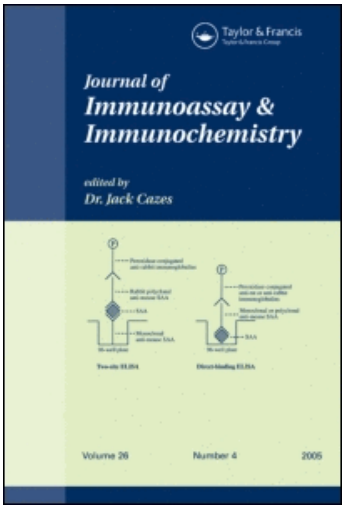

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Rough Lipopolysaccharide of *Brucella abortus* RB51 as a Common Antigen for Serological Detection of *B. ovis*, *B. canis*, and *B. abortus* RB51 Exposure Using Indirect Enzyme Immunoassay and Fluorescence Polarization Assay

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ABSTRACT

Rough lipopolysaccharide (RLPS) antigens were prepared from cultures of *Brucella abortus* RB51, *B. ovis*, and *B. canis*. The preparations were standardized by weight and tested with sera from cattle immunized with *B. abortus* RB51, sheep infected with *B. ovis*, and dogs infected with *B. canis.* Populations of unexposed animals of each species were also tested. The tests used were the indirect enzyme immunoassay (IELISA) using RLPS and the fluorescence polarization assay (FPA) using RLPS core fractions, labeled with fluorescein isothiocyanate. The IELISA using *B. abortus* RB51 RLPS antigen resulted in sensitivity and specificity values of 94.8% and 97.3%, respectively, when testing bovine sera, 98.5% and 97.8% when testing ovine sera, and 95.8% and 100% when testing dog sera. The IELISA using *B. ovis* RLPS antigen gave sensitivity and specificity values of 80.5% and 91.7%, respectively with bovine sera, 98.9% and 93.8% with sheep sera, and 70.8% and 79.8% with dog sera. The IELISA using *B. canis* RLPS antigen resulted in sensitivity and specificity values of 97.0% and 97.4%, respectively, with bovine sera, 96.2% and 96.3% with sheep sera, and 95.8% and 98.8% with dog sera. Labeling RLPS core from *B. ovis* and *B. canis* with fluorescein was not successful. *B. abortus* RB51 core labeled with fluorescein resulted in sensitivity and specificity values of 93.5% and 99.8%, respectively, with bovine sera and 78.1% and 99.0% with sheep sera. It was not possible to test the dog sera in the FPA.

Key Words: Lipopolysaccharide; *Brucella abortus*; Antigen; Serological detection; *Brucella ovis*; *Brucella canis*; Fluorescence polarization.

INTRODUCTION

Serological diagnosis of exposure to the "rough" *Brucella* sp. is difficult, partly because those species of *Brucella* lack the immunodominant *O*-polysaccharide in their lipopolysaccharide, which is the basis for most serological tests for brucellosis, and partly because of the nature of the cell surface antigens, which tend to autoagglutinate and sometimes cause activation of complement in the absence of antibody. Therefore, the agar gel immunodiffusion (AGID) test has been widely used with a hot saline extracted soluble antigen.[1] This assay has been shown to be as accurate as the complement fixation test (CFT) and less cumbersome.[2–5] Both the AGID and the CFT are difficult to standardize and only the CFT is recognized as a prescribed test for international trade.[1]

The indirect enzyme immunoassay for detection of antibody to *B. ovis* has been recognized as a more sensitive diagnostic test.[3,6–11] However, the use

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of a hot saline extracted antigen has been reported to cause cross reaction with sera from animals infected with *B. melitensis*, presumably by sharing epitopes with surface proteins, such as the outer membrane proteins[12] and cytosolic antigens such as the 18 kD antigen[13–15] or the 29 kD antigen.[16] This may be a considerable problem where infection occurs with both *Brucella* sp. or where animals have been vaccinated with smooth *Brucella* sp. such as *B. melitensis* Rev1. Therefore, perhaps a better antigen might be purified rough lipopolysaccharide (RLPS), as fewer epitopes would be shared.

Other assays for detection of antibody to *B. ovis*, including an immunoblotting technique[5,17,18] and a latex coagglutination test[19] for rapid detection of the causative organism, have been developed.

In this communication we have prepared RLPS from *B. ovis*, *B. canis*, and *B. abortus* RB51. Each RLPS preparation was tested with sera from *B. ovis* infected sheep, *B. canis* infected dogs, and *B. abortus* RB51 immunized cattle in IELISA. Core region of RLPS from *B. abortus* RB51 was prepared and labeled with fluorescein isothiocyanate and used to test the same sera in a fluorescence polarization assay (FPA).

EXPERIMENTAL

Serum Samples

Bovine sera: Sequential serum samples ($n = 77$) were obtained from five adult cattle immunized intramuscularly (IM) with 10^{10} heat killed *B. abortus* RB51 incorporated into Freund's complete adjuvant (FCA), followed by a second, similar IM injection 4 weeks later without FCA. Sera from Canadian cattle ($n = 400$), which had not been exposed to *Brucella* sp. were also used.

Sheep sera: Sera ($n = 265$) from flocks in which infection with *B. ovis* was demonstrated by isolation of the bacterium from at least one animal and which were determined to be AGID, CFT, and/or IELISA positive in the laboratory of origin were used. Sera from 405 non-exposed sheep were used as negative controls.

Dog sera: Sera from 23 dogs individually shown to be infected with *B. canis* by bacterial isolation and sera from 304 non-exposed dogs were used.

Non-exposure was determined for all species by lack of clinical, epidemiological, and serological evidence of brucellosis in the area.

Preparation of RLPS

The technique of Galanos et al.[20] was used. Briefly, 250 mL of a mixture of 90% v/v phenol : chloroform : petroleum ether in a ratio of 2 : 5 : 8 was used

to extract the RLPS from 8 gm of dry *B. abortus* RB51, *B. ovis*, or *B. canis* cells at room temperature. The mixtures were homogenized for 2 min using a Virtis Homogenizer and centrifuged at 4000*g* for 10 min at 4°C. The cells were re-extracted with an additional 250 mL of mixture, homogenized, and centrifuged. The two soluble extracts were pooled and the chloroform and ether were removed by evaporation, while the phenol was dialized out by numerous changes of deionized water (until no phenol phase was observable). The RLPS extracts were then freeze dried.

For the FPA, 25 mg *B. abortus* RB51 RLPS was added to 10 mL 4% v/v phosphoric acid and incubated at 100°C for 2 hr. After cooling, the cloudy hydrolysate was adjusted to a pH of 9.0 with 6 M sodium hydroxide and 25 mg fluorescein isothiocyanate dissolved in 0.5 mL 6 M sodium hydroxide was added. The slightly cloudy mixture was incubated at 37°C for 18 hr. The mixture was then applied to a 1 cm × 10 cm column containing DEAE Sephadex A25 equilibrated on 0.01 M phosphate buffer, pH 7.0. A volume of 50–60 mL was obtained after which the buffer was switched to 0.1 M phosphate, pH 7.0, with which a volume of 300 mL was obtained. Finally, a volume of 300 mL of 0.1 M phosphate buffer, pH 7.0 containing 0.1 M sodium chloride was passed through the column. The FPA active component was found in the effluent obtained with 0.1 M phosphate buffer with 0.1 M NaCl. This material was freeze dried.

Serological Tests

Indirect Enzyme Immunoassay

The RLPS (100 μL) was passively attached to NUNC 269620 96 well polystyrene plates at a concentration of 5 μg/mL in 0.06 M carbonate buffer, pH 9.6 by incubation at 18°C for 18 hr. Following four washing cycles, using 0.01 M phosphate buffer, pH 7.2 containing 0.15 M sodium chloride and 0.05% Tween 20 (PBST), 100 μL of sera, diluted 1 : 50 in PBST containing 15 mM EDTA and 15 mM EGTA, pH 6.3 were added for 30 min at 25°C. After four further wash cycles with PBST, 100 μL of a monoclonal antibody against bovine IgG_1 heavy chain, conjugated with horseradish peroxidase (HRPO) and appropriately diluted in PBST, was added when testing bovine and sheep sera. For the dog sera, protein G conjugated with horseradish peroxidase, also appropriately diluted in PBST, was used. Incubation with the detection reagent was 1 hr at 25°C, after which the plates were washed four times with PBST and 100 μL of 1 mM hydrogen peroxide and 4 mM 2,2′-azino-*bis*(3-ethylbenz-thiazoline-6-sulfonic acid) dissolved in 0.05 M citrate buffer, pH 4.5 was added for 10 min with continuous shaking. An optical

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density measurement was obtained at 414 nm and reactivity was calculated relative to the result obtained with a strongly positive serum as 100% positivity, using the formula:

$$\% \text{ Positivity} = \frac{\text{Optical density of the sample}}{\text{Optical density of the strong positive control}} \times 100$$

On each plate, a strongly positive, a weakly positive, and a negative serum sample were tested in duplicate. In addition, two wells containing buffer instead of serum were also included.

Fluorescence Polarization Assay

To 1.0 mL 0.01 M phosphate buffer, pH 6.5 in a 10 mm × 75 mm borosilicate glass tube 40 μL serum was added. After mixing, a blank reading was made using a Sentry FP analyzer (Diachemix Corp., WI), and 20 μL of antigen (RLPS core labeled with FITC) diluted to give an intensity value of between 300,000 and 400,000. After mixing and incubation at 18°C for a minimum of 2 min, a final fluorescence polarization reading was obtained. The result, expressed in millipolarization units, consisted of the final reading with the blank reading subtracted.

All chemicals were obtained from Sigma Fine Chemical Corp., MO.

Data

Results were analyzed using Receiver Operator Characteristic (ROC) analysis,[21] assuming all animals in the positive populations give positive reactions while the negative population was assumed negative. Percent sensitivity and specificity values were determined using cutoff values determined from the ROC analysis.

RESULTS

A summary of the data is presented in Table 1. The relative sensitivity values for testing sheep sera with the *B. ovis*, *B. abortus* RB51, and *B. canis* RLPS preparations using cutoff values of 6%, 21%, and 20% P, respectively, were 98.9%, 98.5%, and 96.2% and the respective relative specificity values were 93.8%, 97.8%, and 96.3%.

For the dog sera, the *B. ovis* RLPS gave a sensitivity value of 70.8% and a specificity value of 79.8% with a cutoff value of 3.0% P. Using *B. abortus* RLPS and a cutoff value of 38% P, the sensitivity and specificity were

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Table 1. Indirect ELISA and FPA results with ovine, bovine, and canine sera from animals exposed to *B. ovis*, *B. abortus* RB51, and *B. canis* and sera from non-exposed animals tested with RLPS prepared from *B. ovis*, *B. abortus* RB51 and *B. canis*.

			IELISA (RLPS)			FPA
Sera		*n*	*B. ovis*	*B. abortus* RB51	*B. canis*	*B. abortus* RB51
Sheep	Sens (%)	265	98.9	98.5	96.2	78.1
	Spec (%)	405	93.8	97.8	96.3	99.0
Dog	Sens (%)	23	70.8	95.8	95.8	ND[a]
	Spec (%)	304	79.8	100	98.8	ND
Cattle	Sens (%)	77	80.5	94.8	97.4	93.5
	Spec (%)	400	91.7	97.3	97.0	99.8

[a]ND, assay could not be performed.

95.8% and 100% while the *B. canis* RLPS gave values of 95.8% and 98.9%, respectively, using a cutoff value of 12% P.

Bovine sera from animals immunized with *B. abortus* RB51 gave sensitivity values of 80.5%, 94.8%, and 97.4% with *B. ovis*, *B. abortus* RB51, and *B. canis* RLPS using cutoff values of 36%, 35%, and 41% P. The specificity values obtained using sera from non-exposed cattle were 91.7%, 97.3%, and 97.0%, respectively.

The FPA results for the ovine sera were 78.1% relative sensitivity and 99.0% specificity. The dog sera could not be tested in the FPA as inconsistent results were obtained, probably due to the high lipid content of the sera. The sensitivity for bovine sera was 93.5%, while the specificity was 99.8% in the FPA.

The sensitivity and specificity values were compiled into a "performance index" for each species with each RLPS antigen in the IELISA and the FPA for comparison in Table 2.

DISCUSSION

A rapid slide agglutination test was developed for detection of antibody to *B. canis* using *B. canis* whole cell antigen rather than *B. ovis* cells, thereby, reducing the false positive rate to 10%.[22] Agglutination tests have not been reported for detection of antibody to *B. ovis* in sheep or *B. abortus* RB51 antibody in vaccinated animals. The most commonly used methods for serological diagnosis of *B. ovis* are precipitin tests, either the AGID[23,24] or radial immunodiffusion,[24] or the complement fixation test.[2,23,25–27] Of these, the

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Table 2. Performance indices (the sum of the sensitivity and specificity values) of the IELISA and FPA tests with RLPS or core region from *B. ovis*, *B. abortus* RB51, and *B. canis* with positive and negative sera of ovine, bovine, and canine origins.

	IELISA (RLPS)			FPA
	B. ovis	*B. abortus* RB51	*B. canis*	*B. abortus* RB51
Sheep	192.7	*196.3*	192.5	177.1
Dog	150.6	*195.8*	194.6	NA
Cattle	172.2	192.1	*194.4*	193.2

Note: The highest value for each species is presented in italics.

precipitin tests appear to be the most accurate, however, both types of tests have several disadvantages in that they are labour intensive, difficult to standardize, slow, require large amounts of reagents, and the results are interpreted subjectively. A counterimmunoelectrophoresis technique was developed for detection of *B. ovis* antibody to reduce test turn around time.[28] Similarly, an immunoblotting technique was developed to increase diagnostic sensitivity,[17,18] as well as a radioimmunoprecipitation test,[29] an immunofluorescence test,[30] and a latex coagglutination test for antigen detection.[19] None of the latter tests have found widespread diagnostic use.

For detection of antibody to *B. abortus* RB51, CFTs were developed.[31,32]

A large number of IELISA procedures for detection of *B. ovis* antibody have been reported.[2,3,5,7,10,11,13–47] No reports of IELISA procedures for detection of antibody to *B. canis* and *B. abortus* RB51 were readily available.

As is the case with all ELISA assays, the accuracy depends on the antigen and the detection system used. Thus, a hot saline extracted antigen contains a number of surface protein antigens as well as RLPS.[5,15,18,31] The presence of surface proteins may cause cross reaction with smooth *Brucella* sp. and possibly other bacteria,[39,43,45,46,48] while RLPS appears to cross react less.[11,18,29,35,39,49,50]

Similarly, the antiglobulin–enzyme conjugate specificity can enhance the accuracy of the IELISA. A specificity value of 84% was obtained using a protein G HRPO conjugate[44] and Marin et al.[46] found protein G conjugate to give better sensitivity than a monoclonal and a polyclonal anti-immunoglobulin conjugate. Similarly, Ficapal et al.[10] found protein G enzyme conjugate to perform better than a polyclonal anti-sheep IgG conjugate, but in this case, neither IELISA were found to improve on the diagnostic accuracy of the CFT or the AGID. A diagnostic specificity value of 100% was obtained with a monoclonal anti-bovine IgG_1–HRPO conjugate along with a diagnostic sensitivity value of 96.4%.[11] These results are similar to those obtained by Gall et al.[47]

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The premise of this study was to try to identify an antigen and a procedure for identification of antibody in important species using single assay format. This was achieved using the *B. abortus* RB51 RLPS antigen in that by reviewing the data presented in Tables 1 and 2, it is clear that this antigen gave the highest sensitivity and specificity results when testing ovine sera for antibody to *B. ovis* and dog sera for antibody to *B. canis*. The bovine sera gave a slightly higher sensitivity value with *B. canis* RLPS, while the specificity values for the *B. abortus* RB51 and *B. canis* RLPS preparations were similar. It is likely that increasing the sample size ($n = 77$) would enhance the sensitivity value obtained with the *B. abortus* RB51 RLPS antigen. Therefore, the only dissimilarity between the three assays was the detection system. Sufficient cross reaction between the bovine and ovine IgG_1 heavy chains allowed the use of a single monoclonal antibody–enzyme conjugate for detection of antibody. However, dog IgG does not cross react, necessitating the use of protein G–enzyme conjugate for detection of dog antibody. Alternately, protein G–enzyme conjugate could be used for all three assays, however, it would increase the assay price.

The FPA using *B. abortus* RB51 RLPS core antigen was unable to detect antibody to *B. ovis* in 21.8% of the sheep sera tested, however, the specificity of the assay was 99.0%, eliminating nearly all false positive reactions. The same FPA did not work when testing dog sera, most likely due to their lipid content causing light scatter. It is interesting to note that human sera could be tested in a similar FPA in spite of their lipid content.[51] The FPA for bovine antibody to *B. abortus* RB51 gave sensitivity and specificity values similar to those obtained with the IELISA and it could, therefore, be used as an assay to detect antibody due to vaccination. These data are in agreement with that of Conde et al.[52]

In summary, a single antigen, *B. abortus* RB51 RLPS was found to be suitable for detection of antibody to *B. ovis, B. abortus* RB51, and *B. canis* in sheep, cattle, and dogs, with a high degree of accuracy in a technically simple assay. The FPA, using *B. abortus* RB51 RLPS core antigen, was found to work well detecting bovine antibody to the homologous bacterium, but not with sheep antibody to *B. ovis* or with dog sera.

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