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

E. DÍAZ-APARICIO,1 V. ARAGÓN,1 C. MARÍN,2 B. ALONSO,1 M. FONT,3 E. MORENO,4 S. FÉREZ-ORTIZ,2 J. M. BLASCO,2 R. DÍAZ,1 AND I. MORIYON1*

Departmento de Microbiologia,1 and Centro de Investigaciones en Farmacobiologia Aplicada,2 Universidad de Navarra, Apartado 273, 31080 Pamplona, Departamento de Producción Animal, Servicio de Investigación Agraria, Diputación General de Aragón, 50080 Zaragoza,3 and Laboratorio Pecuario, Gobierno de Navarra, Serapio Huici s.n., 31610 Villaba,4 Spain, and Programa de Investigación en Enfermedades Tropicales, Escuela de Medicina Veterinaria, Universidad Nacional, Heredia, Costa Rica

Received 4 March 1993/Returned for modification 14 May 1993/Accepted 31 August 1993

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 97.5%), 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 immunochimically characterized polysaccharides for the diagnosis of brucellosis in small ruminants and cattle.

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, PTHK0005 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% NaN₃, 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 glycohydrolase (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.

**B. abortus O-chain polysaccharide.** The hydrolytic method described by Cherwonogradzky 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 thioarbituric acid method (22) was used to measure 2-keto-3-deoxyoctulosonic acid (KDO), with pure KDO (Sigma) used as a standard and deoxyribose used to correct for deoxyxugar interference; absorbance was read in dimethyl sulfoxide at 552 nm for KDO and at 536 nm for deoxy sugars, 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 0.7, 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 quino- vosamine 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. ¹³C 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 aged agglutinins (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 × 10⁹ CFU of *B. abortus* S-19, 16 heifers conjunctivally vaccinated with 5 × 10⁸ CFU of *B. abortus* S-19, and 35 adult cows vaccinated conjunctivally with 1 × 10⁹ 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 × 10⁹ CFU of *B. melitensis* Rev 1, 11 3-month-old rams vaccinated conjunctivally with 2 × 10⁹ CFU of *B. melitensis* Rev 1, 10 adult rams vaccinated subcutaneously with 1.5 × 10⁹ CFU of *B. melitensis* Rev 1, and 10 adult rams vaccinated conjunctivally with 1.5 × 10⁹ 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 (10⁹ 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 10⁹ 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 (¹³C 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-formylpyosamine polysaccharides (¹³C NMR signal or signal sets at 166.98 to 164.05 [formamidino], 102.72 to 99.69 [C-1], 77.20 to 76.16 [C-2], 69.86 to 67.37

Downloaded from http://jcm.asm.org/ on June 19, 2017 by guest
TABLE 1. Characterization of *B. melitensis*, *B. abortus*, and *Y. enterocolitica* O:9 polysaccharide extracts used in the diagnosis of animal brucellosis

<table>
<thead>
<tr>
<th>Strain and extract</th>
<th>% KDO</th>
<th>Quinovosamine&lt;sup&gt;a&lt;/sup&gt;</th>
<th>S-LPS by SDS-PAGE&lt;sup&gt;b&lt;/sup&gt;</th>
<th>% Protein</th>
<th>Perosamine (α linkage)&lt;sup&gt;c&lt;/sup&gt;</th>
<th>Glucan&lt;sup&gt;d&lt;/sup&gt;</th>
<th>Activity&lt;sup&gt;e&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>B. melitensis</em> 16M</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Crude NH</td>
<td>0.09</td>
<td>+</td>
<td>-</td>
<td>9.0</td>
<td>1-2, 1-3</td>
<td>+</td>
<td>300</td>
</tr>
<tr>
<td>Purified NH</td>
<td>&lt;0.02</td>
<td>-</td>
<td>-</td>
<td>&lt;1.0</td>
<td>1-2, 1-3</td>
<td>-</td>
<td>8</td>
</tr>
<tr>
<td><em>B. abortus</em> 2308</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Crude NH</td>
<td>0.10</td>
<td>ND&lt;sup&gt;f&lt;/sup&gt;</td>
<td>+</td>
<td>42.5</td>
<td>1-2</td>
<td>+</td>
<td>600-300</td>
</tr>
<tr>
<td>O chain</td>
<td>0.14</td>
<td>+</td>
<td>-</td>
<td>&lt;1.0</td>
<td>1-2</td>
<td>-</td>
<td>60</td>
</tr>
<tr>
<td><em>B. abortus</em> 19, crude NH</td>
<td>0.15</td>
<td>ND&lt;sup&gt;f&lt;/sup&gt;</td>
<td>+</td>
<td>8.8</td>
<td>ND</td>
<td>ND</td>
<td>1,000-600</td>
</tr>
<tr>
<td><em>Y. enterocolitica</em> O:9 MY79, crude NH</td>
<td>0.17</td>
<td>-</td>
<td>-</td>
<td>20.3</td>
<td>ND</td>
<td>ND</td>
<td>75</td>
</tr>
</tbody>
</table>

<sup>a</sup> Presence (+) or absence (−) of quinovosamine by gas-liquid chromatography-mass spectrometry.

<sup>b</sup> Presence (+) or absence (−) of S-LPS by SDS-PAGE.

<sup>c</sup> N-Formylperosamine polysaccharide in either α-1,2- or α-1,2- plus α-1,3-linkages (from the 13C NMR spectra and known serotype).

<sup>d</sup> Presence (+) or absence (−) of cyclic β-D-glucans by 13C NMR.

<sup>e</sup> 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.

<sup>f</sup> 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 13C 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 13C 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, respectively).

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

<table>
<thead>
<tr>
<th>Host</th>
<th>Infecting species and biotype</th>
<th>No. of animals</th>
<th>Rose bengal test</th>
<th>CF test&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Sensitivity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>RID with:</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Ba 16M NH&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Cattle</td>
<td><em>B. abortus</em> biotype 1</td>
<td>99</td>
<td>100.0</td>
<td>96.0</td>
<td>92.0</td>
</tr>
<tr>
<td></td>
<td><em>B. melitensis</em> biotype 3</td>
<td>13</td>
<td>100.0</td>
<td>100.0</td>
<td>92.4</td>
</tr>
<tr>
<td>Sheep</td>
<td><em>B. melitensis</em> biotype 1</td>
<td>37</td>
<td>94.6</td>
<td>91.9</td>
<td>97.3</td>
</tr>
<tr>
<td></td>
<td><em>B. melitensis</em> biotype 3</td>
<td>48</td>
<td>93.8</td>
<td>85.5</td>
<td>89.6</td>
</tr>
<tr>
<td>Goats</td>
<td><em>B. melitensis</em> biotype 1</td>
<td>53</td>
<td>92.4</td>
<td>94.5</td>
<td>94.5</td>
</tr>
</tbody>
</table>

<sup>a</sup> Titers of ≥8.

<sup>b</sup> Crude NH polysaccharide from *B. melitensis* 16M.

<sup>c</sup> Crude NH polysaccharide from *B. abortus* 2308.

<sup>d</sup> O-chain polysaccharide from *B. abortus* 2308.

<sup>e</sup> Crude native hapten polysaccharide from *Y. enterocolitica* O:9 MY79.

<sup>f</sup> ND, not done.
VOL.
both
the B.

vaccinated
vaccination,
ride
from B. melitensis was at least two times more

results.
both the

animals. With
respectively.
remained
ously

N-formylperosamine

water
5,

S-LPS
(36)
also
that
the

infected animals
also
that
the

S.LPS
is

and

Hypothesis

of
should
since the

11

animals. With

CF

and

1-vaccinated

= 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 prepara-
tions, 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 ani-

Specificity of RID test with sera from vaccinated animals.
Two months after vaccination, none of the sera from 40
heifers vaccinated subcutaneously with 100 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 subcutane-
ously 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 immun-
odominant 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, respec-
tively. This comparative analysis is not consistent with the
hypothesis that NH polysaccharides are degradative prod-
ucts 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 polysac-
charide 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 import-
antly 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 polysaccha-
drides 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 be-
tween 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” polysaccha-
drides (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
formulæ (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. entero-
colitica 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 hapten 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 NHa) 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).

ACKNOWLEDGMENTS

We express our gratitude to J. M. Verger for typing of the Brucella isolates. This research was funded by the Dirección General de Investigación Científica y Tecnológica (GAN 90-0935-CD and 91-0729) of Spain and the Consejo Nacional de Investigaciones Científicas y Tecnológicas (CONICIT) of Costa Rica. Fellowship support from the Consejo Nacional de Ciencia y Tecnología (CONACYT, México) and Ministerio de Educación y Ciencia and Gobierno de Navarra (Spain) is also gratefully acknowledged. E. Díaz-Aparicio is a researcher of the Instituto Nacional de Investigaciones Forestales y Agropecuarias de México.

REFERENCES


BRUCELLA M AND A POLYSACCHARIDES


