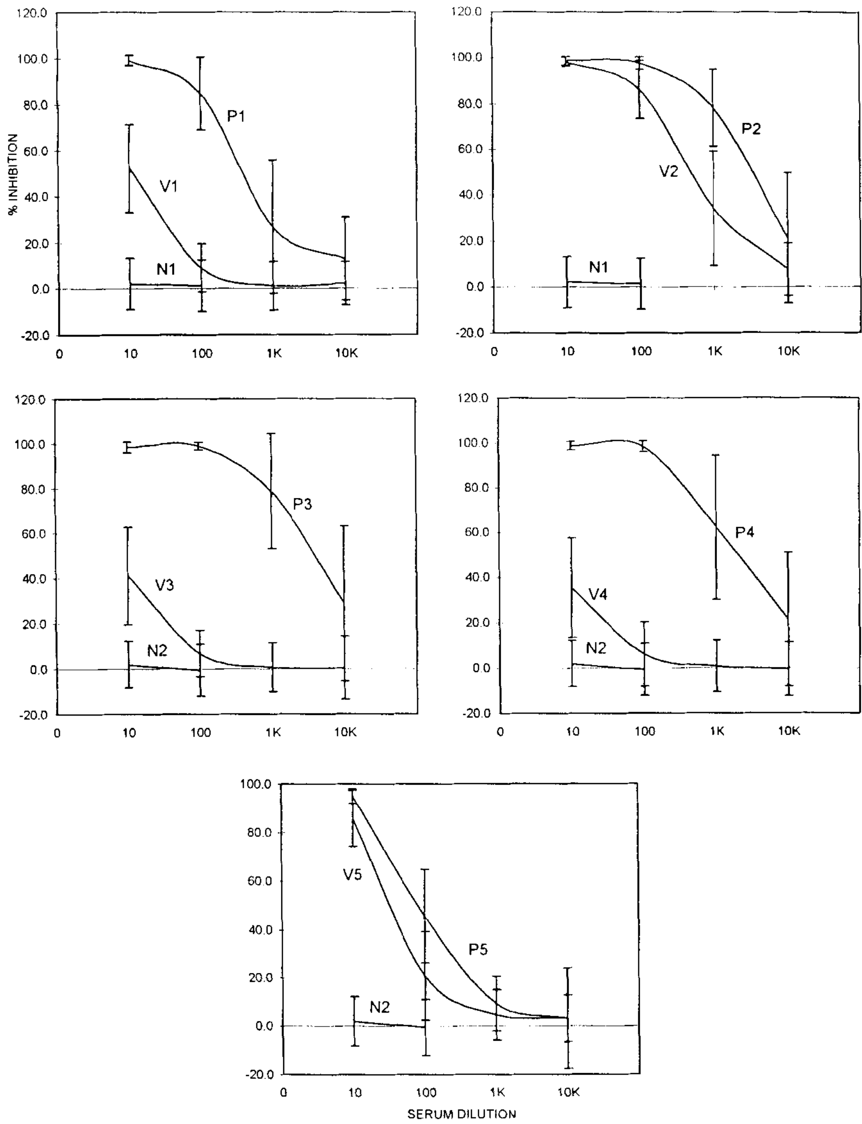

Figure 4

Same legend as Figure 3 but bars represent +/- two standard deviations.

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In the CELISA, the SD values for the lower serum dilutions were generally lower than those of the IELISA while the SD values for the higher dilutions were lower for the IELISA.

Negative results are sometimes obtained with the negative serum and the buffer controls. This is caused by less substrate development than observed in the blanking step. The reason for this is not understood, however, it may result from errors in spectrophotometer readings due to the extremely low optical density values and perhaps due to minor variations in optical densities which are subsequently averaged in diagnostic ELISAs. Similarly, with the current spectrophotometers, optical density values over 2.0 are unreliable as they are not on the linear portion of the spectrophotometer response.

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DISCUSSION

The purpose of this study was to attempt to establish a protocol for standardizing the preparation of smooth lipopolysaccharide from B. abortus used in diagnostic immunoassays. In the past, standardization was based on weight of the SLPS only, however, this was found to give discrepancies in the optical density readings of the I- and CELISAs. Standardization of the antigen preparations could perhaps be based on their biological activity in the ELISAs. Therefore, 8 SLPS preparations prepared periodically since 1991 were tested along with 5 new preparations (prepared individually) with four dilutions of selected sera from B. abortus infected, vaccinated and non-exposed cattle. These sera were selected based on reactivity in the ELISAs, availability of sufficient quantities for future standardization and because an international serum standard is not currently available. It is envisaged that these sera may serve as standards. To accommodate the amount of data generated, the results obtained with each dilution were averaged for all antigen preparations and the SD calculated. From the Figures, it is clear that 1SD provides a very strict criteria and if antigen preparations that gave values outside the 1SD limits were rejected, 6 of the 13 preparations would not be useful. Using 2SD, however, only one antigen preparation was outside the limits. This antigen gave consistently lower values with six of the serum samples and should therefore be rejected for

diagnostic use. An arbitrary criterion of two sera giving results outside the 2SD limits was selected to indicate rejection of that antigen preparation. This should theoretically provide a 95% acceptance of antigen preparations in contrast to 68% acceptance if one SD was used.

In this study, 3 antigen concentrations were used: 0.1, 1.0 and 10 ug/ml. A concentration of 0.1 ug/ml did not give sufficiently high optical density values for the data to be acceptable by the diagnostic criteria established previously. The use of 10 ug/ml gave results nearly identical to those obtained with a concentration of 1.0 ug/ml of antigen. Therefore, it was considered appropriate to use a concentration of 1.0 ug/ml only.

Future SLPS antigen preparations will be tested with this antigen concentration and with the same serum samples and if the data obtained fall within the 2SD limits established with the 13 previous preparations, the antigen would be considered acceptable for diagnostic testing. The results for subsequent antigen preparations would be incorporated into the calculations of the means and SD.

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