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Comparative Analysis of *Brucella* Serotype A and M and *Yersinia enterocolitica* O:9 Polysaccharides for Serological Diagnosis of Brucellosis in Cattle, Sheep, and Goats

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Hapten polysaccharides of *Brucella* smooth M and A serotypes were prepared from *Brucella* sp. and *Yersinia enterocolitica* O:9 by previously described hydrolytic (O chain) or nonhydrolytic (native hapten [NH]) procedures. The purified polysaccharides differed only in the presence (O chain) or absence (NH) of lipopolysaccharide core sugars. The polysaccharides were compared by reverse radial immunodiffusion for the diagnosis of brucellosis in cattle (*Brucella abortus* biotype 1 [A serotype] and *Brucella melitensis* biotype 3 [AM serotype]), sheep (*B. melitensis* biotypes 1 [M serotype] and 3), and goats (*B. melitensis* biotype 1). The reverse radial immunodiffusion test with the NH from *B. melitensis* 16 M (serotype M) showed the highest sensitivity (89.6 to 97.3%), regardless of the host species and the serotype of the infecting *Brucella* sp. *Y. enterocolitica* O:9 NH (A serotype) was useful for diagnosing disease in cattle infected with *B. abortus* biotype 1, but not in cattle infected with *B. melitensis* biotype 3, sheep, or goats. The different results obtained with the serotype M and A polysaccharides and the sera from animals infected with M, A, and AM serotypes of *Brucella* spp. showed that in naturally infected animals, a large proportion of the antibodies are directed to or react with a previously defined common epitope(s) (J. T. Douglas and D. A. Palmer, J. Clin. Microbiol. 26:1353–1356, 1988) different from the A or M epitopes. By using the radial immunodiffusion test with *B. melitensis* 16M NH, it was possible to differentiate infected from vaccinated cattle, sheep, and goats with a sensitivity and specificity similar to that of the complement fixation test.

Brucellosis is a zoonosis that causes great economic losses and human suffering. Most eradication programs are based on vaccination of the animal hosts and the serological identification and culling of infected animals. However, because no single simple test is able to differentiate infected from vaccinated animals, sera are usually screened with a simple test of high sensitivity and positive results are confirmed with a more elaborate test of high specificity. Some indirect (13, 25) and competitive (24, 29, 30) enzyme-linked immunosorbent assays, the complement fixation (CF) test (2, 13, 14), and gel precipitation tests with polysaccharide haptens (3, 7, 9–11, 13–16, 28) have been proposed or used as confirmatory tests. Competitive indirect enzyme-linked immunosorbent assays seem promising, but the necessary monoclonal antibodies are not readily available. Although CF is recommended (2), it cannot be applied to hemolyzed sera, sheep sera often show strong anticomplementary activity, and the test is difficult to standardize under the conditions prevailing in most of the countries where brucellosis is a major problem. Gel precipitation tests with polysaccharide haptens are far simpler. However, there are few studies on their use for the diagnosis of brucellosis in sheep (13) and they have never been evaluated for the diagnosis of brucellosis in goats. Moreover, two basically different (hydrolytic and nonhydrolytic) extraction protocols have been proposed (7, 11), but the corresponding polysaccharides have never been compared. Thus, the goal of the work described here was to compare the usefulness of immunochemically characterized polysaccharides for the diagnosis of brucellosis in small ruminants and cattle.

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MATERIALS AND METHODS

Bacterial strains and cultures. *Brucella melitensis* 16M (biotype 1 [M serotype], virulent), *Brucella abortus* 2308 (biotype 1 [A serotype], virulent), *B. abortus* 19 (biotype 1 [A serotype], vaccine strain), and *Yersinia enterocolitica* O:9 MY79 (*Brucella* A serotype) were used in the present study. The cells were propagated in tryptic soy broth in 2-liter flasks (500 ml per flask) at 37°C on an orbital shaker (200 rpm), inactivated with phenol (0.5% (final concentration, 0.5%) at 37°C for 24 h, harvested by tangential flow filtration (Pellicon Unit, PTHK000C5 filter; Millipore Corp., Bedford, Mass.), and washed twice with saline. *Y. enterocolitica* O:9 MY79 was grown at 26°C in the same medium and under the same conditions described above, but the cells were processed without inactivation.

Extraction of polysaccharides. (i) Crude NH. The nonhydrolytic method of Díaz et al. (11) was used. Briefly, washed cells were extracted with distilled water (30 g [wet weight] in 100 ml) in an autoclave at 120°C for 15 min, and the debris was removed by centrifugation (12,000 × *g*, 30 min). Crude native hapten (NH) was obtained from this water extract by a two-step ethanol precipitation procedure (11).

(ii) Purified NH. Crude NH was digested with nucleases

(10 mg/ml in 0.8% NaCl, 0.05% NaN_3, 0.1 M Tris-HCl [pH 7.0], and 50 μg of DNase II type V and RNase A [Sigma Chemical Co., St. Louis, Mo.] per ml) for 18 h at 37°C. The buffer was replaced by dialysis against 0.1 M sodium acetate (pH 5.0), and the mixture was digested with 50 μg of β-D-glucoside glucohydrolase (EC 3.2.1.21; Sigma) per ml for 18 h at 37°C. After dialysis against the first buffer, proteinase K (50 μg/ml; E. Merck, Darmstadt, Germany) was added, and the mixture was incubated for 1 h at 55°C and for 24 h at room temperature. The proteinase K digestion was repeated twice, and the mixture was ultracentrifuged (6 h, 200,000 × *g*). The supernatant was extracted with an equal volume of phenol at 70°C, the mixture was chilled and then centrifuged (9,000 × *g*, 0°C, 15 min), and the phenol phase was precipitated first with 5 volumes and then with 7 volumes of ethanol at −20°C overnight. The second precipitate was dialyzed and freeze-dried.

(iii) *B. abortus* O-chain polysaccharide. The hydrolytic method described by Cherwonogrodzky and Nielsen (7) for *B. abortus* was used. Briefly, washed cells of *B. abortus* 2308 were extracted with 2.0% acetic acid–10.0% NaCl (20 g [wet weight] in 100 ml) at 120°C for 30 min. The cell debris was removed by centrifugation, and the supernatant was precipitated with methanol–1.0% sodium acetate. The precipitate was then treated with lysozyme, nucleases, and proteinase K, extracted with phenol, ultracentrifuged (100,000 × *g*, 18 h, 4°C), and chromatographed on Sephadex G-50 (Pharmacia, Uppsala, Sweden).

Analytical methods. Total protein was determined by the method of Markwell et al. (19), with bovine serum albumin used as a standard. The thiobarbituric acid method (32) was used to measure 2-keto,3-deoxyoctulosonic acid (KDO), with pure KDO (Sigma) used as a standard and deoxyribose used to correct for deoxysugar interference; absorbance was read in dimethyl sulfoxide at 552 nm for KDO and at 536 nm for deoxysugars, and the interference was corrected as described elsewhere (6) for mixtures of chromogens. Under the conditions of the assay, 1 μM KDO gave an optical density of 50.7, with a sensitivity threshold of 20 μg for *B. melitensis* 16M smooth lipopolysaccharide (S-LPS) (nuclease- and proteinase K-treated fraction 5; 0.78% KDO), which is equivalent to 5% S-LPS in the sample. Gas-liquid chromatography–mass spectrometry for detection of quinovosamine was performed as described before (23). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and periodate silver staining were performed as described elsewhere (31); the sensitivity was 200 ng for the S-LPS preparation described above, which was equivalent to 1% in the sample. ^{13}C nuclear magnetic resonance (NMR) spectra were recorded at room temperature by using a Bruker AC-200E spectrometer (Bruker Analytische Messtechnik, Silberstreifen, Germany) operating at 50.33 MHz for samples (15 to 30 mg) dissolved in deuterium oxide, with tetramethylsilane used as the internal reference standard.

Serological tests. The reverse radial immunodiffusion (RID) test (9) was performed by dissolving the polysaccharides in the appropriate hypertonic buffer and adding agarose (type B; Pharmacia) at 0.8%. As buffer solutions, 10.0% NaCl in 0.1 M glycine (pH 7.8) was used for *B. melitensis* NH (9), while 10.0% NaCl in 0.1 M Tris-HCl (pH 7.2) (7) or 10.0% NaCl in 6 mM borate (pH 8.6) had to be used for optimal precipitation of *B. abortus* and *Y. enterocolitica* O:9 polysaccharides. For cattle, a single concentration of polysaccharide in the gel (30 μg/ml for the O chain and 50 μg/ml for crude NH of *B. abortus* 2308, 50 μg/ml for *Y. enterocolitica* O:9 crude NH, and 20 μg/ml for *B. melitensis* 16M crude NH) was used, while sera from sheep and goats had to be tested with two concentrations for optimal results (5 to 20 μg/ml for *B. melitensis* 16M crude NH, *B. abortus* 2308 O chain, and *Y. enterocolitica* O:9 crude NH and 20 to 50 μg/ml for *B. abortus* 2308 crude NH). At least 48 h before use (sealed plates were stable for 1 month), 1.0- to 1.5-mm-thick gels were poured into 50-by-9-mm Falcon 1006 petri dishes (Becton Dickinson Labware, Lincoln Park, N.J.), and on the day of use, 4.0-mm-diameter wells were punched in the gels and filled with 15 μl of serum. Precipitin rings developed around the wells after 2 to 24 h of incubation in a humid chamber at room temperature. Double gel diffusion was performed in 0.8% agarose (type B; Pharmacia) with 10% NaCl–6 mM borate (pH 8.3); wells of 3 to 4 mm in diameter were punched 4 mm apart. Rose bengal and CF tests were performed as described elsewhere (2). The sensitivities and specificities of the tests were calculated as described by Jones et al. (14).

Sera. Sera from the blood of cattle, sheep, and goats were used.

(i) Cattle. Sera were obtained from 99 cows from whose milk *B. abortus* biotype 1 had been isolated, 13 cows from whose milk *B. melitensis* biotype 3 had been isolated, 95 cows from brucellosis-free flocks, 40 heifers vaccinated subcutaneously with 1×10^{10} CFU of *B. abortus* S-19, 16 heifers conjunctivally vaccinated with 5×10^9 CFU of *B. abortus* S-19, and 35 adult cows vaccinated conjunctivally with 1×10^8 CFU of *B. abortus* S-19. Vaccinated cattle were bled 1, 2, and 6 months after vaccination.

(ii) Sheep. Sera were obtained from 37 sheep (ewes and rams) positive for bacteriological isolation of *B. melitensis* biotype 1, 48 sheep (ewes and rams) positive for bacteriological isolation of *B. melitensis* biotype 3, 77 sheep from *Brucella*-free flocks, 11 3-month-old rams vaccinated subcutaneously with 2×10^9 CFU of *B. melitensis* Rev 1, 11 3-month-old rams vaccinated conjunctivally with 2×10^9 CFU of *B. melitensis* Rev 1, 10 adult rams vaccinated subcutaneously with 1.5×10^9 CFU of *B. melitensis* Rev 1, and 10 adult rams vaccinated conjunctivally with 1.5×10^9 CFU of *B. melitensis* Rev 1. Vaccinated rams were bled 1, 3, and 4 months after vaccination.

(iii) Goats. Sera were obtained from 53 goats culture positive for *B. melitensis* biotype 1, 127 goats from two brucellosis-free flocks, 20 subcutaneously vaccinated young goats (10^9 CFU of *B. melitensis* Rev 1) that were bled 15, 45, 120 and 180 days after vaccination, and 10 young goats that were vaccinated conjunctivally with 10^9 CFU of *B. melitensis* Rev 1 and that were bled 15, 30, 60 and 120 days after vaccination.

Typing of *Brucella* spp. Typing was kindly performed by J. M. Verger (Institut National de la Recherche Agronomique, Nouzilly, France) by standard procedures (2).

RESULTS

Immunochemical characterization of the polysaccharide preparations. The results of immunochemical characterization of the polysaccharide preparations are summarized in Table 1. KDO contents varied from 0.17 to 0.09% in the crude NH extracts. The same extracts contained circular β-1,2-D-glucans (^{13}C NMR signals at 102.65 [C-1], 83.45 [C-2], 77.64 [C-5], 76.56 [C-3], 69.71 [C-4], and 61.57 [C-6] ppm) (18) and *N*-formylperosamine polysaccharides (^{13}C NMR signal or signal sets at 166.98 to 164.05 [formamido], 102.72 to 99.69 [C-1], 77.20 to 76.16 [C-2], 69.86 to 67.37

TABLE 1. Characterization of *B. melitensis*, *B. abortus*, and *Y. enterocolitica* O:9 polysaccharide extracts used in the diagnosis of animal brucellosis

Strain and extract	% KDO	Quinovosamine[a]	S-LPS by SDS-PAGE[b]	% Protein	Perosamine (α linkage)[c]	Glucan[d]	Activity[e]
B. melitensis 16M							
Crude NH	0.09	+	−	9.0	1-2, 1-3	+	300
Purified NH	<0.02	−	−	<1.0	1-2, 1-3	−	8
B. abortus 2308							
Crude NH	0.10	ND[f]	+	42.5	1-2	+	600–300
O chain	0.14	+	−	<1.0	1-2	−	60
B. abortus 19, crude NH	0.15	ND	+	8.8	ND	ND	1,000–600
Y. enterocolitica O:9 MY79, crude NH	0.17	−	−	20.3	ND	ND	75

[a] Presence (+) or absence (−) of quinovosamine by gas-liquid chromatography–mass spectrometry.
[b] Presence (+) or absence (−) of S-LPS by SDS-PAGE.
[c] *N*-Formylperosamine polysaccharide in either α-1,2- or α-1,2- plus α-1,3-linkages (from the ^{13}C NMR spectra and known serotype).
[d] Presence (+) or absence (−) of cyclic β-D-glucans by ^{13}C NMR.
[e] Minimal concentration (in micrograms per milliliter) yielding the characteristic precipitin line by double gel diffusion with a pool of sera from *B. abortus*-infected cattle.
[f] ND, not done.

[C-3], 66.74 [C-5], 51.99 to 51.04 [C-4], and 16.02 [C-6] ppm). Purified *B. melitensis* 16 M NH contained less than 0.02% KDO and did not have detectable quinovosamine, and the ^{13}C NMR spectra showed only the signals of the M-type *N*-formylperosamine polysaccharide (4, 21). The *B. abortus* O-chain polysaccharide preparations contained the S-LPS core markers KDO and quinovosamine, and also showed the ^{13}C NMR spectrum of the A-type *N*-formylperosamine polysaccharide (4, 21). The serological activities (Table 1) of the polysaccharide preparations correlated with the degree of purification, and double gel diffusion with purified NH as a reference confirmed that the component active in the precipitation tests with crude extracts was the *N*-formylperosamine polysaccharide (data not shown).

Sensitivity of precipitation tests with different polysaccharide preparations and sera from infected animals. In preliminary experiments, it was found that double gel diffusion (7) was 10 to 15% less effective than RID (9) in detecting cattle with antibodies to hapten polysaccharides. Also, with sera from 25 infected cattle, only 5 were RID positive with *B. abortus* S-19 crude NH (50 μg/ml), and no further testing was performed with this preparation. The sensitivities obtained with the RID test and the other polysaccharide preparations are presented in Table 2. With the sera of cattle, the NH from *B. melitensis* 16M yielded almost the same results as the NH from *Y. enterocolitica* O:9 and slightly better results than those obtained with the *B. abortus* O chain. However, these differences were not significant ($P = 0.17$). The proportion of positive results in the RID test correlated with CF titers (data not shown), and no animal serum that was RID positive was CF negative. Differences between the CF and RID tests with *B. melitensis* NH were not statistically significant ($P = 0.23$).

With sera from sheep from which *B. melitensis* biotype 1 was isolated, the sensitivities obtained with *B. melitensis* 16M NH were 1.6, 2.2, and 2.8 times higher ($P < 0.001$) than those obtained with *B. abortus* 2308 NH, O chain, and *Y. enterocolitica* O:9 NH, respectively (Table 2), and the differences were particularly clear with sera from animals with low CF titers (data not shown). In the biotype 3-infected group, the best sensitivity was also obtained with the NH of *B. melitensis* 16M, but the sensitivities with *B. abortus* 2308 NH and O chain and *Y. enterocolitica* O:9 NH were considerably increased ($P = 0.048$, 0.077, and <0.001,

TABLE 2. Sensitivities of the rose bengal, CF, and RID tests with *Brucella* polysaccharides in bacteriologically positive cattle, sheep, and goats

Host	Infecting species and biotype	No. of animals	Sensitivity (%)					
			Rose bengal test	CF test[a]	RID with:			
					Bm 16M NH[b]	Ba 2308 NH[c]	Ba 2308 O chain[d]	Ye O:9 NH[e]
Cattle	*B. abortus* biotype 1	99	100.0	96.0	92.0	79.8	85.9	91.0
	B. melitensis biotype 3	13	100.0	100.0	92.4	ND[f]	53.9	84.7
Sheep	*B. melitensis* biotype 1	37	94.6	91.9	97.3	59.5	43.3	35.2
	B. melitensis biotype 3	48	93.8	85.5	89.6	77.1	62.5	73.0
Goats	*B. melitensis* biotype 1	53	92.4	94.5	94.5	32.1	43.5	30.2

[a] Titers of ≥8.
[b] Crude NH polysaccharide from *B. melitensis* 16M.
[c] Crude NH polysaccharide from *B. abortus* 2308.
[d] O-chain polysaccharide from *B. abortus* 2308.
[e] Crude native hapten polysaccharide from *Y. enterocolitica* O:9 MY79.
[f] ND, not done.

respectively) with respect to the corresponding results for the *B. melitensis* biotype 1-infected group (Table 2). For both groups of sheep, positive results in the RID test correlated with CF titers, and when the results were pooled, the average sensitivities of the RID (92.9%) and CF (87.0% at a CF titer of ≥8) tests showed no significant differences ($P = 0.20$).

The results obtained with sera from infected goats (all infected with *B. melitensis* biotype 1) closely matched those obtained with sera from biotype 1-infected sheep, since NH from *B. melitensis* was at least two times more sensitive ($P < 0.001$) in detecting infected animals than any polysaccharide from *B. abortus* or than the A-type polysaccharide from *Y. enterocolitica* O:9 (Table 2). With sera from goats, the CF and RID tests with *B. melitensis* 16 NH gave identical results.

Specificity of RID test with sera from healthy unvaccinated animals. The 95 serum samples from cattle free of brucellosis were RID test negative with all the polysaccharide preparations, and similar negative results were obtained with sera from 77 brucella-free sheep and 127 brucella-free goats. Therefore, the specificity was 100% in unvaccinated animals.

Specificity of RID test with sera from vaccinated animals. Two months after vaccination, none of the sera from 40 heifers vaccinated subcutaneously with 10^{10} CFU of *B. abortus* S-19 was positive with any of the polysaccharides tested, while CF titers were ≥8 for sera from 21 of these animals. With sera from the 16 conjunctivally vaccinated heifers, both the RID and the CF test results were negative 2 months after vaccination. Sera from the 35 adult cattle vaccinated conjunctivally were RID test negative 2 months after vaccination, while sera from 14 of them had CF test titers of ≥8. For sera from all groups of cattle, both tests were negative (100% specificity) 6 months after vaccination.

Sera from Rev 1-vaccinated sheep and goats were tested only with the NH of *B. melitensis* 16M. Four months after vaccination, both CF and RID were negative for sera from the 11 young sheep vaccinated conjunctivally, while sera from 11 and 2 of the 11 young sheep vaccinated subcutaneously remained positive by the RID and CF (titer, ≥8) tests, respectively. At the same time of bleeding, serum from one adult vaccinated conjunctivally was positive by both the CF and the RID tests, and sera from 2 and 4 of the 10 adults vaccinated subcutaneously were also positive by the RID and CF (titer, ≥8) tests, respectively. One month after vaccination, the RID test was negative for sera from the 10 young goats vaccinated by the conjunctival route, while sera from 3 young goats had CF titers of ≥4. Among the 20 young goats vaccinated subcutaneously, serum from 1 animal was RID test positive and sera from 5 animals were CF test positive (titer, ≥4) 4 months after vaccination.

DISCUSSION

The results presented here show that the nonhydrolytic water extraction (11) and the acetic acid extraction (7) protocols yield polysaccharides that contain the immunodominant *N*-formylperosamine section of the S-LPS (1, 4, 5, 20, 21, 34) but differ in the absence and presence of LPS core markers in NH and O-chain polysaccharides, respectively. This comparative analysis is not consistent with the hypothesis that NH polysaccharides are degradative products of the S-LPS (36) and also suggest that a more exact terminology should be used for the "O-chain" extracts, since the polysaccharide includes sugars of the LPS core. However, the presence of the immunodominant section explains (see also below) why in cattle hydrolytic and nonhydrolytic polysaccharides yielded similar results. From a practical point of view, crude NH was simpler to obtain than the O chain, and NH from *B. melitensis* 16M performed better than any *B. abortus* or *Y. enterocolitica* O:9 polysaccharide in the diagnosis of brucellosis in sheep and goats. Although *B. melitensis* 16M is a virulent strain, both the better serological activity of the 16M NH and the fact that the A polysaccharides (*B. abortus* or *Y. enterocolitica* O:9) were not useful in diagnosing brucellosis in sheep or goats show that strain 16M cannot be replaced by the attenuated *B. abortus* S-19 strain (35). Our results also show that if the RID test is to be applied only to cattle and safety in antigen production cannot be implemented, extraction of antigen from *Y. enterocolitica* O:9 would be a clear option.

A significant difference between the results obtained with animals infected with *B. abortus* (cattle) and those infected with *B. melitensis* (sheep and goats) was that in the latter the serological specificity of the polysaccharide was important in the precipitation test. Studies with monoclonal (5, 12, 27) and polyclonal (22) antibodies have shown that the A and M antigens are not simultaneously present in the O polysaccharides of *B. abortus* and *B. melitensis* biotype 1 strains, a result contrary to the hypothesis of Wilson and Miles (33) that quantitative differences in the distribution of the A and M antigens explain by themselves the cross-reactivity between the smooth *Brucella* serotypes. The same studies have identified a C epitope common to *B. abortus*, *B. melitensis*, and *Y. enterocolitica* O:9 (5, 8, 12, 20), and the NMR analysis has shown that, while C epitopes do exist, a true A epitope cannot be found in the "M-dominant" polysaccharides (5, 17). Our observation that the sensitivity obtained with the hapten polysaccharides did not correlate with the serotype of the infecting biotype (A "dominant" or AM in cattle, M "dominant" or AM in sheep or goats) provides evidence for both the actual value of the A-C and M-C formulae (12, 26) in animals with natural infection and the relevance of the C epitope(s) in the diagnosis of infection. In cattle, *B. melitensis* 16M NH (M-C) was at least as efficient as the polysaccharides from *B. abortus* 2308 and *Y. enterocolitica* O:9 (A-C), and the simplest explanation is that antibodies specific for or reacting with the C epitope(s) are at least as efficient in precipitating hapten polysaccharides as those of true A-epitope specificity. In sheep and goats, antibodies to both the M and C epitopes would be important, because the *B. melitensis* 16M NH (M-C) is more efficient in detecting *B. melitensis* biotype 1 (M-C)-infected animals than the polysaccharides from *B. abortus* 2308 and *Y. enterocolitica* O:9 (A-C). An intermediate situation can be postulated for sheep infected with *B. melitensis* biotype 3. Since organisms with this biotype contain both the A and M epitopes (as well as the C epitope), the relative increase in the sensitivities of *B. abortus* 2308 and *Y. enterocolitica* O:9 (A-C) polysaccharides in the *B. melitensis* biotype 3 group is fully consistent with the explanations given above. All those results also show that the expressions "A dominant" or "M dominant," which are often used to designate the main smooth *Brucella* serotypes, are misleading in the sense that in naturally infected animals, the C epitope(s) seems to be of at least equal importance.

With respect to the value of the serological tests, it is noteworthy that the rose bengal test was not 100% sensitive with sera from sheep and goats. This result suggests that rose bengal test standardization (2) must be modified for the latter species. Finally, the present study extends to goat and

sheep brucellosis the conclusions of previous work on the value of the precipitation tests with *Brucella* polysaccharide haptens as confirmatory tests (3, 9–11, 14–16, 28), provided that a polysaccharide of the appropriate serological specificity is used. For sera from infected sheep and goats, the RID and CF tests had similar sensitivities, and the RID test became negative faster than the CF test for sera from sheep vaccinated subcutaneously and for sera from vaccinated adult sheep and goats. These results closely paralleled those obtained with cattle, in which the RID test (with the *B. melitensis* 16M or the *Y. enterocolitica* O:9 NHs) had a sensitivity of 91.9 to 90.9% and sera from the vaccinated animals became RID test negative faster than they became CF test negative. The simplicity of the extraction protocol and test, the fact that the same polysaccharide preparation (*B. melitensis* 16M NH) can be used for cattle, sheep, and goats, and the repeatability of the test stress the practical value of the RID test, a conclusion supported by the fact that eradication of cattle brucellosis in a large area has been achieved by using the RID test as the only confirmatory test (3).

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REFERENCES

1. **Alonso-Urmeneta, B., I. Moriyón, R. Díaz, and J. M. Blasco.** 1988. Enzyme-linked immunosorbent assay with *Brucella* native hapten polysaccharide and smooth lipopolysaccharide. J. Clin. Microbiol. **26:**2642–2646.
2. **Alton, G. G., L. M. Jones, R. D. Angus, and J. M. Verger.** 1988. Techniques for the brucellosis laboratory. Institut National de la Recherche Agronomique, Paris.
3. **Asarta, A.** 1989. Erradicación de la brucellosis en el ganado vacuno de Navarra, p. 371–375. Actas del XII Congreso Nacional de Microbiología, vol. I. Sociedad Española de Microbiología, Madrid.
4. **Bundle, D. R., J. W. Cherwonogrodzky, M. Caroff, and M. B. Perry.** 1987. The lipopolysaccharides of *Brucella abortus* and *Brucella melitensis*. Ann. Inst. Pasteur Microbiol. **138:**92–98.
5. **Bundle, D. R., J. W. Cherwonogrodzky, M. A. J. Gidney, P. J. Meikle, M. B. Perry, and T. Peters.** 1989. Definition of *Brucella* A and M epitopes by monoclonal typing reagents and synthetic oligosaccharides. Infect. Immun. **57:**2829–2836.
6. **Chaplin, M. F.** 1986. Monosaccharides, p. 1–36. *In* M. F. Chaplin and J. F. Kennedy (ed.), Carbohydrate analysis: a practical approach. IRL Press, Oxford.
7. **Cherwonogrodzky, J. W., and K. Nielsen.** 1988. *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. J. Clin. Microbiol. **26:**1120–1123.
8. **Cloeckaert, A., I. Jacques, P. de Wergifosse, G. Dubray, and J. N. Limet.** 1992. Protection against *Brucella melitensis* or *Brucella abortus* in mice with immunoglobulin G (IgG), IgA, and IgM monoclonal antibodies specific for a common epitope shared by the *Brucella* A and M smooth lipopolysaccharides. Infect. Immun. **60:**312–315.
9. **Díaz, R., P. Garatea, L. M. Jones, and I. Moriyón.** 1979. Radial immunodiffusion test with a *Brucella* polysaccharide antigen for differentiating infected from vaccinated cattle. J. Clin. Microbiol. **10:**37–41.
10. **Díaz, R., M. D. Salvo, L. Fernández-Lago, and M. Zorraquino.** 1980. Diagnóstico serológico de la brucellosis bovina. Estudio comparativo de la inmunodifusión radial con la fijación de complemento. Rev. Diagn. Biol. **29:**433–438.
11. **Díaz, R., J. Toyos, M. D. Salvo, and M. L. Pardo.** 1981. A simple method for the extraction of polysaccharide B from *Brucella* cells for use in the radial immunodiffusion test diagnosis of bovine brucellosis. Ann. Rech. Vet. **12:**35–39.
12. **Douglas, J. T., and D. A. Palmer.** 1988. Use of monoclonal antibodies to identify the distribution of A and M epitopes on smooth *Brucella* species. J. Clin. Microbiol. **26:**1353–1356.
13. **Jiménez de Bagües, M. P., C. M. Marín, J. M. Blasco, I. Moriyón, and C. Gamazo.** 1991. An ELISA with *Brucella* lipopolysaccharide antigen for the diagnosis of *B. melitensis* infection in sheep and for the evaluation of serological responses following subcutaneous or conjunctival *B. melitensis* Rev 1 vaccination. Vet. Microbiol. **30:**233–241.
14. **Jones, L. M., D. T. Berman, E. Moreno, B. L. Deyoe, M. J. Gilsford, J. D. Huber, and P. Nicoletti.** 1980. Evaluation of a radial immunodiffusion test with polysaccharide B antigen for diagnosis of bovine brucellosis. J. Clin. Microbiol. **12:**753–760.
15. **Lord, V. R., M. R. Rolo, and J. W. Cherwonogrodzky.** 1989. Evaluation of humoral immunity to *Brucella* sp. in cattle by the use of an agar-gel immunodiffusion test containing a polysaccharide antigen. Am. J. Vet. Res. **50:**1813–1816.
16. **Lord, V. R., and J. W. Cherwonogrodzky.** 1992. Evaluation of polysaccharide, lipopolysaccharide, and beta-glucan antigens in gel immunodiffusion tests for brucellosis in cattle. Am. J. Vet. Res. **53:**389–391.
17. **L'vov, V. L., V. E. Malikov, A. S. Shaskov, E. A. Dranovskaya, and B. A. Dmitriev.** 1985. Somatic antigens of the genus *Brucella*: structure of the O-specific polysaccharide chain of the lipopolysaccharide of *Brucella melitensis*. Bioorg. Khim. **11:** 963–969.
18. **L'vov, V. L., G. N. Pluzhnikova, E. B. Lapina, A. S. Shashkov, S. A. Askerova, E. Malikov, E. A. Dranovskaya, and B. A. Dimitriev.** 1987. Molecular nature of the polysaccharide B antigen from *Brucella* (poly-B). Bioorg. Khim. **13:**1070–1074.
19. **Markwell, M. A. K., S. M. Haas, L. L. Bieber, and N. E. Tolbert.** 1978. A modification of the Lowry procedure to simplify protein determination in membrane lipoprotein samples. Anal. Biochem. **87:**206–210.
20. **Meikle, P. J., M. B. Perry, J. W. Cherwonogrodzky, and D. R. Bundle.** 1989. Fine structure of A and M antigens from *Brucella* biovars. Infect. Immun. **57:**2820–2828.
21. **Moreno, E., D. Borowiak, and H. Mayer.** 1987. *Brucella* lipopolysaccharides and polysaccharides. Ann. Inst. Pasteur Microbiol. **43:**779–782.
22. **Moreno, E., H. Mayer, and I. Moriyón.** 1987. Characterization of a native polysaccharide hapten from *Brucella melitensis*. Infect. Immun. **55:**2850–2853.
23. **Moreno, E., E. Stackebrandt, M. Dorsch, J. Wolters, M. Busch, and H. Mayer.** 1990. *Brucella abortus* 16S rRNA and lipid A reveal a phylogenetic relationship with members of the alpha-2 subdivision of the class *Proteobacteria*. J. Bacteriol. **172:**3569–3576.
24. **Nielsen, K., J. W. Cherwonogrodzky, J. R. Duncan, and D. R. Bundle.** 1989. Enzyme-linked immunosorbent assay for differentiation of the antibody response of cattle naturally infected with *Brucella abortus* or vaccinated with strain 19. Am. J. Vet. Res. **50:**5–9.
25. **Nielsen, K. H., P. F. Wright, W. A. Kelly, and J. H. Cherwonogrodzky.** 1988. A review of enzyme immunoassay for detection of antibody to *Brucella abortus* in cattle. Vet. Immunol. Immunopathol. **18:**331–347.
26. **Olitzki, A. L., and J. Gurevitch.** 1933. Über die serologischen typen der Brucellagruppe und ihre Variationsmöglichkeiten. Zentralbl. Bakteriol. Parasitenkd. Infektionskr. Hyg. Abt. 1 Orig. **128:**112–124.
27. **Palmer, D. A., and J. T. Douglas.** 1989. Analysis of *Brucella* lipopolysaccharide with specific and cross-reacting monoclonal antibodies. J. Clin. Microbiol. **27:**2331–2337.
28. **Pinochet, L., P. Abalos, M. L. Sánchez, I. Palavicino, and M. A.**

Vent. 1989. Preparación y evaluación de un antígeno para descartar la respuesta post-vacunal a *Brucella abortus* cepa 19. Av. Cien. Vet. **4**:43–48.

29. **Rylatt, D. B., D. M. Wyatt, and P. G. Bundsen.** 1985. A competitive enzyme immunoassay for the detection of bovine antibodies to *Brucella abortus* using monoclonal antibodies. Vet. Immunol. Immunopathol. **8**:261–271.
30. **Sutherland, S. S.** 1985. An enzyme-linked immunosorbent assay for detection of *Brucella abortus* in cattle using monoclonal antibodies. Aust. Vet. J. **62**:264–268.
31. **Tsai, C., and C. E. Frasch.** 1982. A sensitive stain for detecting lipopolysaccharides in polyacrylamide gels. Anal. Biochem. **119**:115–119.
32. **Warren, L.** 1959. The thiobarbituric acid assay of sialic acids. J. Biol. Chem. **243**:1971–1975.
33. **Wilson, G. S., and A. A. Miles.** 1932. The serological differentiation of smooth strains of the *Brucella* group. Br. J. Exp. Pathol. **13**:1–13.
34. **Wu, A. M., and N. E. Mackenzie.** 1987. Structural and immunochemical characterization of the O-haptens of *Brucella abortus* lipopolysaccharides from strains 19 and 2308. Mol. Cell. Biochem. **75**:103–111.
35. **Zygmunt, M. S., and G. Dubray.** 1987. Preparation by ultrafiltration and control by high-performance liquid chromatography of the native hapten of *Brucella abortus* for use in radial immunodiffusion diagnostic test. J. Clin. Microbiol. **25**:1860–1863.
36. **Zygmunt, M. S., G. Dubray, D. R. Bundle, and M. B. Perry.** 1988. Purified native haptens of *Brucella abortus* B19 and *B. melitensis* 16M reveal the lipopolysaccharide origin of the antigens. Ann. Inst. Pasteur/Microbiol. **139**:421–423.