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Comparison of the Antibody Response in Adult Cattle Against Different Epitopes of *Brucella abortus* Lipopolysaccharide

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With 4 figures

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Summary

The comparison of serological responses in a sample of adult, vaccinated and field-infected bovines with *Brucella abortus* is reported. Indirect enzyme immunoassay (EIA) titration curves and Western blotting tests for smooth-type lipopolysaccharide (S-LPS), rough-type LPS (R-LPS) and lipid A were performed. In the initial screening of sera, an overall prevalence of 20.5 % was found, which corresponds to a country with a high incidence of brucellosis. End-point EIA titres against LPS antigens from vaccinated and field-infected cows were not significantly different. However, the absorbance values in the titration curves were significantly higher for S-LPS as compared with the other antigens. A high correlation coefficient ($r = 0.933$) was obtained when the titres to R-LPS versus lipid A were compared. Western blotting reactions of vaccinated and field-infected animals were indistinguishable. S-LPS, R-LPS and lipid A epitopes were recognized in a heterogeneous manner. In general, the number of bovines that reacted against LPS was higher in the field-infected group, with a stronger binding to S-LPS. Based on our observations, the vaccinated and field-infected bovines are capable of producing similar antibody responses to the *Brucella* main outer surface antigen, LPS. It should be emphasized that the humoral response of cattle to *Brucella* LPS contains significant amounts of antibodies to other antigenic moieties of this important surface molecule, which may contribute to the immunity to brucellosis.

Introduction

Bovine brucellosis is caused by the Gram-negative, facultative intracellular bacterium *Brucella abortus* which is responsible for considerable economic losses due to abortion, low milk production and infertility in cattle, as well as for severe chronic human health problems (Corbel, 1997). The vaccination of young animals with *B. abortus* S19 has been used for many years in the control of brucellosis in many countries, including Costa Rica (Gall et al., 1998). Moreover, many farmers are now re-vaccinating herds every 2 years.

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Consequently, it can be difficult to distinguish between S19-vaccinated and naturally infected cattle using serologic tests when attempting to identify and remove cattle with brucellosis from vaccinated herds (Nielsen et al., 1996). Antibody detection has been a useful tool in the bovine brucellosis eradication programmes established in many countries. The majority of the tests employ whole cells and smooth-type lipopolysaccharide (S-LPS) preparations as binding antigens, the latter with variable amounts of contaminant proteins, which further complicates the interpretation of the results (Nielsen et al., 1988). Other antigens used in serological assays include the carbohydrates polysaccharide B and native hapten (Díaz et al., 1981; Moreno et al., 1987).

LPS from *Brucella* is composed of a lipid A portion immersed in the outer leaflet of the outer membrane, a core oligosaccharide, and a long polysaccharide of N-formylperosamine, the O-chain (Cherwonogrodzky et al., 1990). Because LPS is the major antigenic component of the outer membrane in *Brucella*, a large proportion of the humoral response of cattle is directed to LPS, particularly to its more exposed region, the O-chain. However, little is known about the recognition of 'deeper' domains in the LPS molecule, such as the core oligosaccharide and lipid A (Rojas et al., 1994). In order to investigate the specificity of bovine sera to the different domains of *B. abortus* LPS, several immunochemical tests were performed with positive sera, and the results were evaluated considering previous findings on the epitopic distribution of the molecule.

Materials and Methods

Bacterial strains

Brucella abortus strain S19 (vaccine, attenuated, smooth strain) and *B. abortus* 45/20 (avirulent, rough strain) were grown on blood agar plates and on tripticase-soy broth for batch production at 37°C under an atmosphere of 5% CO_2 for 48 h.

Preparation of bacterial antigens

LPS from *B. abortus* S19 (S-LPS) was extracted and purified following the hot phenol/water method described previously (Moreno et al., 1981). LPS from rough *B. abortus* 45/20 (R-LPS) was extracted using the phenol–chloroform–petroleum ether method (Galanos et al., 1969). R-LPS was further purified by nuclease and proteinase treatment followed by ultracentrifugation and gel filtration chromatography. Lipid A from strain 45/20 was obtained by acetic acid hydrolysis (2% acetic acid at 100°C for 3 h) of LPS. Lipid A was further purified by thin-layer chromatography (Freer, 1990).

Sera

A total of 176 sera from adult bovines distributed in dairy farms of the Valle Central, Costa Rica were collected. Animals were divided into two groups: (i) non-vaccinated, field-infected ($n = 67$); and (ii) vaccinated cows ($n = 114$). The disease status of the cattle was determined by reviewing historical vaccination and clinical records, and by assessing serodiagnostic results. All sera were stored at –20°C in 50 % glycerol.

Serological assays

All fluid phase assays indicated below were performed in triplicate on two or more separate occasions. Indirect enzyme immunoassay (EIA) was carried out as preliminary screening for antibodies to *B. abortus* using the method of the Joint FAO/IAEA Programme (1992). Sera positive in the initial screening were diluted serially starting at 1:10 and an EIA was performed. Briefly, polystyrene 96-well plates (Nunc-Immunoplate, Rochester, NY, USA) were coated with 100 μl of S-LPS (5 μg/ml) or R-LPS and lipid A (10 μg/ml) in 0.1 M carbonate buffer (pH 9.6) overnight at

37°C. The next day, the plates were washed three times with 0.01 M phosphate-buffered saline (PBS), pH 7.2, containing 0.05 % Tween 20 and 0.1 % casein (wash buffer). After washing, 100 μl of serum diluted in PBS–0.05 % Tween 20 was added in triplicate and incubated for 1 h at 37°C. After washing three times as above, 100 μl of anti-bovine immunoglobulin (Ig) serum conjugated to horseradish peroxidase (HRPO) was added for 1 h at 37°C. Bound antibodies were detected by the addition of 100 μl of 1 mM hydrogen peroxide and 10 mM 2,2′-azinobis(3-ethylbenzthiazoline)-6-sulphonic acid (ABTS, Sigma, St Louis, MO, USA) in 0.05 M citrate buffer, pH 4.5. The plates were read in a Titertek Multiscan (Titertek Instruments Inc., Huntsville, AL, USA) microplate reader at 405 nm after 30 min. The end-point EIA titres were estimated as the highest dilution giving an absorbance equal to 0.200. The results from the EIA were expressed as mean $\log_{10}$ titre $\pm$ standard deviation (SD).

Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) for the separation of LPS and lipid A was carried out in 12 and 15 % discontinuous gels in a Mini Protean II unit (Bio-Rad, Richmond, CA, USA). The transfer of the antigens to nitrocellulose membranes was performed as previously described (Rojas et al., 1994). Briefly, the gels were electrotransferred in a semidry transblot cell (Bio-Rad) for 15 min at 15 V. After blocking overnight at 4°C with PBS–0.05 % Tween 20–2 % casein, the membranes were incubated for 2 h at 37°C with sera diluted 1:500, followed by washing as above. After incubation for 1 h at 37°C with the same conjugate as in the EIA, the membranes were washed and the reaction was visualized by adding 0.06 % 4-chloro-1-naphthol in 0.02 M triethanolamine buffer, pH 7.5.

Statistical analysis

Student's *t*- or *Z*-tests were performed for comparison of the results from the EIA tests.

Results

S-LPS extracted from *B. abortus* S19 contained approximately 5 % protein, and further purification reduced its concentration to 1–2 %. R-LPS from *B. abortus* 45/20 showed little amounts of protein (< 1 %). Lipid A yielded less than 0.1 % protein. Nucleic acids were not detectable in all antigens tested. Because considerable amounts of O-polysaccharide epitopes have been found in lipid A preparations from S-LPS, lipid A from the rough 45/20 strain was used (Freer et al., 1995). Electrophoretic analysis of the LPS preparations showed characteristic patterns; the S-LPS was composed of a wide smear of high molecular weight (100–200 kDa) and two to four bands of low molecular weight (30–80 kDa). The R-LPS showed only the low molecular weight bands and the lipid A was observed as a discrete, low molecular weight, broad band migrating at the bottom of the gel (less than 10 kDa).

A total of 176 animals were screened for antibody to *B. abortus* S-LPS. Thirty-six (20.5 %) sera were positive, considering as positive an absorbance greater than 0.200 at 405 nm after 30 min, in the indirect EIA test of the Joint FAO/IAEA Programme (1992). From these, 11/114 (9.64 %) were animals vaccinated in the preceding year, and 25/67 (37.31 %) were non-vaccinated, field-infected animals. Positive sera were selected for further immunochemical assays.

The reactivity of these 36 sera against the three domains of *B. abortus* LPS was evaluated by their titre in the indirect EIA, using the antigens prepared from LPS of smooth and rough strains (Fig. 1). The results showed a wide variation in the titres, ranging from 2.920 to 8.680 log of reciprocal dilution. Titres from vaccinated and field-infected cows were not significantly different. In general, the values were significantly higher for S19 LPS as compared with the other antigens, and the differences were maintained throughout the dilutions higher than 1:100 (Fig. 2). To evaluate this difference further, coefficients of correlation (r) for the EIA titres against the different LPS moieties were calculated. The highest correlation ($r = 0.933$) was obtained when the titres to R-LPS versus lipid A moieties were compared. The other comparisons of EIA titres

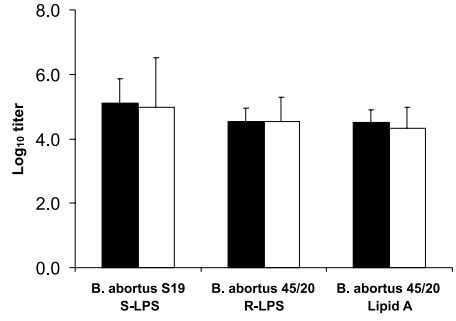

Fig. 1. Comparison of antibody titres from vaccinated and non-vaccinated bovines directed against the three *Brucella abortus* lipopolysaccharide (LPS)-derived antigens. Titres are expressed as $\log_{10}$ of the maximum dilution positive of at least three independent experiments.

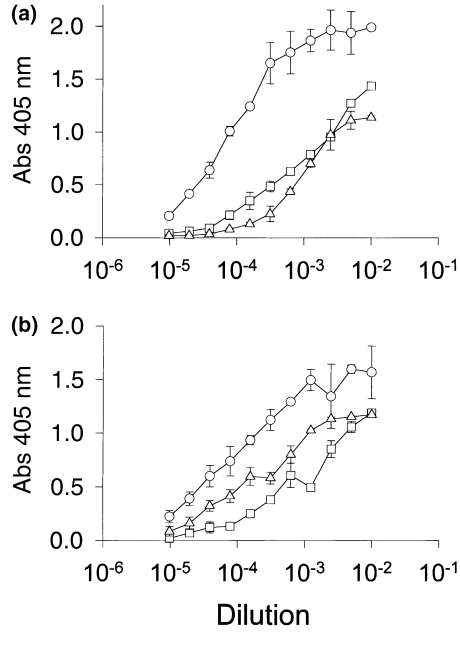

Fig. 2. Antibody titration curves from 11 vaccinated (a) and 25 field-infected (b) bovine sera directed against the three *Brucella abortus* lipopolysaccharide (LPS)-derived antigens: smooth-type (S)-LPS (○); rough-type (R)-LPS (□); lipid A (△). Points represent the mean of triplicate readings ± standard deviation of at least three separate experiments.

generated coefficients significantly different, that is 0.459 when S-LPS versus R-LPS, and 0.562 when S-LPS versus lipid A were compared.

Electrophoresis and immunoblotting for each of the three antigens used in the EIA were performed. Representative reactions are shown in Fig. 3. Reactions of vaccinated and field-infected animals were indistinguishable. Typical reactions were directed to S-LPS epitopes only (Fig. 3, lanes 4–6), S-LPS and R-LPS epitopes (Fig. 3, lanes 1–3), S-LPS and lipid A epitopes (Fig. 3, lanes 7–9) and, less frequently, S-LPS, R-LPS and lipid

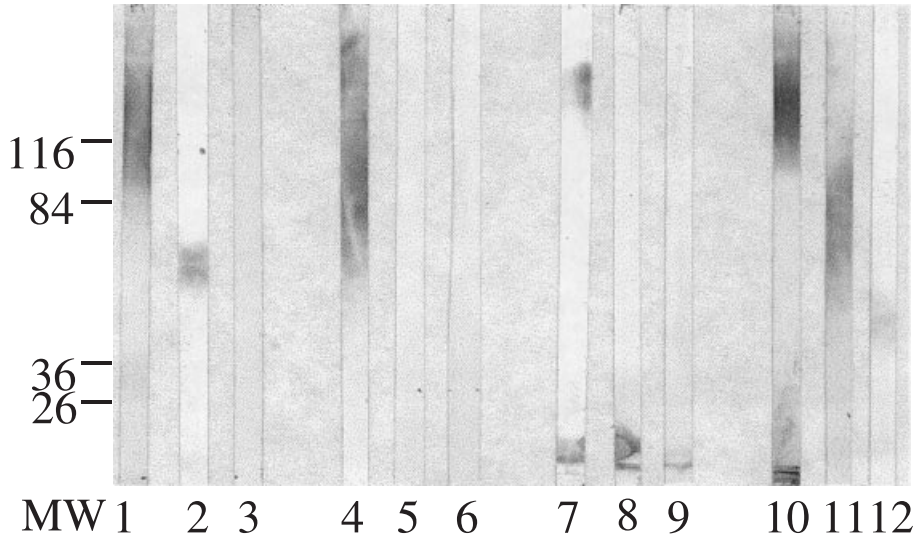

Fig. 3. Western blot of representative bovine sera against *Brucella abortus* lipopolysaccharide (LPS) antigens. Triplets of lanes (1–3, 4–6, 7–9, 10–12) represent four different animals. Lanes 1, 4, 7, and 10 are smooth-type (S)-LPS; lanes 2, 5, 8, and 11 are rough-type (R)-LPS; lanes 3, 6, 9, and 12 are lipid A. Molecular weight markers (MW) are indicated on the left.

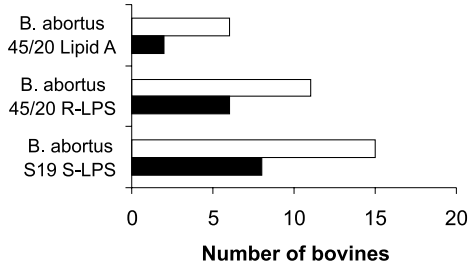

Fig. 4. Comparison of Western blot reactions of bovine sera against *Brucella abortus* lipopolysaccharide (LPS) antigens. Open bars represent the number of sera from field-infected animals ($n = 25$) and filled bars represent the number of sera from vaccinated bovines ($n = 11$).

A epitopes (Fig. 3, lanes 10–12). In general, the number of bovines that reacted against LPS was higher in the field-infected group, with a stronger binding to S-LPS. Also, a decrease in binding was observed when R-LPS and lipid A antigens were used (Fig. 4). Only one serum from a field-infected animal showed a negative reaction to S-LPS, but it bound to R-LPS and lipid A.

Discussion

In this work we have compared the humoral immune response of vaccinated and field-infected bovines from Costa Rican herds against *B. abortus* LPS antigens. We found an overall prevalence of 20.5 % in the sample evaluated, which corresponds to a country with a high incidence of brucellosis (Gall et al., 1998).

Independent of the type of exposure to the pathogen, i.e. by vaccination or by natural infection, bovines generated similar amounts of antibodies against LPS antigens, as supported by both the antibody titres and immunoblotting reactions. However, it has been suggested that in a natural infection, the pathogen remains viable and immunogenically active for a longer time than in vaccination, so this might explain the shorter duration of immunity after vaccination (Nielsen and Duncan, 1988). Given the intracellular nature of *Brucella*, it may be possible that the avidity of antibodies matured after natural infection is higher than that generated after vaccination, but a deeper analysis of the cellular response is needed to support this hypothesis.

The EIA results have also shown that although the majority of the antibody response is directed to S-LPS, a significant amount is also directed to the inner components of the molecule. We found that all positive sera contained antibodies against the three LPS antigens, as shown by end-point titres. In addition, the titration curves and the correlation coefficients indicate that the avidity for the outermost portion of LPS, the O-polysaccharide, is different from that directed to inner epitopes. In addition, the high correlation between the reaction to R-LPS and lipid A suggests that these antigens stimulate a similar antibody response. It has been demonstrated that four major isotypes (i.e. IgG_2, IgG_1, IgM and IgA) are produced in *Brucella* vaccination or infection (Nielsen and Duncan, 1988). Therefore, it may be hypothesized that IgG_2 and IgG_1 of high avidity are predominant in the binding to O-polysaccharide epitopes, and IgM and IgA predominate in the binding to R-LPS and lipid A epitopes, with less avidity than the former. Our previous results with the production of murine monoclonal antibodies showed that the major isotypes for O-polysaccharide epitopes were IgG_2 and IgG_1, whereas IgM and IgG_3 were produced against R-LPS (core oligosaccharide) and lipid A epitopes (Rojas et al., 1994).

The Western blotting results clearly showed a significantly higher frequency of field-infected bovines with antibodies that bound to LPS antigens. Also, a more evident decrease in the strength of binding and the number of sera reactive to inner epitopes was observed. Nevertheless, the difference in the conformation of antigens in fluid or solid phase should be considered when analysing the relative strength of binding. The patterns of reactivity vary extensively in the sera studied, with no obvious difference between vaccinated and field-infected animals, suggesting individual differences in humoral immune responses among cattle.

Based on our observations, the vaccinated and field-infected bovines are capable of producing similar antibody responses to the *Brucella* main outer surface antigen, LPS. The reactions against deeper domains of LPS are not sufficient to discriminate infection from vaccination, although significant differences in EIA curves and Western blotting reactions were found. However, it should be emphasized that the humoral response of cattle to *Brucella* LPS contains significant amounts of antibodies to other antigenic moieties of this important surface molecule, which may contribute to the immunity to brucellosis.

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