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Evaluation of Serological Tests for Diagnosis of *Brucella melitensis* Infection of Goats

E. DÍAZ-APARICIO,[1]† C. MARÍN,[2] B. ALONSO-URMENETA,[1] V. ARAGÓN,[1] S. PÉREZ-ORTIZ,[3] M. PARDO,[1] J. M. BLASCO,[2] R. DÍAZ,[1] AND I. MORIYÓN[1]*

Departamento de Microbiología, Universidad de Navarra, 31080 Pamplona,[1] Departamento de Producción Animal, Servicio de Investigación Agraria, Diputación General de Aragón, 50080 Zaragoza,[2] and Laboratorio Pecuario, Gobierno de Navarra, Serapio Huici s.n., 31610 Villaba,[3] Spain

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Five serological assays were evaluated for the diagnosis of brucellosis in goats: the rose bengal test (RBT), complement fixation test (CFT), radial immunodiffusion (RID) with *Brucella* and *Yersinia enterocolitica* O:9 polysaccharides, counterimmunoelectrophoresis (CIEP) with cytosol, and enzyme-linked immunosorbent assay (ELISA) with polyclonal and protein G conjugates and smooth lipopolysaccharide (S-LPS), native hapten polysaccharide (NH), or cytosol antigens. For optimal sensitivity, RBT had to be used with sera-antigen at a 3:1 dilution. In the RID test, *Brucella melitensis* biotype 1 NH could not be replaced by *Brucella abortus* biotype 1 or *Y. enterocolitica* 0:9 polysaccharides. In the ELISA, S-LPS and NH gave similar results and the protein G conjugate increased the specificity. With the sera from 55 *B. melitensis* culture-positive goats, the sensitivity was 100% for RBT, CFT (titer ≥ 4), and ELISA with S-LPS or NH; 94% for RID; and 93% for CIEP. All tests were negative (100% specific) when testing the sera from 127 brucella-free goats. Larger discrepancies among the results of the serological tests were obtained with sera from goats of areas where brucellosis is endemic. When the sera of 20 young goats vaccinated subcutaneously (10^9 CFU of *B. melitensis* Rev 1) and bled 6 months later were examined, the specificities were as follows: NH ELISA, 60%; CFT and S-LPS ELISA, 75%; RBT, 80%; CIEP, 90%; and RID, 94%. With the sera from 10 young goats vaccinated conjunctivally (10^9 CFU of *B. melitensis* Rev 1) all tests were 100% specific 4 months after vaccination. The proportion of goats giving a positive reaction after vaccination decreased faster in RID than in other tests.

Brucellosis is an infectious disease of worldwide importance in domestic ruminants, and the causative bacteria (*Brucella abortus* in cattle and *Brucella melitensis* in sheep and goats) are transmitted to humans through contact with infected livestock or by consumption of contaminated dairy products. Because of the economic importance of cattle in developed countries, means for *B. abortus* diagnosis and prophylaxis have been widely investigated (6, 32, 36, 42), and several serological tests developed for cattle brucellosis have been found useful for the diagnosis of *B. melitensis* infection in sheep (4, 14, 21). By contrast, although goats are crucial in the economy of developing countries and *B. melitensis* is a common cause of human infection, the brucellosis of goats has received comparatively little attention. With respect to serological diagnosis, there have been studies on the use of conventional tests with whole-cell antigens (tube serum agglutination, rose bengal test [RBT] [4, 18, 40], and complement fixation test [CFT] [4, 40]) and also on agar immunodiffusion tests with uncharacterized trichloroacetic acid or sonic extracts (7, 40). However, those studies have not been performed with sera from goats shown to be actually infected or free of brucellosis, and therefore, the actual value of those tests is unknown. Moreover, tests using fractions known to be antigenically relevant by studies on cattle and sheep brucellosis (16), including immunoenzymatic tests, have not been investigated. Due to such a paucity of data, the purpose of the present work was threefold: (i) to standardize for goat brucellosis the most used conventional tests (i.e., RBT and CFT) and to adapt or develop some selected tests with subcellular antigens; (ii) to compare those tests using control sera from infected and brucella-free goats and sera from goats from areas where brucellosis is endemic; and (iii) to evaluate the interference of the postvaccinal serological response in the same tests.

* Corresponding author. Mailing address: Depto. Microbiología, University of Navarra, Aptdo. 273, 31080 Pamplona, Spain. Phone: 34-48-252150. Fax: 34-48-175500.

† Present address: Departamento de Microbiología, Centro Nacional de Investigacion y Desarrollo, 11001 México DF, México.

MATERIALS AND METHODS

Bacterial strains and cultures. *B. melitensis* 16M (biotype 1 [M serotype], virulent), *B. melitensis* Rev 1 (biotype 1, [M serotype], vaccine strain, attenuated), *B. melitensis* 115 (rough, avirulent), *B. abortus* 2308 (biotype 1 [A serotype], virulent), and *Yersinia enterocolitica* O:9 MY79 (*Brucella* A serotype) have been used in previous works (5, 6, 20). To ensure that they were in the smooth (S) phase, the S *Brucella* strains were inoculated into mice, recovered from the spleen 2 weeks later, and tested for dissociation (6). For cell fractionation (see below), cells were grown in tryptic soy broth in 2-liter flasks (500 ml per flask) at 37°C (26°C for *Y. enterocolitica* O:9 MY79) on an orbital shaker (200 rpm), harvested by tangential flow filtration (Pellicon Unit, PTHKOOOC5 filter; Millipore Corp., Bedford, Mass.), and washed twice with saline. Virulent *Brucella* strains were inactivated with phenol (0.5%, final concentration) at 37°C for 24 h before harvesting.

Cell fractions. (i) Native haptens (NH). Washed cells of *B. melitensis* 16M and Rev 1, *B. abortus* 2308, and *Y. enterocolitica* O:9 MY79 were extracted with distilled water (30 g [wet weight] in 100 ml) at 120°C for 15 min. The extract was clarified by centrifugation and precipitated first with 3 volumes of ethanol and then with 2 additional volumes of the same solvent (13). The second ethanol precipitate is enriched in NH, a homopolymer of *N*-formyl-perosamine immunologically re-

lated to the O chain of the S lipopolysaccharide (S-LPS) (14, 30), and also contains cyclic glucans devoid of immunological activity (14, 27) and trace amounts of S-LPS (14). NH was purified by digestion with nucleases and proteinase K followed by ultracentrifugation, phenol extraction, and ethanol precipitation (14).

(ii) *B. abortus* S-LPS polysaccharide. Briefly, washed cells of *B. abortus* 2308 were suspended in 2.0% acetic acid–10.0% NaCl and autoclaved at 120°C for 30 min. After removal of the cell debris by centrifugation, the polysaccharide was precipitated with methanol and purified by digestion with lysozyme, nucleases, and proteinase K followed by phenol extraction, ultracentrifugation, and gel filtration (10). The final product had no protein, glucans, or S-LPS, but it contained the O chain and core sugars of the S-LPS (14).

(iii) S-LPS-rich fractions. The material obtained in the first ethanol precipitation of the hot water extract from *B. melitensis* 16M (see section i above) was used without further purification, since comparison with purified S-LPS did not show significant differences in the enzyme-linked immunosorbent assay (ELISA) described below (2). By colorimetric methods (14, 28, 41), this extract contained 0.7% 2-keto-3-deoxyoctonic acid (equivalent to 80% S-LPS prepared by the phenol-water method [26]) and 18% protein (bovine serum albumin was used as the standard). By sodium dodecyl sulfate-polyacrylamide gel electrophoresis (24) and silver staining for S-LPS or proteins (29, 38), it showed the characteristic S-LPS pattern plus three major bands corresponding to *Brucella* group 3 outer membrane proteins (39).

(iv) Cytosolic fraction. Logarithmic-phase cells of *B. melitensis* 115 were washed once with cold saline and resuspended in 10 mM phosphate-buffered saline (PBS) (pH 7.2) supplemented with 50 μg each of DNase and RNase (Sigma Chemical Co., St. Louis, Mo.) per ml. The cells were disintegrated with glass beads in an MSK cell homogenizer (B. Braun Mesulgen AG, Leinfelden, Germany) with liquid CO_2 cooling, glass beads were decanted, cell envelopes were sedimented (80,000 × *g*, 2 h, 4°C), and the supernatant was held at 4°C for 24 h before being ultracentrifuged again under the same conditions. The supernatant (cytosol) was dialyzed against deionized distilled water (previously adjusted at pH 7.0) and stored frozen at −20°C. This fraction lacks cell envelope or S-LPS markers but contains cytoplasmic markers (31) and 50 to 60% protein. The NH-related polysaccharide B present in some *B. melitensis* 115 variants (11) was not detected.

Serological tests. (i) RBT and CFT. RBT was performed with a commercial cell suspension of *B. abortus* 1119-3 (Rhone-Merieux, Lyon, France) standardized according to the European Community guidelines (6, 9). CFT was performed by the standard micromethod technique, using a standard *B. abortus* 1119-3 cell suspension and the cold fixation protocol (6); a titer equal to 4 contained 20 international CFT units per ml.

(ii) Radial immunodiffusion (RID) test. *B. melitensis* 16M crude NH (second ethanol precipitate; see above) was dissolved in 10.0% NaCl–0.1 M glycine (pH 7.8) (11), and *B. abortus* and *Y. enterocolitica* O:9 polysaccharides were dissolved in 10.0% NaCl–6 mM borate (pH 8.6) (14). Agarose (Indubiose A37HAA; IBF-Biotechnics, Villeneuve la Garenne, France) at 0.8% was used as the gelling agent. At least 48 h before use, 1.0- to 1.5-mm-thick gels were poured into Falcon 1006 petri dishes (50 by 9 mm; Becton Dickinson Labware, Lincoln Park, N.J.). On the day of use (sealed plates were stable for 1 month), 4.0-mm-diameter wells were punched and filled with 15 μl of serum. Sera developing a characteristic precipitin ring after 2 to 24 h of incubation at room temperature were scored as positive.

(iii) Counterimmunoelectrophoresis (CIEP). Glass slides (9 by 12 cm) were coated with 2.5-mm-thick gels of 1% agarose (Indubiose A37HAA; IBF-Biotechnics) in 40 mM sodium barbital (pH 8.6), and 3-mm-diameter antigen and serum wells were punched 1 cm apart (12). Anodic wells were filled up with the sera, and 12 μl of cytosol (at 2 mg of protein per ml) was dispensed in the cathode wells. Electrophoresis was performed at 8 V/cm with paper wicks and with the above-described barbital buffer. After 60 min, power was turned off and the slides were incubated for 30 min in 5% monosodium citrate to remove unspecific bands.

(iv) ELISA. The following conditions were determined in preliminary experiments. For the ELISA with S-LPS and NH (both from *B. melitensis* 16M), coating was performed as described by Abalos et al. (1) since by this method NH bound to polystyrene without previous acylation (2). A 2.5-μg/ml solution in 10 mM PBS (pH 7.2) of either S-LPS-rich extract or purified NH was dispensed (100 μl per well) in standard 96-well polystyrene plates (Inotech-ELISA; Bioreba, Basel, Switzerland), and the plates were sealed and incubated overnight at 4°C. Nonadsorbed material was removed with four washings of PBS–0.05% Tween 20 and stored dry at 4°C (under these conditions sealed plates were stable for at least 6

TABLE 1. Results of serological tests with sera from *B. melitensis*-infected goats

CFT titer	No. of serum specimens						
	With indicated titer	Positive by:					
		RBT	RID[a]	CIEP	ELISA with[b]:		
					S-LPS	NH	Cytosol
4	3	3	1	2	3	3	1
8	6	6	5	4	6	6	3
16	10	10	10	9	10	10	6
32	16	16	16	16	16	16	16
64	13	13	13	13	13	13	11
≥128	7	7	7	7	7	7	7
Total	55	55	52	51	55	55	44
Sensitivity (%)	100	100	94.5	92.7	100	100	80.0

[a] With crude NH from *B. melitensis* 16M.

[b] ELISA with protein G-peroxidase conjugate and the following cutoffs: 15 to 40% for *B. melitensis* 16M S-LPS, 35 to 65% for *B. melitensis* 16M NH, and ≥40% for the cytosol of *B. melitensis* 115.

months). For the cytosolic fraction, Maxisorp (Nunc A/S, Roskilde, Denmark) polystyrene plates were used and coating was performed with a solution of 2.5 μg of protein per ml in carbonate buffer (pH 9.2) at 37°C overnight. Nonadsorbed antigens were removed as described above, and plates were used immediately. Aminated covalent binding plates (Costar, Cambridge, Mass.) and the cross-linker bis(sulfosuccinimidyl) suberate (Pierce Chemical Co., Rockford, Ill.) did not improve the results obtained with the Maxisorp plates.

Sera (diluted in PBS plus 0.05% Tween 20) were dispensed in the plates and incubated for 1 h at 37°C, and plates were washed four times with the same diluent. Two different peroxidase conjugates were used: polyclonal (rabbit) anti-sheep immunoglobulin G (IgG) of heavy- and light-chain specificity (Pierce) and recombinant protein G (Pierce). The polyclonal conjugate was used at a 1:2,000 dilution, and the protein G was used at 0.2 μg/ml, both in PBS–0.05% Tween. One hundred microliters of the conjugate solution was dispensed in each well, and after 1 h at 37°C, plates were washed and developed by incubation for 15 min at 20°C with, per well, 100 μl of 0.1% 2,2′-azino-bis-3-ethylbenzothiazoline sulfonic acid diammonium salt (Sigma) in 0.05 M citrate buffer (pH 4) with 0.004% H_2O_2. Tests of control negative and positive reference sera were repeated in all plates, and the results were expressed as the percentage of the absorbance of the positive control serum (1.360 to 1.480 at 405 nm) at the dilution giving the best discrimination between the 55 positive and 127 negative goats (see below).

(v) Sensitivity and specificity. The sensitivity and specificity of the tests were calculated with respect to the infected and brucella-free groups (see below) as described by Jones et al. (22). For comparisons, the relative sensitivity with respect to that of the CFT was calculated with the sera from the group of goats from an area where brucellosis is endemic (see below).

Sera. The blood sera of the following animals were used.

(i) *B. melitensis*-infected goats. Fifty-five goats with field infections shown by isolation of *B. melitensis* (53 with biotype 1 and 2 with biotype 3) came from areas in northern Spain where vaccination had not been used. No previous selection of the animals was made on the basis of any serological test.

(ii) *Brucella*-free goats. One hundred twenty-seven goats were from two flocks free of brucellosis in which vaccination had not been performed.

(iii) Vaccinated goats. Twenty young goats (3 to 6 months old) were vaccinated subcutaneously (10^9 *B. melitensis* Rev 1 CFU), and blood serum samples were taken 15, 45, 120, and 180 days after vaccination. In addition, 10 young goats (3 to 6 months old) were vaccinated conjunctivally (10^9 *B. melitensis* Rev 1 CFU) and bled 15, 30, 60, and 120 days after vaccination.

(iv) Unvaccinated goats from areas in which brucellosis is endemic. One hundred sixty-one goats were from Gafsa (Tunisia), an area where brucellosis is endemic and vaccination had never been used.

Bacteriological procedures. The mammary glands, uterus, spleen, and cranial, iliac, mammary, precrural, and scapular lymph nodes were taken at necropsy. They were homogenized in sterile saline, and 0.5 ml of each homogenate was seeded onto each of two petri dishes with blood agar base (Biolife, Milano, Italy) with 7% sheep blood and vancomycin (3 mg/liter), colistin methate (7.5 mg/liter), and nystatin (3 mg/liter) (Sigma). Incubation was carried out for up to 10 days at 37°C in 10% CO_2, and identification and typing of the isolates were performed by standard procedures (6).

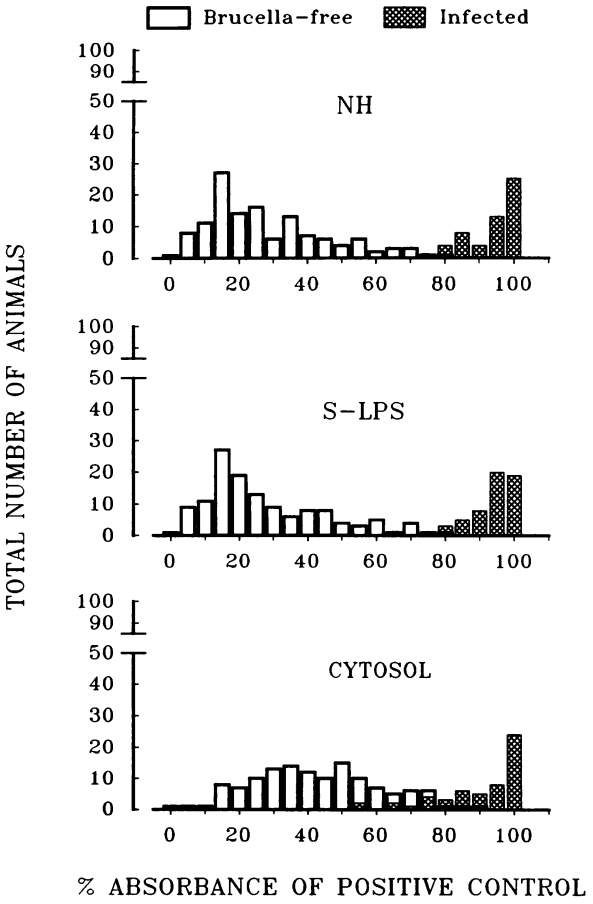

FIG. 1. ELISA with polyclonal rabbit anti-goat IgG-peroxidase conjugate and sera from 127 healthy unvaccinated goats (*Brucella*-free) and 55 goats bacteriologically positive for *B. melitensis* (Infected). Plates were coated with either NH from *B. melitensis* 16M, an extract rich in S-LPS from the same strain, or the cytosol from *B. melitensis* 115. The results correspond to a 1:200 dilution of the sera.

RESULTS

Standardization of serological tests. RBT performed as currently recommended failed to detect 6 of the 55 infected goats (90% sensitivity). In contrast, when the proportion of serum was increased (25 μl of antigenic suspension and 75 μl of serum), RBT yielded no false-negative results (Table 1). In the RID test, a single concentration (5 μg/ml of gel) of NH from either *B. melitensis* 16M or Rev 1 would detect 52 (Table 1) and 51 (not shown), respectively, of the 55 infected goats. On the other hand, the optimal concentration of the A-type polysaccharides was comparatively higher (50 μg/ml), and only 24 (*B. abortus* polysaccharide), 18 (*B. abortus* NH), and 16 (*Y. enterocolitica* O:9 NH) of the infected goats were positive. Thus, no further evaluation of the *B. abortus* and *Y. enterocolitica* 0:9 polysaccharides was carried out.

The results obtained with the ELISAs at the dilution giving the best discrimination between the sera of the infected and brucella-free goats are presented in Fig. 1 and 2. Overlapping of the results of the two sorts of sera occurred with the polyclonal conjugate, with either NH, S-LPS, or cytosol adsorbed to the plates (Fig. 1). By contrast, the sera of the infected and brucella-free goats were clearly resolved when the protein G conjugate was used in combination with NH or

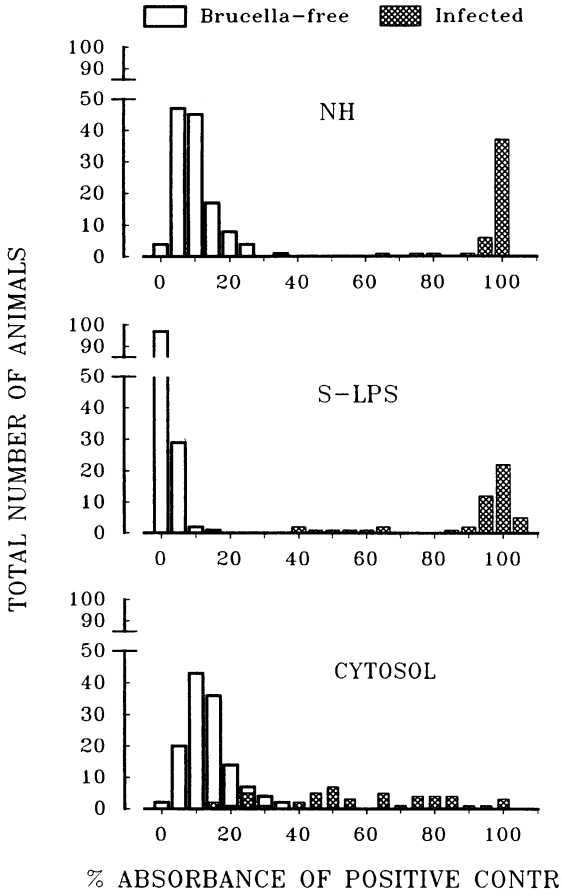

FIG. 2. ELISA with protein G-peroxidase conjugate and sera from 127 healthy unvaccinated goats (Brucella-free) and 55 goats bacteriologically positive for *B. melitensis* (Infected). Plates were coated with either NH from *B. melitensis* 16M, an extract rich in S-LPS from the same strain, or the cytosol from *B. melitensis* 115. The results correspond to a 1:100 serum dilution for LPS and a 1:50 serum dilution for NH and cytosol.

S-LPS (Fig. 2). However, even with the protein G conjugate, separation of the positive and negative sera was not achieved with the cytosol adsorbed to the plates (Fig. 2). Finally, the experiments showed a close parallelism between the results of ELISAs performed with the S-LPS-rich fraction or with the purified NH, no matter which the conjugate was used (Fig. 1 and 2).

Evaluation of the serological tests with sera from infected and brucella-free goats, and goats from areas where brucellosis is endemic. Table 1 contains a summary of the results obtained with the sera of the infected goats. It can be seen that at a titer of ≥4 CFT detected as positive the 55 infected goats (100% sensitivity) and that a diagnostic titer of ≥8 reduced the sensitivity to 94.5%. Also, the modified RBT had 100% sensitivity. The RID test with *B. melitensis* 16M NH showed a sensitivity of 94.5%, close to that of CIEP with cytosol (92.7%). It can also be noted that as CFT titers increased, there was a closer correlation between the results of the CFT, RID test, and CIEP. With the sera from the 127 brucella-free goats none of the above tests gave a false-positive result (100% specificity in these animals).

The sensitivity and specificity of the ELISA depended on the cutoff chosen. For the protein G conjugate, values between 35 and 65% (of the absorbance of the positive reference serum) for the NH or between 15 and 40% for the S-LPS discriminated between the sera from the infected and the brucella-free goats with 100% sensitivity and specificity (Fig. 2 and Table 1). By contrast, the ELISA with cytosol did not show simultaneously 100% specificity and sensitivity; for a cutoff of ≥40%, the specificity was 100% and the sensitivity was 80% (Fig. 2 and Table 1).

Table 2 summarizes the results obtained with sera from goats from an area in which brucellosis is endemic. In contrast with the close agreement observed with the sera from the infected and brucella-free groups, a large proportion of the sera with CFT titers of ≥4 were negative in the RID test and in the CIEP. With respect to the ELISA (only the data of the S-LPS and the protein G conjugate are presented), even with the more stringent cutoff (≥20%; Fig. 2), the assay had a reduced relative sensitivity compared with that of the CFT (Table 2). For the highest possible cutoff (40%; Fig. 2), the

TABLE 2. Results of serological tests with sera from goats from an area where brucellosis is endemic

CFT titer	No. of serum specimens						
	With indicated titer	Positive by:					
		RBT	RID[a]	CIEP	S-LPS ELISA[b]		
					≥20%	≥30%	≥40%
<4	78	8	0	1	8	2	0
4	18	14	2	1	10	4	3
8	25	24	6	7	20	17	11
16	21	21	17	12	20	18	16
32	8	8	8	8	8	8	8
64	8	8	8	8	8	8	8
≥128	3	3	3	3	3	3	3
Total	83[c]	86	44	40	77	60	49
Relative sensitivity[d]	100	103.6	53.0	49.4	92.7	72.3	59.0

[a] With crude NH from *B. melitensis* 16M.
[b] With protein G-peroxidase conjugate and *B. melitensis* 16M S-LPS and the indicated cutoff.
[c] Total number with a CFT titer of ≥4.
[d] Percentage of sera positive with respect to the number with a CFT titer ≥4.

TABLE 3. Results of serological tests with sera from goats vaccinated with 10^9 CFU of *B. melitensis* Rev 1

Vaccination route	Days after vaccination	No. of serum specimens						
		Total	Positive by:					
			RBT	CFT[a]	ELISA with[b]:		CIEP	RID
					NH	LPS		
Subcutaneous	14	20	20	20	15	18	10	20
	42	20	20	20	20	20	13	14
	130	17	12	12	11	8	7	1
	180	20	4	5	8	5	2	1
Conjunctival	14	10	9	3	0	0	1	1
	28	10	5	3	4	1	1	0
	60	10	2	0	3	0	1	0
	120	10	0	0	0	0	0	0

[a] Titer ≥4.
[b] Cutoffs were ≥30% (LPS) and ≥55% (NH) of the positive control.

relative sensitivity of the ELISA would be close to that of the RID or CIEP test (Table 2).

Interference of the postvaccinal serological response in the serological tests. Table 3 shows the numbers of Rev 1 vaccinated goats whose sera gave a positive result in the above-described serological tests. In the subcutaneously vaccinated group, the specificities at the end of the follow-up period were as follows: RID test, 95%; CIEP, 90%; RBT, 80%; ELISA with S-LPS and CFT, 75%; and ELISA with NH, 60%. In the conjunctivally vaccinated group, the serological response was clearly less intense (Table 2). The RID test was the first to become negative (30 days), followed by ELISA with S-LPS and CFT (60 days). All tests were negative (100% specific) 120 days after vaccination.

DISCUSSION

Serological tests used in the diagnosis of animal brucellosis can be classified, depending on the antigens used, as conventional tests (i.e., those using suspensions of whole cells as antigens) and tests using antigenic extracts. Conventional tests, RBT and CFT in particular, are widely used for cattle and sheep brucellosis (6), but gel precipitation with selected *B. abortus* and *B. melitensis* polysaccharides (3, 9, 10, 11, 13, 14, 21, 22, 27, 35) and several ELISAs with S-LPS-rich extracts (3, 21, 33) have also been proposed. All the above tests have in common that they detect mostly antibodies to antigenic determinants present in the O chain of the S-LPS. In the present work, the above tests have been evaluated for the first time using sera from goats with brucellosis proved by bacteriological isolation of bacteria, and the results show that they are all potentially useful for the diagnosis of *B. melitensis* infection in goats. However, the RBT had to be modified by increasing the serum/antigen ratio to reach 100% sensitivity. This confirms (9, 14) that present guidelines for the standardization of the RBT are not adequate for goat brucellosis. The CFT had 100% sensitivity, and the diagnostic titer (≥4) coincides with that found by Renoux in experimentally infected goats (37). Therefore, the lower sensitivity reported before for RBT and CFT (18, 40) is possibly due both to the use of sera from animals of unknown infectious status and, for the RBT, to the use of the standard method. Also, the RID test described for cattle (11, 13, 14, 22) had to be modified in two ways. First, a lower polysaccharide concentration was necessary (5 μg/ml versus the 15 to 20 μg/ml used for cattle [13, 14]), suggesting that the average antibody response of goats to the *B. melitensis* M and C epitopes is somewhat lower than that of cattle to the A and C epitopes of *B. abortus* (15). Second, an M-type NH was necessary in the RID test for *B. melitensis*-infected goats, and we have suggested (14) that this relates to the practical absence of the M epitope in the *Brucella* A serotype.

To the best of our knowledge, an ELISA for the diagnosis of goat brucellosis had not been presented before. The assay described here with NH and S-LPS is not essentially different from some indirect ELISAs used for animal brucellosis (1, 3, 21, 33, 42), but several methodological aspects deserve further comments. First, it was found that the protein G conjugate reduced the reactivity of the negative sera (i.e., it increased the ELISA specificity). In cattle brucellosis, Wright and Nielsen (42) have found that an anti-IgG1 monoclonal conjugate increases the specificity of the indirect ELISA (with S-LPS) with respect to conjugates prepared with polyclonal antibodies to IgG heavy and light chains or with a monoclonal antibody to the light chain. Since the polyclonal and the anti-light chain monoclonal antibodies, but not the anti-IgG1 monoclonal antibody, react with IgM (42), it was suggested that the specificity of the ELISA for brucellosis increases when IgM is not detected (42). Protein G binds strongly to the Fc of goat IgG (8), and in the commercial reagent, the serum albumin and other binding sites of protein G have been removed (34). Thus, the results with protein G should have the same explanation as those obtained in cattle with the anti-IgG1 monoclonal antibody. Second, the ELISAs with pure NH and extracts enriched in S-LPS yielded almost identical results. This is consistent with the identity in immunodominant sugars of the NH and the O chain of the S-LPS (14, 30) and with previous results with cattle in experiments in which acylated NH and S-LPS were compared in an indirect ELISA (3).

Serological tests that detect antibodies to *Brucella* proteins have been used in the diagnosis of human brucellosis (12) but seldom in the diagnosis of *B. abortus* and *B. melitensis* animal brucellosis. Studies performed by gel precipitation methods have shown that cattle develop antibodies to some *Brucella* soluble proteins (for a review, see reference 16), but no information is available for sheep or goats. The results of the CIEP test show that such an antibody response is common in infected goats. The fact that CIEP was more sensitive than ELISA with cytosol can be explained by a low adherence of the relevant antigens to polystyrene. Thus, an improvement of the ELISA with cytosolic proteins would require further research to define the relevant proteins and to achieve its binding to the solid phase.

When sera from unvaccinated goats in areas where brucellosis is endemic were tested, a large disagreement was found

between the RID test and CIEP and between RBT and CFT. The discrepancy was a roughly 50% difference in relative sensitivity, a value much larger than the 7 to 5% that could be expected from the results obtained with the sera from the 55 infected control goats. Presently, there is no objective criterion to decide whether those results represent false-negative reactions of the RID test and CIEP in the animals with low CFT titers, detection in the CFT and RBT of antibodies developed by goats in which the disease did not become established after exposure to *B. melitensis*, or both. By itself, the first possibility is unlikely to account for the discrepancy, because if we consider the sera from the 55 infected goats which had CFT titers of ≤16, the RID test detected 16 of these 19 animals (Table 1), only 16% fewer than the CFT. Also, there is evidence that sera with specific IgM but without significant levels of IgG are positive in RBT and CFT and negative in RID and CIEP (2). Thus, those discrepant results could correspond to animals that were in the early stages of the host-parasite interaction before the infection was (or was not) established. The interpretation of the ELISA results presents the same problems since it is not possible to decide which value in the 20 to 40% range (Table 2) should be applied. Those results illustrate the problems that would be encountered in the evaluation of indirect tests without the use of a direct test (i.e., a thorough bacteriological search) as a reference.

Since live attenuated vaccines are powerful tools in the eradication of brucellosis, the interference of the postvaccinal response in the serological diagnosis is a major problem and should be considered whenever serological tests are evaluated. Jones et al. (23) reported that the card test (equivalent to RBT) and CFT become negative by the 4th month after subcutaneous vaccination of goats with 10^9 CFU of Rev 1, and the more protracted positive serological response found in our work is likely to be due to the use of a different CFT diagnostic titer (4 versus 10 in reference 23) and the modified RBT. Also, our results suggest that ELISA with S-LPS or NH would not outperform the CFT or RBT after subcutaneous vaccination. Jones et al. (23) also observed that vaccination with a very reduced dose (5×10^4 CFU) of Rev 1 shortened the postvaccinal response to less than 2 months. However, there is evidence that vaccination with reduced doses confers a less solid immunity (17), and therefore, the conjunctival route (19, 25, 36, 43) seems a better alternative. The results presented here confirm for RBT and CFT (19), and illustrate for ELISA, CIEP, and the RID test, that the serological response after vaccination of goats is considerably reduced by this route, with the RID test followed by the ELISA with S-LPS as the tests that become negative faster. This reduction of the postvaccinal serological response by the use of the conjunctival route has also been shown in cattle (*B. abortus* 19 vaccine) and sheep (*B. melitensis* Rev 1 vaccine) (21, 36).

Although larger numbers of bacteriologically positive and vaccinated goats should be tested to reach a definite conclusion, the results of this work suggest that the modified RBT and the CFT have the same advantages and drawbacks for the diagnosis of goat brucellosis as those described for cattle and sheep. The ELISA with S-LPS and conjugates detecting IgG could be an appropriate test in areas where no vaccination or vaccination by the conjunctival route is used, laboratories are equipped with automatic ELISA systems, and the incidence of the disease is low. It is remarkable that in both conjunctivally and subcutaneously vaccinated goats the RID test was the test that became negative faster or showed the highest specificity. Thus, regardless of the route of vaccination, a combination of the modified RBT as a screening test and the RID as a confirmatory test would be an appropriate choice for those laboratories that need economical and simple diagnostic tests.

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