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Brucella abortus 1119-3 O-Chain Polysaccharide To Differentiate Sera from *B. abortus* S-19-Vaccinated and Field-Strain-Infected Cattle by Agar Gel Immunodiffusion†

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Purified *Brucella abortus* 1119-3 and *Brucella melitensis* 16M lipopolysaccharide O-chain polysaccharides were not precipitated in agar gel immunodiffusion by any of 24 sera from vaccinated cattle but were precipitated by 18 of 24 sera from infected cattle. This difference can be used to differentiate sera of cattle vaccinated with *B. abortus* S-19 from sera of some field-strain-infected cattle.

Brucella abortus is a bacterium that can cause abortions in cattle and a debilitating fever (undulant fever) that may persist intermittently for years in humans. In many parts of the world, vaccination of cattle is done by inoculating calves with *B. abortus* S-19, an attenuated strain (11). Although *B. abortus* S-19, is effective as a vaccine against brucellosis, protection is not absolute (1), and the attenuated strain has antigenicity similar to that of virulent strains of *B. abortus*, a fact which may complicate serodiagnostic interpretations (6).

Methods have been reported for the discrimination of *B. abortus* S-19-vaccinated and field-strain-infected cattle. Diaz et al. (7) described polysaccharide (PS) B (poly-B), which was isolated from rough-strain *B. melitensis* B115 and which was precipitated by sera of *B. abortus*-infected cattle but not by sera of those vaccinated with *B. abortus* S-19. Raybould and Chantler (14) reported that sodium dodecyl sulfate extracts of *B. abortus* 544/W contained antigen-X which, when bound nonspecifically to Sepharose beads, was precipitated by sera of infected but not vaccinated cattle. Lawman et al. (9) found that the immunoglobulin G2 anti-*Brucella* antibody response of cattle was considerably higher for infected than for vaccinated animals. The work reported here shows that the O-chain PS of *B. abortus* or *B. melitensis* can be used in an agar gel immunodiffusion (AGID) test to differentiate between some *B. abortus* S-19-vaccinated and field-strain-infected cattle.

MATERIALS AND METHODS

Antigens. For purification of *B. abortus* 1119-3 O-chain PS, killed cells (in 2% phenol) were washed and suspended (100 g [wet weight] of cells per 500 ml) in 2% (vol/vol) acetic acid–10% NaCl. The suspension was autoclaved (15 lb/in^2, 121°C, 30 min), cooled (room temperature), and centrifuged, and the supernatant was saved. Five volumes of methanol containing 1% sodium acetate were added and, after chilling (4°C, 18 h), the precipitate was recovered by centrifugation. The pellet was dissolved in and dialyzed against Tris-saline (1% NaCl, 0.02% NaN_3, 0.12% Tris hydrochloride [pH 7]) at 4°C. The preparation was digested with 25 μg each of lysozyme, RNase, and DNase (Sigma Chemical Co.) per ml for 6 h (room temperature, continuous stirring) and then with 50 μg of proteinase K per ml for 48 h. An equivalent volume of 90% aqueous phenol was added, extraction was done at 70°C (30 min, with stirring), and the solution was allowed to stand at 4°C overnight. The PS was precipitated from the phenol layer, washed twice with methanol-acetate (suspension was done with a Polytron [Brinkman Instruments, Canada, Ltd.]), and dissolved in and dialyzed against Tris-saline. Particulate material and lipopolysaccharide (LPS) were removed by ultracentrifugation (100,000 × *g*, 18 h, 4°C). The supernatant was dialyzed against double-distilled water and lyophilized (yield, 700 mg [dry weight] of PS per 100 g [wet weight] of cells). The powder was dissolved in 0.05 M pyridinium acetate at pH 4 (350 mg of PS per 3.5 ml) and eluted through a Sephadex G-50 column (2.5 cm [diameter] by 100 cm [height]), and the voided-volume fraction was collected and lyophilized. The final yield was 500 mg (dry weight) of PS per 100 g (wet weight) of cells. The preparation contained less than 1% protein (as determined with gamma-chain immunoglobulin G fraction III as the standard by the method of Lowry et al. [10]). *Yersinia enterocolitica* O:9 PS (4) and *B. melitensis* 16M PS were purified as previously described (2).

Smooth LPS (S-LPS) of *B. abortus* 1119-3 was prepared as described previously (5). Alkali-treated S-LPS was prepared by dissolving 10 mg of S-LPS in 1 ml of double-distilled water, adding 0.25 ml of 1 N NaOH, and heating the mixture in a water bath (60°C, 15 min) with occasional swirling. The mixture was cooled to room temperature, 0.25 ml of 1 N HCl was added, as was 0.25 ml of 0.1 M Tris hydrochloride (pH 7.2), and the pH was adjusted with NaOH or HCl to 7.2. Tris-saline was added so that the final concentration of alkali-treated S-LPS was 4 mg/ml.

Rough LPS (R-LPS) was prepared from *B. abortus* 45/20 grown on agar medium (5). The aqueous phase of a hot phenol water extraction was dialyzed against Tris-saline, digested with lysozyme, RNase, DNase, and proteinase K, and ultracentrifuged (100,000 × *g*, 18 h, 4°C). The clear gel was R-LPS, as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis followed by silver nitrate staining (16).

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† We dedicate this paper to the memory of the late L. M. Jones, whose contributions included preparations of polysaccharide B and encouraging discussions.

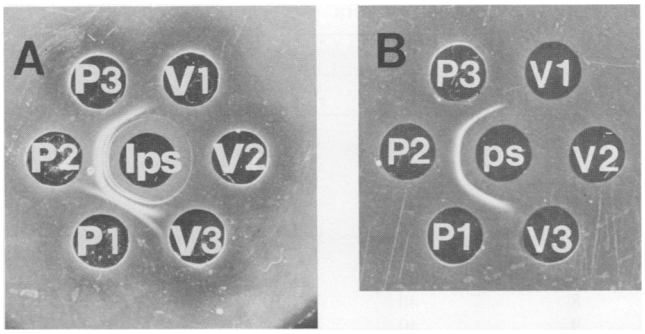

FIG. 1. AGID of cattle sera against *B. abortus* 1119-3 antigens. Undiluted sera of S-19-vaccinated cattle are V1, V2, and V3; sera of field-strain-infected cattle are P1, P2, and P3. Antigens were alkali-treated S-LPS (lps) at 4 mg/ml (A) or O-chain PS (ps) at 1 mg/ml (B). The gels contained 10% NaCl.

Purified poly-B, identified as a cyclic 1,2-linked β-D-glucan, was prepared from *B. melitensis* 16M and *B. melitensis* B115 as described by Bundle et al. (2). Poly-B preparations from these same strains were gifts from L. M. Jones (University of Wisconsin—Madison, Madison).

Sera. Sera from 10 *B. abortus*-negative cattle (as judged by serology and postmortem bacterial culturing of tissues), 24 S-19-vaccinated (with 3×10^{10} *B. abortus* S-19 cells) cattle (16 as 8-month-old calves and 8 as 2-year-old adults, all bled from 3 weeks to 6 months postinoculation), and 24 *B. abortus*-positive cattle (as determined by serology and confirmed by culturing of the bacterium from tissues) had been stored at −20°C. A pool of positive sera was formed by combining equivalent amounts of high-titer sera from five *B. abortus*-positive cattle.

Cross-absorbed sera were prepared by suspending phenol (2%)-killed cells of *Y. enterocolitica* O:3 or *Y. enterocolitica* O:9 (washed in Tris-saline buffer) in the pool of positive sera (1:5 [vol/vol]) and incubating the mixture in a 37°C water bath for 1 h with occasional stirring. The mixture was then centrifuged, and the supernatant was absorbed four more times.

Mouse monoclonal antibodies. The preparation and characterization of these antibodies have been described previously (3). Mouse ascites fluids containing monoclonal antibodies were used undiluted.

Serological tests. AGID medium (13) was prepared with 10% NaCl, 0.1 M Tris hydrochloride [pH 7.2], and 0.7% agarose. Five-millimeter-diameter wells were cut into the gel, and 20-μl test samples were loaded into each well. All antigens were applied at 1 mg/ml, except for alkali-treated *B. abortus* 1119-3 S-LPS, which was applied at 4 mg/ml. Cattle sera were applied undiluted. Incubation was for 24 h at room temperature (no significant differences were observed at 3 days of incubation). Following 24 h of washing in 0.15 M NaCl, the agar films were dried and stained with Coomassie blue R250 in acidified alcohol.

The complement fixation test was done as described by Samagh and Boulanger (15). The standard tube agglutination test was carried out by doubly diluting serum samples starting from 1:50 to 1:3,200. The indirect enzyme-linked immunosorbent assay (ELISA) was performed as outlined by Nielsen et al. (12) with a heavy-chain-specific antibody to bovine immunoglobulin G1 as the detection reagent and target, as described by Wright et al. (17).

RESULTS AND DISCUSSION

Alkali-treated S-LPS was precipitated by sera of vaccinated and infected cattle (Fig. 1A), but a second precipitin band seen in reactions with sera from infected cattle was not evident in reactions with sera from vaccinated cattle. Neither infected- nor vaccinated-cattle sera precipitated R-LPS (1 mg/ml) (data not shown), indicating that their antibodies were directed against the O-chain PS region of S-LPS rather than the core or lipid A moieties. When tested against purified *B. abortus* 1119-3 PS, the sera from 10 uninfected and 24 *B. abortus* S-19-vaccinated cattle produced no detectable precipitation, while 18 of the 24 sera from *B. abortus*-infected cattle precipitated this antigen (Fig. 1B). Similar results were observed with *B. melitensis* 16M PS (data not shown). Therefore, PS or alkali-treated *B. abortus* 1119-3 S-LPS was able to differentiate the sera of vaccinated cattle from the sera of some infected cattle. Two explanations are possible for the observed differentiation; either there were differences in the amounts of antibodies against *B. abortus* in the sera of the vaccinated and infected cattle or these antibodies differed in their specificities and/or affinities for *B. abortus* antigens. Table 1 shows that both explanations are likely. Sera of some field-strain-infected cattle had low anti-*Brucella* titers (as judged by the complement fixation test, standard tube agglutination test, and ELISA results; e.g., sera 1 and 2) and did not precipitate PS in the AGID. However, other infected cattle with low anti-*Brucella* titers (e.g., *B. abortus*-infected-cattle sera 3 and 5) precipitated PS, while vaccinated cattle with high anti-*Brucella* titers (e.g., *B. abortus* S-19-vaccinated-cattle sera 23 and 24) did not precipitate PS. These latter results suggest that the antibodies of *B. abortus* field-strain-infected cattle differ qualitatively from those of *B. abortus* S-19-vaccinated cattle by their specificity and/or affinity for PS.

The above-described precipitation patterns were similar to those observed for two types of mouse monoclonal antibodies raised against the PS of *Y. enterocolitica* O:9, which has an LPS O-chain identical to that of *B. abortus* 1119-3 (3). Figure 2 shows that mouse monoclonal antibodies YsT9-2 and -4 precipitated PS and S-LPS (which contains PS) of *B. abortus* 1119-3; YsT9-1, -3, and -7 precipitated only S-LPS. These differences have been suggested as being due to

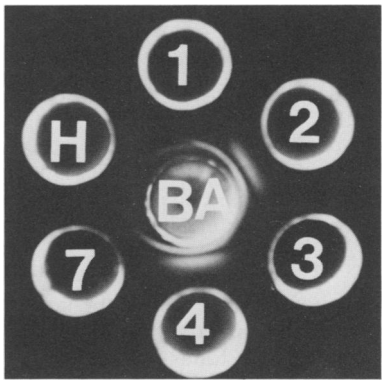

FIG. 2. AGID of mouse monoclonal antibodies against *B. abortus*1119-3 antigens. Undiluted mouse ascites fluids with antibodies of YsT9 hybridomas are 1, 2, 3, 4, and 7 (H, hyridoma F5-3-E4 raised against human blood group B, served as a control). BA is a mixture of 1 mg of O-chain PS and 4 mg of S-LPS (untreated with alkali) per ml. The gel contained 1% NaCl (10% was found inhibitory for the formation of an antibody-antigen precipitate).

TABLE 1. Comparison of serological tests used to evaluate cattle sera

B. abortus-infected-cattle serum	Result in:				*B. abortus* S-19-vaccinated-cattle serum	Result in:			
	CF[a]	SAT[b]	AGID[c]	ELISA[d]		CF[a]	SAT[b]	AGID[c]	ELISA[d]
1	10	200	−	0.55	1	20	100	−	0.63
2	20	400	−	0.75	2	20	100	−	0.70
3	40	200	+	0.85	3	20	100	−	0.81
4	40	400	−	1.23	4	20	200	−	0.55
5	40	800	+	0.92	5	20	200	−	0.85
6	80	800	−	1.25	6	20	200	−	0.59
7	80	800	+	1.06	7	20	200	−	0.73
8	80	1,600	−	1.33	8	40	200	−	1.03
9	80	1,600	+	1.30	9	40	200	−	0.85
10	160	800	+	1.45	10	40	200	−	0.96
11	160	1,600	+	1.48	11	80	400	−	1.25
12	320	400	+	1.55	12	80	400	−	1.37
13	320	3,200	−	1.90	13	80	800	−	1.50
14	320	≥3,200	+	≥2.00	14	80	1,600	−	1.69
15	320	1,600	+	≥2.00	15	160	400	−	1.94
16	640	800	+	≥2.00	16	160	800	−	1.83
17	640	3,200	+	≥2.00	17	160	800	−	2.00
18	640	≥3,200	+	≥2.00	18	160	1,600	−	≥2.00
19	≥640	≥3,200	+	≥2.00	19	320	1,600	−	≥2.00
20	≥640	≥3,200	+	≥2.00	20	320	1,600	−	≥2.00
21	≥640	≥3,200	+	≥2.00	21	320	1,600	−	≥2.00
22	≥640	≥3,200	+	≥2.00	22	320	3,200	−	≥2.00
23	≥640	≥3,200	+	≥2.00	23	640	1,600	−	≥2.00
24	≥640	≥3,200	+	≥2.00	24	640	1,600	−	≥2.00

[a] CF, Complement fixation test. Titers represent the reciprocal of the serum dilution which reduced complement activity by 50% or more.
[b] SAT, Standard tube agglutination test. Titers represent the reciprocal dilution that agglutinated 50% or more of the antigen.
[c] +, Precipitation of serum with 1 mg of PS per ml; −, no precipitation.
[d] Values are the optical densities at 414 nm. Normal negative cattle sera yielded values lower than 0.200.

"length" and "tip" specificities, respectively; i.e., antibodies of the former specificity can bridge PS (whether purified or as part of S-LPS) to form an antibody-antigen precipitate, whereas antibodies of the latter specificity can only precipitate PS as part of S-LPS, owing to the participation of micelle interaction (3). These differences may explain the different characteristics of cattle sera used in this study. Cattle chronically infected with field strains of *B. abortus* may raise antibodies with both of the noted specificities. Cattle vaccinated with whole cells of the attenuated strain S-19 are exposed to the bacterium only briefly and may produce only antibodies to the surface tip O-chain epitopes.

Only sera of some infected cattle were observed to precipitate PS in the AGID (only 18 of 24 positive results were detected). Greater sensitivity has been obtained in an ELISA which uses considerably fewer materials. The latter assay is currently being developed for the improved discrimination of sera from infected and vaccinated cattle (K. H. Nielsen, J. W. Cherwonogrodzky, J. R. Duncan, and D. R. Bundle, Am. J. Vet. Res., in press). Extensive trials are currently under way to determine the diagnostic usefulness of this ELISA.

Purified poly-B is a cyclic 1,2-linked β-D-glucan (2). It was not precipitated by sera from any of the field-strain-infected or *B. abortus* S-19-vaccinated cattle. Preparations of poly-B (kindly supplied by L. M. Jones) which were precipitated by sera from infected cattle (8) were found to contain *Brucella* O-chain PS (about 5 to 10%) by nuclear magnetic resonance analysis (data not shown). The presence of O-chain PS was probably responsible for the observed precipitin reaction with pooled sera from *B. abortus*-infected cattle. This conclusion was supported by the observation that poly-B was precipitated by the pooled infected-cattle sera cross-absorbed with *Y. enterocolitica* O:3 cells but not by that cross-absorbed with *Y. enterocolitica* O:9 cells. The latter organism has O-chain PS identical to that of *B. abortus* 1119-3 (4, 5).

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