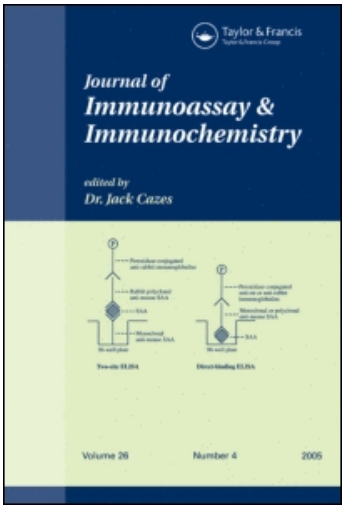

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Improvements to the Competitive Elisa for Detection of Antibodies to Brucella Abortus in Cattle Sera

D. Gall[ab]; K. Nielsen[a]
[a] Agriculture Canada, Animal Diseases Research Institute, Station H, Nepean, Ontario, Canada [b] Immunology Section, Animal Diseases Research Institute, Nepean, Canada

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IMPROVEMENTS TO THE COMPETITIVE ELISA FOR DETECTION OF ANTIBODIES TO BRUCELLA ABORTUS IN CATTLE SERA

D. Gall and K. Nielsen,
Agriculture Canada, Animal Diseases Research Institute,
Box 11300, Station H, Nepean, Ontario, Canada K2H 8P9.

ABSTRACT

This paper reports improvements in the competitive ELISA (cELISA) for the detection of serum antibodies to Brucella abortus through modification of the coating of antigen and of the enzyme labelling of the monoclonal antibody conjugate. The covalent linkage of poly-L-lysine to the o-polysaccharide antigen of Brucella abortus enhanced its binding to the polystyrene matrix which resulted in a more efficient and reliable cELISA. Optimal discrimination between vaccinated and infected cattle was achieved by adjustment of the ratio between the monoclonal antibody used in the cELISA and the horseradish peroxidase. Both modifications resulted in a refined, more efficient and reliable cELISA with potential as a routine serodiagnostic assay for Brucellosis.
KEYWORDS: (Brucella, cELISA, HRPo, monoclonal, o-polysaccharide, yersinia)

INTRODUCTION

A cELISA which was capable of distinguishing antibody induced in cattle by vaccination with Brucella abortus strain 19 from that

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Requests for reprints should be sent to:

David Gall, Immunology Section, Animal Diseases Research Institute, 3851 Fallowfield Road, Nepean, Ontario, K2H 8P9, Canada.

Tel: (613) 998-9320, Fax: (613) 952-2285.

developed following natural infection was described earlier (8). While it seemed initially that the cELISA using unmodified Brucella abortus o-polysaccharide worked excellently (9), further experience with new lots of antigen and a variety of sera revealed some problems. It became evident that the results were not reproducible and had high fluctuations, probably due to poor binding of the o-polysaccharide, as seen with other polysaccharides (3). Also, the use of horseradish peroxidase (HRPo) conjugated monoclonal antibody (Mab) with a high ratio of HRPo seemed to result in an inappropriately high sensitivity. Thus, the effect of these two factors resulted in an infrequent occurrence of false positive and negative reactions with sera in general and as one would expect with high fluctuations, an occasional reactivity of sera from certain vaccinated animals and with sera with heavy bacterial contamination. In addition, occasional false positive reactions occurred with sera from cattle vaccinated with a full dose of B. abortus strain 19 tested within the first month after vaccination. However, considering that this would result in the approximate peak of antibody response, such a result is not unexpected (7). In the case of the false positive reactions observed with contaminated sera, it is speculated that the o-polysaccharide antigen was displaced by bacterial lipopolysaccharides which bind better to polystyrene (3). Of the false negative reactions, a few were apparent when sera from recently infected (within one month) cattle were tested. This was presumably due to the antibody produced being of lesser affinity and unable to compete with the Mab or due to the low titre of the antibody produced in the early stage of infection. We have improved the performance of the cELISA by developing a more efficient, reliable antigen and optimizing the ratio of Mab to HRPo.

This paper describes the procedures for binding poly-L-lysine to the o-polysaccharide antigen and the adjusting of the ratio of Mab to HRPo, used in the assay.

MATERIALS AND METHODS

Antigen

The O-polysaccharide was prepared from B. abortus strain 1119.3 (standard strain used for antigen preparation) by hydrolysing heat killed cells with 2% v/v glacial acetic acid at 121°C for 15 min. The cell slurry was cooled and neutralized with 5N NaOH. The particulate matter was removed by centrifugation at 10,000 x g for 20 min. The supernatant solution was dialysed against 10mM sodium phosphate buffer, pH 6.0 and containing 100mM NaCl. The dialysate was applied to a 1x15 cm column containing polymyxin B immobilized on a bead matrix (BioRad Laboratories Ltd., 5149 Bradco Blvd., Mississauga, Ontario L4W 2A6) equilibrated in the same buffer. Lipopolysaccharide contained in the dialysate was bound to the polymyxin B via lipid A while free O-polysaccharide was eluted in the void volume. The O-polysaccharide was dialysed against distilled water and lyophilized.

Conjugation of O-polysaccharide with poly-L-lysine

The technique described by Gray (3) was used with some modifications. The O-polysaccharide was reconstituted to 1.0 mg/ml with 0.06M carbonate buffer, pH 9.6. Two ml of O-polysaccharide were dispensed into 10 ml 0.01N NaOH, mixed gently for 5 minutes, and then transferred to a vial containing 13 mg cyanuric chloride (Aldrich Chemical Company Inc., 1001 West Saint Paul Avenue, Milwaukee, Wisconsin 53233 USA). The pH of the solution was monitored as the cyanuric chloride dissolved with gentle stirring over 2 to 2.5 hours. When the pH reached 8.8, immediately 2.0 ml of 0.1% v/v poly-L-lysine (Sigma Chemical Company, P.O. Box 14508, St.

Louis, MO. 63178, USA) was dispensed into the mixture and agitated gently for 5 minutes. The mixture was incubated at 4°C for 18 hours and stored at -20°C for one week prior to use. Subsequently, the antigen was lyophilized.

Sera

Control sera from animals of known status of exposure to B. abortus were used. The control sera consisted of a serum from an naturally infected cow (QC3) which had a very high titre on other diagnostic tests for brucellosis, including the tube agglutination, the complement fixation and the indirect ELISA tests. A second control serum (QC1) was obtained from a cow immunized with heat killed B. abortus by intramuscular injection. This serum was chosen as a control from a vaccinated animal (out of many tested) to ensure and demonstrate on a continuous basis that the cELISA was discriminating between animals that were naturally infected and those that were immunized. This serum gave positive reactions with the other serological tests for brucellosis and a negative reaction on the cELISA. A third control reagent (QC2) was a pool of sera obtained from a herd from the Central Experimental Farm, Agriculture Canada. This herd had no clinical, serological or epidemiological evidence of exposure to B. abortus (including strain 19). These sera were used to titrate the antigen and to determine the most useful Mab to HRPo ratio.

Monoclonal antibody

The Mab antibody used was that produced by a murine IgG_3 hybridoma cell line established from balb/c mice immunized with

Yersinia enterocolitica serotype O:9 and fused with the murine plasmacytoma cell line SP/2, according to the technique described by Kennett et al (6). This hybrid cell line was developed at the National Research Council of Canada and Mab from the cell line was shown to have specificity for an epitope of the O-polysaccharide of B. abortus (1). Mab from ascites fluid was purified by anion exchange high pressure liquid chromatography by conventional methods (9). The purified Mab was dialysed against 0.15M NaCl and concentrated to 2 mg/ml and stored frozen. Aliquots of the Mab were conjugated with HRPo by the technique described by Henning and Nielsen (4). The HRPo conjugated Mab was separated from unbound HRPo by gel filtration chromatography.

An elution profile is presented in Figure 1A. The first protein peak (peak 1) contained the Mab with or without enzyme attached to it. The second protein peak contained unattached HRPo. The HRPo conjugated Mab was collected and the protein and peroxidase contents measured using the formulas below after scanning from 240 to 440 nm (Figure 1B) and using an extinction coefficient of 13.7 (2) for the IgG and a factor of 0.4 (11) for determining the concentration of HRPo.

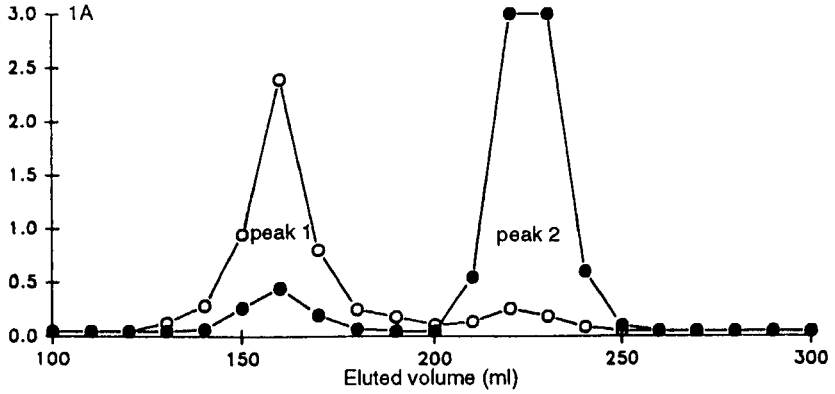
$$\text{Total protein (mg/ml)} = \text{optical density}_{280nm} \div 1.37$$

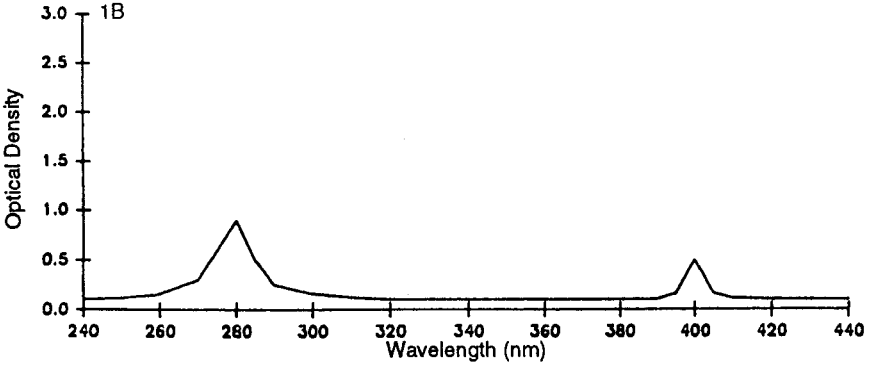
$$\text{HRPo (mg/ml)} = \text{optical density}_{403nm} \times 0.4$$

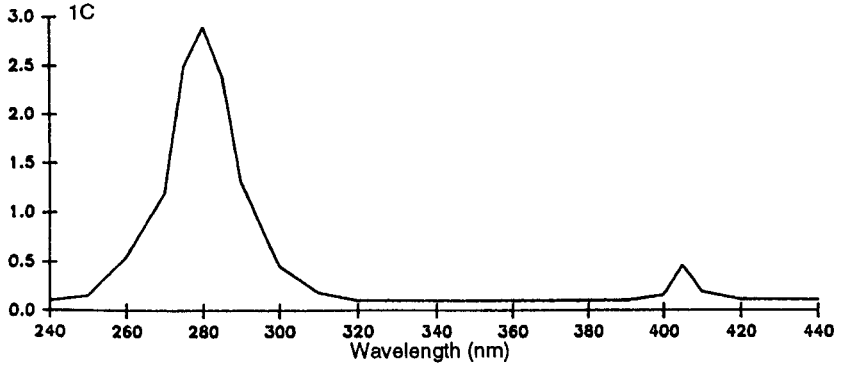
The ratio of Mab to HRPo was based on the molecular weights of 160,000 for IgG and 40,000 for HRPo and the concentration of each reagent (in mg/ml) in the mixture using the following formula:

$$\text{Ratio of IgG : HRPo} = \frac{\text{(conc. of total protein} \div \text{mol. wt. of IgG)}}{\text{(conc. of HRPo} \div \text{mol. wt. of HRPo)}}$$

Unlabelled Mab was added to the HRPo conjugated Mab in various quantities and the mixtures were tested in the cELISA. An example of the dilution effect on the ratio of Mab to HRPo is presented in Figure 1C.







Competitive enzyme immunoassay

The method of Nielsen et al (9) was used. The antigen, reconstituted in 0.06M carbonate buffer, pH 9.6, was passively coated onto 96 well polystyrene plates (NUNC 2-69620 plates from GIBCO-BRL, 2270 Industrial St., Burlington,Ontario L7P 1A1) using 100 ul of 2.0 ug/ml amounts/well and incubated overnight at 25°C. Plates were also coated with antigen and then stored frozen at -20°C. After removing unbound antigen by washing five times with 0.1M sodium phosphate buffer, containing 0.15M NaCl and 0.05% Tween 20, pH 7.2 (PBS/T) using an automated plate washer (Titertek Microplate Washer 120, Labsystems, Pulttitie 8, P.O. Box 8, SF-00881 Helsinki, Finland), 50 ul of serum, diluted 1:50 and 50 ul of HRPo conju-

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FIGURE 1

A. Elution pattern of HRPo conjugated Mab using gel filtration chromatography. The Y-axis represents the optical density at 280 nm (open circles) and at 403 nm (close circles) and the X-axis is the eluted volume. Peak 1 contains the HRPo conjugated Mab as well as Mab without HRPo. Peak 2 contains unattached HRPo.

B. Pattern obtained when the HRPo conjugated Mab is scanned in an ultraviolet spectrophotometer from 240 to 440 nm. Peak 1, at 280 nm, represents proteins in solution (IgG and enzyme) while the peak at 403 nm represents HRPo. Concentration of each (mg/ml) can be determined from the area under the curves.

C. Parameters are the same as for B, but unconjugated Mab has been added to alter the Mab to HRPo ratio.

gated Mab both diluted in PBS/T were added to the microplate coated with O-polysaccharide in quadruplicate for the controls and in duplicate for the test samples. A quadrant plate lay out as described previously (10) was used with a targeting protocol (12) to control day to day variation throughout this study. The serum and HRPo conjugated Mab mixture was agitated for three minutes on an orbital shaker and incubated at 25°C for two hours. After, five further wash cycles were performed as above and the wells filled with a solution containing 100 ul of 1mM H_2O_2 (substrate) and 0.5 ml of 4mM ABTS (chromogen) in 20 ml of freshly prepared 0.05M citrate buffer, pH 4.5. The microplates were then shaken continuously for four minutes at room temperature (20°C ± 2oC). Development of colour was assessed in a spectrophotometer (Multiscan MCC 340, Labsystems Pulttitie 8, P.O. Box 8, SF-00881 Helsinki, Finland) and the time for the buffer control (50 ul of PBS/T and 50 ul of HRPO conjugated Mab) to achieve an optical density of 1.0 in approximately 10 minutes of development was calculated as described by Wright et al, 1985. The plate was re-read for colour development at that time. All readings were done at 414nm and room temperature. The cELISA results were expressed as percent inhibition (%I) and were derived from the mean optical density (OD) values for each sample by the following formula:

$$\%I = 100 - [(ODt \div ODc) \times 100]$$

where %I = percent inhibition

ODt = mean OD of test sample

ODc = mean OD of buffer control

Antigen titration

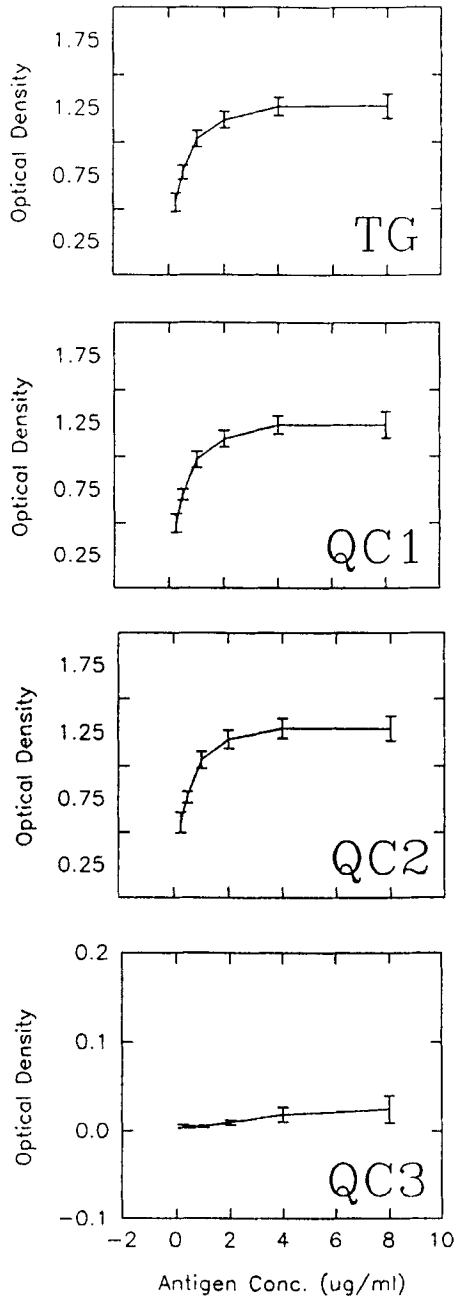
Antigen reconstituted as above was passively attached to the polystyrene matrix at concentrations of 0.25, 0.5, 1.0, 2.0, 4.0

and 8.0 ug/ml using the same conditions as above. Sera from QC3, QC1 and QC2 were tested and a buffer control was included. The targeting protocol was not used in this instance but rather, optical density readings were taken 10 min after the substrate\chromogen was added.

RESULTS

Repeat titrations of ten preparations (N = 40) of poly-L-lysine linked O-polysaccharide antigen with control sera QC3, QC1 and QC2 revealed that an antigen concentration of 2 ug/ml was optimal for the refined cELISA. These data are presented in Figure 2 (A to D). The 95 % confidence limit bars indicated that the binding was reproducible. This approach allows the analyst to track and produce consistent batches of antigen. If the titration values fall outside the confidence limits, the reagent may be rejected for use. The minor variations noted are in part caused by the methods of colour assessment used in that these titrations were not targeted to one reagent but read at 10 minutes of development. Thus day to day environmental conditions cause minor fluctuations. These fluctuations would normally be minimized by the targeting protocol in which all optical density readings are corrected by using an uninhibited buffer control as a standard (0% inhibition).

Using an antigen concentration of 2 ug/ml, cELISAs were performed with HRPo conjugated Mab to O-polysaccharide to which various ratios of Mab to HRPo were added (Table 1). The mixtures were prepared by adding unlabelled Mab to the HRPO conjugated Mab and then determining the total protein content (and subtracting the protein contributed by the HRPo) and the amount of HRPo present by scanning on a spectrophotometer (see Figure 1 for an example). The control sera used for antigen titration were also used for this



assessment and the results were expressed as %I values (Table 1). A low Mab to HRPo ratio resulted in rapid enzymatic degradation in the assay but its discriminative value for antibodies induced by vaccination and infection was not great and results with the vaccinated cow serum would indicate it as originating from an infected animal (the cutoff value has between positive and negative reactions was tentatively set at 20% inhibition). Conversely, a high Mab to HRPo ratio resulted in a delay of enzymatic degradation and considerable negative inhibition. An intermediate ratio of 2.66:1 appeared to be the most suitable in that the QC3 control (positive serum) gave almost total inhibition while neither the QC1

FIGURE 2

Titration of O-polysaccharide from Brucella abortus lipopolysaccharide using a Mab, specific for the O-polysaccharide, conjugated with HRPo. The Y-axis represents optical density units at 414 nm and the X-axis concentrations of antigen (ug/ml) used for passive attachment to the polystyrene matrix.

A. (TG) is the uninhibited control.
B. (QC1) is a serum obtained from a cow, immunized with heat killed B. abortus cells.
C. (QC2) is a serum from a cow that had not been exposed to B. abortus.
D. (QC3) represents inhibition by a serum from a cow infected with a field strain of B. abortus.

The 95% confidence limit bars represents data from 10 separate titrations.

TABLE 1

Effect of various ratios of Mab to HRPo on the sensitivity and development time of the cELISA for differentiation of cattle vaccinated with B. abortus strain 19 from field infected animals.

	Ratio of Mab to HRPo					
	0.66:1	1.33:1	2.00:1	2.66:1	3.32:1	3.98:1
TG[1]	0[6]	0	0	0	0	0
QC1[2]	20	16	12	4	-7	-21
QC2[3]	16	14	12	-1	-3	-10
QC3[4]	99	99	99	99	94	89
Time[5]	9.05	9.54	10.36	12.21	15.66	22.43

[1] Target = buffer (uninhibited) control.

[2] QC1 = serum from a cow immunized with heat killed **B. abortus** cells. This reagent behaves as serum from animals vaccinated with B. abortus strain 19. The % inhibition should normally be the range of 0 to 10%.

[3] QC2 = serum from an animal that had not been exposed to B. abortus. The % inhibition should be around 0%.

[4] QC3 = serum from a cow infected with B. abortus. The % inhibition should be between 95 and 100%.

[5] Time = number of minutes required for the buffer control to reach an optical density of 1.0. This time should be around 10 minutes.

[6] = % inhibition.

(serum from a vaccinated cow) or the QC2 (serum from a negative cow) sera gave significant inhibition.

DISCUSSION

The cELISA described previously (8) was intended to replace the less sensitive precipitation test developed by Jones et al (5), as the test for differentiating the antibodies resulting from vaccination or infection with B. abortus. However, application of the cELISA using purified unlinked O-polysaccharide from lipopolysaccharide as antigen resulted in poor binding capacity to solid matrix and subsequently resulting in variability in the assay performance. We have demonstrated that by attaching an 'anchor' to the O-polysaccharide in the form of a defined peptide (poly-L-Lysine) more efficient (avid) antigen binding can be produced for a reliable assay.

In addition, it was observed that highly purified Mab conjugated with HRPo had a decreased capacity to differentiate between the antibody responses of vaccinated and infected cattle. This decreased differentiation was hypothesized to result from minute amounts of highly conjugated Mab being displaced by non-specifically binding serum proteins present in both sera from vaccinated and non-exposed animals causing a measurable decrease in colour development. To overcome this problem, either the conjugation efficiency (the amount of HRPo conjugated to Mab) could be decreased or non-conjugated Mab could be added. The latter was chosen because it was found to be easier to control. Thus by reducing the sensitivity, the assay could differentiate between infected and non-infected cattle. However, if the sensitivity was reduced too much (using a higher Mab to HRPo ratio), negative inhibition occurred. This observation of more HRPo conjugated Mab

binding in the presence of diluted serum than in the uninhibited buffer control has not been explained. It is clear that for cELISA procedures, the Mab to HRPo ratio can influence the results and the interpretation of the results and therefore, when developing an assay of this type, this parameter should be carefully considered.

By improving the antigen binding, the quality control of antigen production and decreasing the sensitivity of the cELISA and increasing ratio of Mab to HRPo we have clearly improved the performance of the assay. These modifications have made it possible to use the cELISA in a diagnostic context for discrimination of bovine antibodies raised by vaccination and infection.

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