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Efficacy of Several Serological Tests and Antigens for Diagnosis of Bovine Brucellosis in the Presence of False-Positive Serological Results Due to *Yersinia enterocolitica* O:9

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***Yersinia enterocolitica* O:9 bears a smooth lipopolysaccharide (S-LPS) of *Brucella* sp. O-chain A + C/Y epitopic structure and is a cause of false-positive serological reactions (FPSR) in standard tests for cattle brucellosis. *Brucella* S-LPS, cross-reacting S-LPSs representing several O-chain epitope combinations, *Brucella* core lipid A epitopes (rough LPS), *Brucella abortus* S-LPS-derived polysaccharide, native hapten polysaccharide, rough LPS group 3 outer membrane protein complexes, recombinant BP26, and cytosolic proteins were tested in enzyme-linked immunosorbent assays (ELISA) and precipitation tests to detect cattle brucellosis (sensitivity) and to differentiate it from FPSR (specificity). No single serological test and antigen combination showed 100% sensitivity and specificity simultaneously. Immunoprecipitation tests with native hapten polysaccharide, counterimmunoelectrophoresis with cytosolic proteins, and a chaotropic ELISA with *Brucella* S-LPS were 100% specific but less sensitive than the Rose Bengal test, complement fixation, and indirect ELISA with *Brucella* S-LPSs and native hapten or S-LPS-derived polysaccharides. A competitive ELISA with *Brucella* S-LPS and M84 C/Y-specific monoclonal antibody was not 100% specific and was less sensitive than other tests. ELISA with *Brucella suis* bv. 2 S-LPS (deficient in C epitopes), *Escherichia hermannii* S-LPSs [lacking the contiguous α-(1–2)-linked perosamine residues characteristic of *Y. enterocolitica* S-LPS], BP26 recombinant protein, and *Brucella* cytosolic fractions did not provide adequate sensitivity/specificity ratios. Although no serological test and antigen combination fully resolved the diagnosis of bovine brucellosis in the presence of FPSR, some are simple and practical alternatives to the brucellin skin test currently recommended for differential diagnosis.**

Brucellosis is a disease caused by members of the genus *Brucella* that affects animals and humans. The species that infects cattle most often is *Brucella abortus*, but cattle infections by *Brucella melitensis* are not rare in areas where there is contact with infected sheep and goats (63, 64). Both *B. abortus* and *B. melitensis* are termed smooth (S) because they bear a S-type lipopolysaccharide (S-LPS). Many serological tests have been proposed for the diagnosis of brucellosis caused by S brucellae, and they can be broadly classified as those detecting antibodies to the S-LPS and those detecting antibodies to proteins (21, 45). The former tests use either suspensions of S brucellae as antigens (3) or S-LPS extracts. The Rose Bengal test (RBT) and the complement fixation test (CFT) belong to the first group, and are recommended by the Office International des Épizooties for international trade (4). In addition, indirect enzyme-linked immunosorbent assays (ELISA) using S-LPS extracts or its O-chain have been extensively studied (47) and may replace the RBT and CFT. S-LPS tests are the most sensitive for detecting cattle brucellosis, but they may yield false positive results for cattle vaccinated with *B. abortus* S19 or exposed to gram-negative bacteria with LPS O-chains similar to those of S brucellae. These bacteria include *Vibrio cholerae* O1, *Escherichia coli* O:157, some strains of *Escherichia hermannii* and *Stenotrophomonas maltophila*, *Salmonella* group N (O:30), and *Yersinia enterocolitica* O:9 (41–43, 50), but only *Yersinia enterocolitica* O:9 is a significant cause of false-positive serological reactions (FPSR) in the diagnosis of bovine brucellosis (29). Orally acquired *Y. enterocolitica* O:9 seldom induces high levels of antibodies to *Brucella* spp. S-LPS and the responses are usually transient in cattle (28, 43), but titers in blood serum and milk may be high and persistent (43). Accordingly, the sporadic appearance of positive serological results with brucellosis tests in countries free of brucellosis or with advanced eradication programs calls for an immediate differential diagnosis (30). A high proportion of FPSR due to *Y. enterocolitica* O:9 have emerged in the European Union since 1990, affecting up to 15% of the herds in regions free from brucellosis (29, 52, 53, 58, 67). Thus, *Y. enterocolitica* O:9 infections in cattle are troublesome and generate considerable additional costs in surveillance programs.

The cross-reactivity between *Y. enterocolitica* O:9 and S brucellae is due to a strong similarity of the LPS O-chains (32). According to nuclear magnetic resonance studies, the O-chain of S brucellae is a homopolymer of *N*-formyl-perosamine either exclusively in α-(1-2) linkages (for example, in *B. abortus*

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bv. 1) or in α-(1-2) plus α-(1-3) in a ≥4:1 proportion (4:1 in *B. melitensis* bv. 1) (50). These O-chains carry three basic types of overlapping epitopes: C (common to all types of *Brucella* O-chains), M [present in O-chains with α-(1-3) linkages], and A [present in O-chains with no α-(1-3) linkages or with a proportion of α-(1-2) to α-(1-3) linkages higher than 4:1] (16, 25, 66). The O-chain of *Yersinia enterocolitica* O:9 is a homopolymer of *N*-formyl-perosamine in α-(1-2) linkages that is indistinguishable from the O-chain of *B. abortus* biotype 1 (50). However, whereas some monoclonal antibodies (MAb) of O-chain specificity react equally with S brucellae and *Y. enterocolitica* O:9 (C/Y epitopes), others recognize epitopes common to S brucellae but not to *Y. enterocolitica* O:9 (C epitopes) (16, 25, 66), strongly suggesting subtle structural differences. Other cross-reacting bacteria also carry perosamine in their O-chains but differ in the presence of additional sugars and linkages, the types of N-substitutions, and the proportions of α-(1-2) to α-(1-3) linkages (49, 50).

Although the closely related structures of *Brucella* sp. and *Y. enterocolitica* O:9 O-chains make differential diagnosis using S-LPS tests extremely difficult, a strategy has been proposed based on the displacement of the cross-reacting antibodies (presumed to be of lower avidity in yersiniosis) in ELISA by means of MAb of C/Y specificity (46, 65) or a chaotropic agent (59). A second approach is based on the use of antigens not shared by these bacteria. The enterobacterial common antigen (40), *Y. enterocolitica* flagellar antigens (40), and outer membrane proteins (30, 36, 68) have been found to be of little usefulness, and the existence of dual infections by *Y. enterocolitica* O:9 and *B. abortus* (36, 42) further reduces the value of *Y. enterocolitica*-specific antigens. On the other hand, the immunoresponse to *Brucella* proteins is highly specific (7, 8, 10–12, 13–15, 17, 18, 20, 30, 35), and on the basis of present evidence, the best available strategy to solve the FPSR problem is the use of a skin test with *Brucella*-soluble proteins (brucellin) (8, 11, 12, 52, 30). This test is officially recommended in the European Union to discriminate FPSR in areas where vaccination has been discontinued. However, the skin test is cumbersome and expensive, so cheaper and simpler diagnostic tests would be preferable.

The aim of this work was to reevaluate in a FPSR context the above-summarized approaches by using serological test and antigen combinations that differ in threshold avidity and in the nature (LPS or protein) of the antigens. Moreover, the possibilities offered by the two main sections of *Brucella* S-LPS (core lipid A and O-polysaccharide) were systematically studied by including complete S-LPS molecules, core O-polysaccharide, and core lipid A molecules plus structural variants of the O-polysaccharide. Sera from cattle infected by either *B. abortus* or *B. melitensis* were included in these evaluations.

MATERIALS AND METHODS

Bacterial strains. The relevant characteristics of the S and rough (R) *Brucella* and *E. hermannii* strains used are summarized in Table 1. They were grown for antigen extraction as described elsewhere (3, 5).

Antigens. *B. abortus* cell suspensions for RBT and CFT (3) were provided by the Laboratorio Nacional de Referencia para la Brucelosis (Santa Fe, Granada, Spain). S-LPSs were obtained from the phenol fraction of phenol-water extracts (5) (Table 1). A crude S-LPS fraction containing group 3 Omps and native hapten (NH) polysaccharide and pure NH (Table 1) were prepared as described previously (1, 5, 23). To obtain the LPS core O-polysaccharide (PS), cells of the appropriate strain (Table 1) were hydrolyzed in 5.0% acetic acid–10% NaCl for 30 min. at 120°C, and PS was purified by enzymatic digestion, ultracentrifugation, and gel filtration (9). *E. hermannii* S-LPS was a generous gift of M. B. Perry (Institute of Biological Sciences, Ottawa, Ontario, Canada). R-LPS was obtained from *B. abortus* strain 9.49, a transposon mutant in the *per* (perosamine synthetase) gene (44), by using the phenol-chloroform-light petroleum method (26). The hot saline extraction method was applied to *Brucella ovis*, and the extract was ultracentrifuged to sediment the R-LPS group 3 Omps complexes (R-LPS-Omps) characteristic of these extracts (54). The cytosolic fractions were obtained from the appropriate strain (Table 1) by disintegration in a 40K French pressure cell press (SLM Instruments Inc., Urbana, Ill.), digestion with nucleases, and ultracentrifugation (8). The BP26 recombinant protein (6, 57) was kindly provided by O. Rossetti (INTA, Buenos Aires, Argentina). The relevant characteristics of the antigens are summarized in Table 1. The methods used in their characterization were those reported previously (5, 8, 24, 44, 54, 57, 61).

Animals and sera. The blood sera of 112 unvaccinated cows from *Brucella*-free herds were used as the reference samples for the *Brucella*-free population that was not exposed to *Y. enterocolitica* O:9, and the sera from 189 cows naturally infected by brucellae were used as the positive control population samples. The 189 cows were first selected by a positive result by RBT and CFT (Table 2) in routine serological surveys, and the infection was confirmed in all cases by culture of milk samples, vaginal swabs after abortion, and/or selected necropsy samples (3). By standard typing procedures (3), 64 of the 189 isolates were identified as *B. abortus* bv. 1, 50 of the isolates were identified as *B. abortus* bv. 3, and 75 of the isolates were identified as *B. melitensis* bv. 3.

Ten nonpregnant unvaccinated heifers of 18 to 24 months of age belonging to two flocks free of both *Brucella* and FPSR during the 5 previous years were used for experimental infection with *Y. enterocolitica* O:9 (28). All animals were negative by both RBT and CFT, and no *Y. enterocolitica* was isolated from their feces before experimental infection. Eight heifers were inoculated once per day on 5 days a week for 9 weeks by drenching with capsules containing 4×10^9 CFU of a *Y. enterocolitica* O:9 strain isolated from naturally infected cattle (29), and two heifers were given empty capsules and kept in a separate pen as controls. All animals were bled before the experiment and then twice a week for 11 weeks (28) to obtain a total of 228 serum samples. Samples taken before inoculation and from the two uninfected control heifers were negative in all serological tests. Twenty-eight samples from inoculated animals were both RBT and CFT positive (no sample was positive in only one of these two tests) and were used as the *Brucella*-free population experimentally infected with *Y. enterocolitica* O:9. Moreover, 130 serum samples from the same number of cows from unvaccinated *Brucella*-free herds affected by FPSR were used as a third *Brucella*-free control population. Of these animals, 14% were positive by RBT and/or CFT. The epidemiological characteristics of these FPSR herds have been reported (53).

Serological tests. (i) RBT and CFT. The RBT was performed according to standard procedures (3). The CFT was performed by using the standard warm microtechnique (3); sera showing 50% or less hemolysis at 1/4 dilution (20 international complement fixation test units [ICFTU]/ml) were considered positive (4).

(ii) Indirect ELISA. Stock solutions of antigens (Table 1) were prepared at 1 mg/ml in distilled water, sonicated briefly, and used directly or stored at −20°C. Standard 96-well polystyrene plates (MaxiSorp Nunc A/S, Roskilde, Denmark) were coated with antigens in phosphate-buffered saline (PBS) at 4°C overnight, except for BP26 and *B. abortus per* R-LPS, for which the coating was made in 60 mM carbonate buffer (pH 9.6) at 37°C overnight. Optimal antigen concentrations were 10 μg/ml for *E. hermannii* S-LPS, 1 μg/ml for BP26, and 2.5 μg/ml for the remaining antigens. Nonadsorbed material was removed with three washings of 0.05% Tween 20 in PBS. Serum dilutions were made in 0.05% Tween 20 in PBS or, for BP26-coated plates, in the same diluent supplemented with 3% skim milk. Serum dilutions giving the largest differences in optical density (OD) between sera from culture positive and *Brucella*-free controls were 1/10 for *E. hermannii* LPS-coated plates, 1/50 for plates coated with cytosolic proteins, BP26, R-LPS, and R-LPS-Omps, and 1/200 for plates coated with NH, PS, crude S-LPS, and *B. suis* S-LPS. One hundred microliters was added to duplicate wells, the plates were incubated for 1 h at 37°C, the sera were removed, and the wells were washed three times with 0.05% Tween-PBS before adding the conjugate. The chaotropic ELISA with *B. melitensis* crude S-LPS was performed likewise, but after removal of the sera, 100 μl of either 1 M, 2 M, or 3 M KSCN was dispensed into each well and the plates were incubated for 15 min at room temperature before washing. Recombinant protein G-peroxidase (Pierce Chemical Co., Rockford, Ill.) (100 μl/well of a solution containing 0.2 μg of protein G per ml, in 0.05% Tween in PBS [0.05% Tween and 3% skim milk when testing BP26]) was added, and the plates were incubated for 1 h at 37°C, washed three times

TABLE 1. Denomination, source, and main characteristics of the antigens used in the different diagnostic tests

Antigen	Source	Characteristics	Test	Reference
Crude S-LPS	*B. melitensis* 16M bv. 1	S-LPS (lipid A and core epitopes). Over 90% formylated perosamine O-polysaccharide in α-(1-2) and α-(1-3) linkages in a 4:1 proportion bearing M, C, and C/Y epitopes. NH and group 3 Omps present.	Double gel immunodiffusion, indirect and chaotropic ELISA	1
S-LPS	*B. abortus* S19 bv. 1	S-LPS (lipid A and core epitopes). Over 90% formylated perosamine O-polysaccharide in α-(1-2) linkages bearing A, C, and C/Y epitopes. Traces of NH and group 3 Omps.	Competitive ELISA	1
	B. suis Thomsen bv. 2	S-LPS (lipid A and core epitopes). Perosamine O-polysaccharide of structure presumed to be similar to that of *B. abortus* but with markedly reduced reactivity with MAb 12G12; this MAb reacts with other *Brucella* S-LPSs but not with *Y. enterocolitica* O:9 S-LPS).	Indirect ELISA	3, 65
	E. hermannii NRCC 4298	S-LPS. O-polysaccharide of N-acetylated perosamine in α-(1-2) and α-(1-3) linkages in a 2:3 ratio [no contiguous α-(1-2) linkages].	Indirect ELISA	49
PS	*B. abortus* S19 bv. 1	O-polysaccharide of *B. abortus* bv. 1 (see above) plus core epitopes.	Indirect ELISA	25
NH	*B. melitensis* 16M bv. 1	About 60% N-formylated perosamine polysaccharide in α-(1-2) and α-(1-3) linkages in a 4:1 proportion.	Indirect ELISA, NH-RID	5; G. Widmalm and I. Moriyón (unpublished results)
R-LPS	*B. abortus per* (R mutant)	R-LPS (lipid A and core epitopes).	Indirect ELISA	44
R-LPS-Omps	*B. ovis* Reo198 (R mutant)	R-LPS (lipid A and core epitopes) and group 3 Omps.	Indirect ELISA	54
BP26	*B. abortus* S19	Recombinant BP26 (*Brucella* periplasmic protein).	Indirect ELISA	6, 13, 57
Cytosolic fraction	*B. melitensis* 115 (R mutant)	Protein mixture soluble fraction of French press-disrupted bacteria.	Indirect ELISA, counterimmunoelectrophoresis, protein-RID	8, 20

with 0.05% Tween in PBS, and developed with 0.1% 2,2′-azinobis(3-ethylbenzothiazolinesulfonic acid) (ABTS) diammonium salt (Sigma Chemical Co., St. Louis, Mo.) and 0.004% hydrogen peroxide in 0.05 M citrate buffer (pH 4). The reaction was not stopped, and the OD at 405 nm was measured (Multiskan RC; Thermo Labsystems, Vantaa, Finland) after 15 min. (for *Brucella* S-LPSs, O-chain, R-LPS, and cytosol) or 30 min. (for other antigens) at room temperature. Duplicate tests of the same negative and positive control sera were repeated for each plate as internal controls, and the results were expressed as percentages of average ODs with respect to the average OD of the positive control serum.

(iii) Competitive ELISA. The competitive ELISA was performed by following the procedures described in the Brucellosis ELISA kit manual (Competitive enzyme immunoassay for detection of antibody to *Brucella abortus*. Bench protocol, version cELISA prototype 2, October 1994. Joint FAO/IAEA Programme, Seibersdorf, Austria). Mouse MAb M84 of C/Y specificity (International Atomic Energy Agency, Vienna, Austria) was obtained from E. Moreno (Universidad Nacional, Heredia, Costa Rica) and used as a competitive reagent. Horseradish peroxidase-conjugated goat anti-mouse immunoglobulin G (heavy plus light chain specificity), ABTS substrate, buffer substances, and negative and strong, intermediate, and weak positive bovine control sera (27) were used as described in the kit manual. The results were expressed as the percent inhibition of binding of MAb M84 {[(1− mean absorbance value of the duplicate test sample)/mean absorbance value of the duplicate test sample with the MAb alone] × 100}.

(iv) Double gel immunodiffusion. Double gel immunodiffusion was performed in 1% Noble agar (Difco Laboratories, Detroit, Mich.) with 10% NaCl–0.1 M $NaOH$-H_3BO_4 (pH 8.3) with 20 μl of serum and antigen in wells set 3 mm apart (39). In this test, the antigen used (*B. melitensis* crude S-LPS [Table 1] at 1 to 2 mg/ml) develops both the NH and S-LPS precipitation bands (5, 23). The plates were read after 24 and 48 h of incubation in a moist chamber at room temperature. Immediately before the 48-h reading, unspecific precipitation lines were removed by soaking the plates in 5% sodium citrate solution for 1 h.

TABLE 2. Sensitivities and specificities of tests using polysaccharide or S-LPS antigens for the serological diagnosis of bovine brucellosis in the presence of interferences due to *Y. enterocolitica* O:9[a]

Test	Antigen	% Sensitivity (95% CI) and no. of sera tested for cattle infected with:			Cutoff[b]	% Specificity (95% CI) and no. of sera for *Brucella*-free cattle:		
		B. abortus	*B. melitensis*	Either brucella		Not exposed to *Y. enterocolitica* O:9	Experimentally infected with *Y. enterocolitica* O:9	From FPSR herds
RBT	*B. abortus* whole cells	100 (97.1–100), 114	100 (95.7–100), 75	100 (98.2–100), 189		100 (96.7–100), 112	Not applicable	86.4 (79.1–91.9), 125
CFT	*B. abortus* whole cells	100 (97.1–100), 114	100 (95.7–100), 75	100 (98.2–100), 189	20	100 (96.7–100), 112	Not applicable	94.4 (88.8–97.7), 125
Indirect ELISA	*B. melitensis* crude S-LPS	100 (96.8–100), 114	100 (95.2–100), 75	100 (98–100), 189	>31.12	100 (96.7–100), 112	42.9 (24.5–62.8), 28	58.4 (49.2–67.1), 125
	B. melitensis NH	100 (96.8–100), 114	100 (95.2–100), 75	100 (98–100), 189	>35.29	100 (96.7–100), 112	42.9 (24.5–62.8), 28	94.4 (88.8–97.7), 125
	B. abortus PS	100 (96.8–100), 114	100 (95.2–100), 75	100 (98–100), 189	>23.07	100 (96.7–100), 112	7.1 (1.1–23.5), 28	36.8 (28.4–45.9), 125
	B. suis S-LPS	100 (96.8–100), 114	100 (95.2–100), 75	100 (98–100), 189	>15.87	100 (96.7–100), 112	0 (0.0–12.5), 28	20 (13.4–28.1), 125
	E. hermanni S-LPS	95.3 (84.2–99.3), 43	100 (91.7–100), 43	97.7 (91.8–99.7), 86	>75.04	100 (91.3–100), 41	100 (80.3–100), 17	29.6 (13.8–50.2), 125
Chaotropic ELISA								
1 M KSCN	*B. melitensis* crude S-LPS	100 (96.8–100), 114	100 (95.2–100), 75	100 (98–100), 189	>12.62	100 (96.7–100), 112	64.3 (44.1–81.3), 28	84 (76.4–89.9), 125
2 M KSCN	*B. melitensis* crude S-LPS	97.4 (92.5–99.4), 114	98.7 (92.8–99.8), 75	97.9 (94.7–99.4), 189	>25.79	100 (96.7–100), 112	100 (87.5–100), 28	97.6 (93.1–99.5), 125
3 M KSCN	*B. melitensis* crude S-LPS	89.5 (82.3–94.4), 114	88 (78.4–94.4), 75	88.9 (83.5–93), 189	>21.22	100 (96.7–100), 112	100 (87.5–100), 28	100 (97.1–100), 125
Competitive ELISA	*B. abortus* S-LPS	90.5 (83.2–95.3), 105	76 (64.7–85.1), 75	84.4 (78.3–89.4), 180	>35.73	100 (95.9–100), 90	85.7 (67.3–95.9), 28	88.8 (81.9–93.7), 125
Double gel diffusion	*B. melitensis* crude S-LPS	85.1 (77.2–91.1), 114	93.3 (85.1–97.8), 75	88.4 (82.9–92.6), 189		100 (96.7–100), 112	100 (87.5–100), 28	100 (97.1–100), 125
NH-RID	*B. melitensis* NH	92.9 (85.1–97.3), 84	94.2 (85.8–98.4), 69	93.5 (88.3–96.8), 153		100 (96.7–100), 112	100 (87.5–100), 28	100 (97.1–100), 125

[a] Cutoffs for ELISAs were selected to result in 100% specificity with the sera from *Brucella*-free animals not exposed to *Y. enterocolitica* O:9.
[b] See Materials and Methods for the definition of the cutoff for each test.

TABLE 3. Sensitivities and specificities of tests using R-LPS or protein antigens for the serological diagnosis of bovine brucellosis in the presence of interferences due to *Y. enterocolitica* O:9[a]

Test	Antigen	% Sensitivity (95% CI) and no. of sera tested for cattle infected with:			Cutoff[b]	% Specificity (95% CI) and no. of sera for *Brucella*-free cattle:		
		B. abortus	*B. melitensis*	Either brucella		Not exposed to *Y. enterocolitica* O:9	Experimentally infected with *Y. enterocolitica* O:9	From FPSR herds
Indirect ELISA	*B. ovis* R-LPS-Omps	83.3 (75.2–89.7), 114	81.3 (70.7–89.4), 75	82.5 (76.4–87.7), 189	>37.45	100 (96.7–100), 112	32.1 (15.9–52.3), 28	56.8 (47.6–65.6), 125
	B. abortus per R-LPS	91.1 (84.2–95.6), 112	96 (88.7–99.1), 75	93 (88.4–96.2), 187	>38.47	100 (96.7–100), 112	0 (0–12.5), 28	3.2 (0.9–8), 125
	B. abortus BP26	70.7 (61.5–78.8), 114	80.5 (70.3–88.4), 75	74.7 (68.1–80.6), 198	>64.58	100 (96.7–100), 112	84.6 (65.1–95.5), 26	98.4 (94.3–99.8), 124
	B. melitensis 115 cytosol	90.4 (83.4–95.1), 114	100 (95.2–100), 75	93.1 (88.5–96.3), 189	>62.42	100 (96.7–100), 112	42.9 (24.5–62.8), 28	68.8 (59.9–76.8), 125
Counterimmuno-electrophoresis	*B. melitensis* 115 cytosol	85.7 (71.4–94.5), 42	95.9 (86–99.4), 49	91.2 (83.4–96.1), 91		100 (96.7–100), 112	100 (87.5–100), 28	100 (97.1–100), 125
Protein-RID	*B. melitensis* 115 cytosol	81.0 (65.9–91.4), 42	91.8 (80.4–97.7), 49	86.8 (78.1–93), 91		100 (96.7–100), 112	100 (87.5–100), 28	100 (97.1–100), 125

[a] Cutoffs for ELISAs were selected to result in 100% specificity with the population of sera from *Brucella*-free animals not exposed to *Y. enterocolitica* O:9.
[b] See Materials and Methods for the definition of the cutoff for each test.

(v) Counterimmunoelectrophoresis. Counterimmunoelectrophoresis was performed in 0.8% low electroendosmosis agarose (Indubiose A37HAA; IBF-Biotechnics, Villeneuve la Garenne, France) in 20 mM Veronal buffer (pH 8.6) (20). Sera were placed on the anode side and the cytosolic fraction (Table 1) at a concentration of 1 to 2 mg/ml on the cathode side. The electrophoresis was run for 2 h at 2 V/cm with paper wicks and 40 mM Veronal (pH 8.6) as the vessel buffer. Unspecific bands were removed as described above.

(vi) RID. The use of the radial immunodiffusion (RID) precipitation test with NH (NH-RID) or *Brucella* proteins has been described in detail (18, 19, 21, 22, 23, 24, 33, 34). For the NH-RID, a commercial kit (Ingenasa, Madrid, Spain) was used. For proteins, the cytosolic fraction (Table 1) was dissolved at 10 μg/ml in 10% NaCl–0.1 M glycine buffer (pH 7.8) and mixed with an equal volume of 1.6% agarose (Indubiose A37HAA) in the same buffer previously equilibrated at 42°C. The gel-antigen mixture was poured into 50- by 9-mm Falcon 1006 petri dishes (Becton Dickinson Labware, Lincoln Park, N.J.) to generate 1.0- to 1.5-mm-thick gels in which 4.0-mm-diameter wells were filled with 15 μl of serum. Positive sera develop a characteristic precipitin ring(s) after 2 to 24 h of incubation at room temperature.

Sensitivity, specificity, and statistical analyses. For each test, the sensitivity (percentage of sera from culture-positive animals scoring positive), the specificity (percentage of sera from *Brucella*-free animals scoring negative), and the 95% confidence intervals (CI) were calculated (MedCalc 7.2.0.0). Specificities were estimated with respect to (i) *Brucella*-free animals not exposed to *Y. enterocolitica* O:9, (ii) *Brucella*-free animals experimentally infected with *Y. enterocolitica* O:9, and (iii) animals from *Brucella*-free herds affected by FPSR. For quantitative tests, results from the infected (culture-positive) animals and the three different *Brucella*-free populations were used to perform receiver-operating characteristic (ROC) analyses and to determine appropriate cutoff values, and the overall test performance was evaluated as the area under the specificity-sensitivity curve (AUC) (MedCalc 7.2.0.0). These analyses provide a useful estimate of test accuracy that is independent of specific cutoff values and prevalence (31). Comparisons between sensitivities and specificities were performed as described in reference 2a by using Microsoft Excel 2002.

RESULTS

Tables 2 and 3 summarize the results obtained with the different test and antigen combinations. For quantitative tests, cutoffs were adjusted to yield 100% specificity when testing the *Brucella*-free population not exposed to *Y. enterocolitica* O:9. Since the RBT and CFT had been used in the selection of these animals, they resulted in 100% sensitivity with the sera from culture-positive cattle and in 100% specificity with the sera from *Brucella*-free animals not exposed to *Y. enterocolitica* O:9. However, both tests yielded positive reactions with the sera from *Brucella*-free animals that had been experimentally infected with *Y. enterocolitica* O:9 or belonged to FPSR herds (Table 2). Confirming previous analyses (1), the epitopic structure of the S-LPS (Table 1) did not significantly affect the ELISA results with regard to the species (*B. melitensis* or *B. abortus*) infecting the animals (Table 2).

FPSR becomes particularly significant when brucellosis prevalence is low, a context requiring highly specific tests. With cutoffs adjusted to 100% specificity with the sera from *Brucella*-free animals not exposed to *Y. enterocolitica* O:9, the indirect ELISA with crude S-LPS, NH, and PS yielded 100% sensitivity. Moreover, as illustrated in Fig. 1 for ELISA with *B. melitensis* crude S-LPS, the sera from *Brucella*-infected and *Brucella*-free animals not exposed to *Y. enterocolitica* O:9 were clearly separated, thus making possible a wide range of OD cutoff values resulting in 100% specificity in the discrimination of *Brucella*-free animals not exposed to *Y. enterocolitica* O:9. However, independent of the cutoff used, these ELISA were not 100% specific when testing the sera of the cattle that had been experimentally infected with *Y. enterocolitica* O:9 or that belonged to FPSR herds (Table 2). In these two groups, the

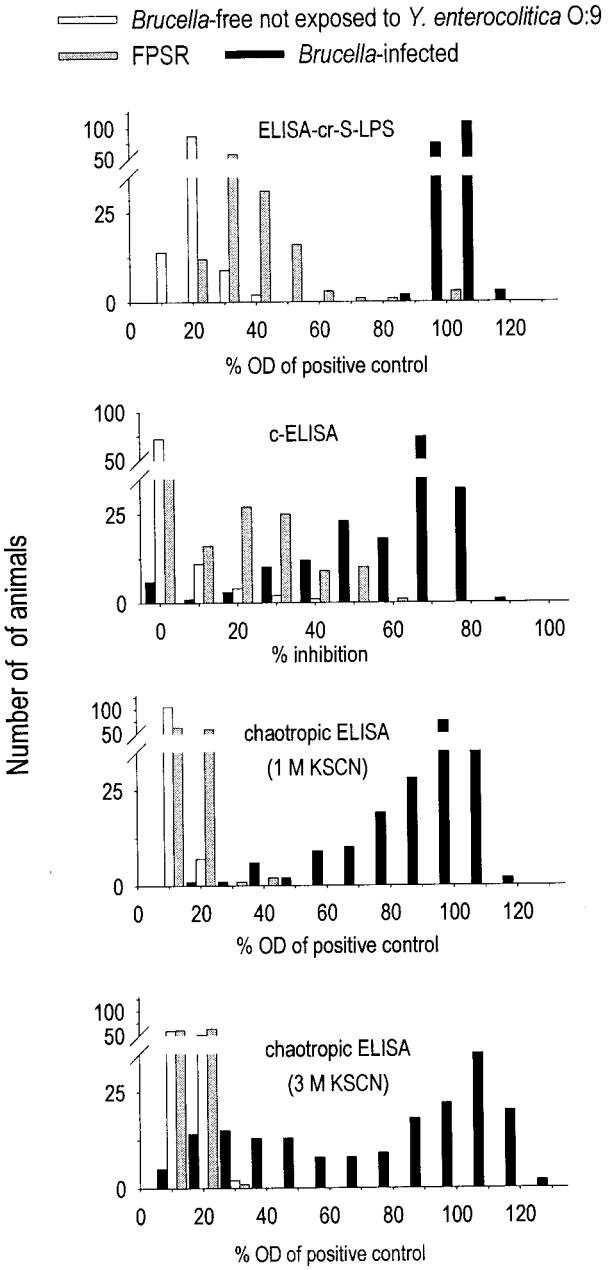

FIG. 1. Distribution of the sera from *Brucella*-infected cattle (black bars), *Brucella*-free cattle not exposed to *Y. enterocolitica* O:9 (white bars), and *Brucella*-free cattle from FPSR herds (gray bars) in four ELISA.

possible cutoffs resulted in a range of specificities. Table 2 also shows that the use of S-LPSs of *B. suis* bv. 2 and *E. hermannii* (with epitopic structures departing from those of *Y. enterocolitica* O:9 or other S *brucellae* [Table 1]) resulted in adequate sensitivities but not specificities for cattle experimentally infected with *Y. enterocolitica* O:9 or cattle with FPSR.

The chaotropic and competitive ELISA are designed to remove antibodies of comparatively low avidity. However, both protocols resulted in a marked overlapping of the *Brucella*-infected and *Brucella*-free populations not exposed to *Y. enterocolitica* O:9 (Fig. 1) and, accordingly, none afforded 100% specificity and 100% sensitivity simultaneously (Table 2). The chaotropic ELISA using 3 M KSCN reduced considerably the reactivity of sera from *Y. enterocolitica* O:9 experimentally infected and FPSR groups (Fig. 1) and, at a given cutoff, resulted in 100% specificity in tests of both groups of sera (Table 2). However, these conditions resulted in a significant ($P < 0.01$) decrease in the diagnostic sensitivity with respect to the crude S-LPS indirect ELISA (Table 2). Although this effect was less intense when 1 M (Fig. 1) or 2 M (data not shown) KSCN was used, these chaotropic protocols did not outperform the specificity of CFT in the FPSR group (Table 2). Owing to the marked overlapping in the populations tested (Fig. 1), the competitive ELISA was less sensitive than the 3 M KSCN chaotropic assay and, moreover, was not 100% specific when the sera from animals infected with *Y. enterocolitica* O:9 or those of animals from FPSR herds were tested (Table 2). With a cutoff adjusted to 100% specificity for the sera from *Brucella*-free animals not exposed to *Y. enterocolitica* O:9, the competitive ELISA was significantly less sensitive than the RBT, CFT, and most indirect ELISA (Table 2).

The NH-RID test had a sensitivity higher than those of the 3 M KSCN chaotropic ELISA ($P < 0.05$) and the competitive ELISA ($P < 0.01$), although its sensitivity was lower ($P < 0.001$) than those of the RBT, CFT, and indirect ELISA with crude S-LPS, NH, or PS as the antigen. Although not statistically significant, the sensitivity of the NH-RID test was somewhat higher than that of the double gel immunodiffusion test (Table 2). Both precipitation tests showed 100% specificity with the sera from *Brucella*-free cattle that had been experimentally infected with *Y. enterocolitica* O:9 or belonged to FPSR herds.

The possibility that LPS epitopes other than those in the O-chain of the S-LPS could discriminate brucellosis from *Y. enterocolitica* O:9 infections was tested by using the R-LPS of a *B. abortus per* mutant or the *B. ovis* R-LPS-Omps complex (Table 1). When adjusted to 100% specificity for the sera from *Brucella*-free animals not exposed to *Y. enterocolitica* O:9, these ELISA resulted in moderate sensitivities (Table 3), with no significant differences between the *B. abortus*- and *B. melitensis*-infected subgroups. Moreover, their specificities were poor when the sera from *Brucella*-free cattle experimentally infected with *Y. enterocolitica* O:9 or members of FPSR herds were tested.

The antibody response to *Brucella* soluble proteins was examined in a variety of tests. The counterimmunoelectrophoresis and protein-RID precipitation tests showed 100% specificity with all *Brucella*-free populations no matter whether the animals had been exposed to *Y. enterocolitica* O:9 (Table 3). Both tests had a sensitivity similar to that of NH-RID, but not all sera reacted simultaneously with NH and proteins, so the combined sensitivities of RID or counterimmunoelectrophoresis with proteins and NH-RID increased to about 5% over that of the individual tests. ELISA with the cytosolic protein fraction resulted in sensitivities and specificities similar to those of the gel precipitation tests with the same antigen in the sera from animals not exposed to *Y. enterocolitica* O:9. However, this ELISA was significantly less specific than gel precipitation tests for the *Brucella*-free animals infected with *Y. enterocolitica* O:9 or members of FPSR herds (Table 3). The use of BP26 recombinant protein considerably improved the specificity of

TABLE 4. Overall test performance (AUC) after ROC analysis and resulting sensitivities of quantitative tests when used on three different *Brucella*-free populations as negative controls[a]

Test	Antigen	Results for *Brucella*-free populations with characteristic indicated								
		Not exposed to *Y. enterocolitica* O:9			Experimentally infected with *Y. enterocolitica* O:9			Member of FPSR herds		
		AUC (95% CI)	Cutoff	Se (95% CI)	AUC (95% CI)	Cutoff	Se (95% CI)	AUC (95% CI)	Cutoff	Se (95% CI)
Indirect ELISA	*B. melitensis* crude S-LPS	1 (0.988–1)	>31.12	100 (98–100)	1 (0.983–1)	>79.57	100 (98–100)	1 (0.988–1)	>93.1	98.9 (96.2–99.8)
	B. melitensis NH	1 (0.988–1)	>35.29	100 (98–100)	0.999 (0.98–1)	>90.6	97.9 (94.7–99.4)	0.999 (0.987–1)	>94.7	96.3 (92.5–98.5)
	B. abortus PS	1 (0.988–1)	>23.07	100 (98–100)	0.911 (0.865–0.945)	>100.74	30.7 (24.2–37.8)	0.987 (0.968–0.996)	>102.5	19 (13.7–25.4)
	B. suis S-LPS	1 (0.988–1)	>15.87	100 (98–100)	0.983 (0.955–0.995)	>98.6	71.4 (64.4–77.7)	0.999 (0.987–1)	>94.7	97.9 (94.7–99.4)
	E. hermanni S-LPS	0.988 (0.95–0.998)	>75.04	97.7 (91.8–99.7)	0.997 (0.959–1)	>143.78	0 (0–4.2)	0.712 (0.62–0.794)	>141.1	0 (0–4.2)
	B. ovis R-LPS-Omps	0.994 (0.978–0.999)	>37.45	82.5 (76.4–87.7)	0.714 (0.649–0.773)	>74.02	42.9 (35.7–50.2)	0.827 (0.78–0.867)	>82.9	39.2 (32.2–46.5)
	B. abortus per R-LPS	0.997 (0.983–0.999)	>38.47	93 (88.4–96.2)	0.484 (0.415–0.553)	>85.04	8.6 (5–13.5)	0.678 (0.623–0.729)	>114.34	1.1 (0.2–3.8)
	B. abortus BP26	0.973 (0.949–0.988)	>64.58	74.7 (68.1–80.6)	0.859 (0.806–0.901)	>66.96	70.7 (63.8–76.9)	0.96 (0.932–0.979)	>67.6	70.7 (63.8–76.9)
	B. melitensis 115 cytosol	0.999 (0.985–1)	>62.42	93.1 (88.5–96.3)	0.9 (0.852–0.936)	>89.9	69.3 (62.2–75.8)	0.941 (0.908–0.964)	>88.5	71.4 (64.4–77.7)
Chaotropic ELISA										
1 M KSCN	*B. melitensis* crude S-LPS	1 (0.988–1)	>12.62	100 (98–100)	0.999 (0.981–1)	>19.35	99.5 (97.1–99.9)	0.999 (0.987–1)	>39.9	95.8 (91.8–98.1)
2 M KSCN	*B. melitensis* crude S-LPS	0.994 (0.977–0.999)	>25.79	97.9 (94.7–99.4)	0.996 (0.975–0.999)	>19.65	98.9 (96.2–99.8)	0.994 (0.977–0.999)	>52.5	85.7 (79.9–90.4)
3 M KSCN	*B. melitensis* crude S-LPS	0.961 (0.932–0.98)	>21.22	88.9 (83.5–93)	0.978 (0.949–0.993)	>13.44	95.8 (91.8–98.1)	0.973 (0.948–0.988)	21.2	88.9 (83.5–93)
Competitive ELISA	*B. abortus* S-LPS	0.977 (0.951–0.991)	>35.73	84.4 (78.3–89.4)	0.930 (0.886–0.961)	>45.23	75.0 (68.0–81.1)	0.932 (0.897–0.957)	>57.2	61.7 (54.1–68.8)

[a] Cutoffs were selected to result in 100% specificity for each independent *Brucella*-free population. Se, sensitivity (%).

ELISA with cytosolic fraction in these sera, but the sensitivity was low (Table 3).

Table 4 shows the results of ROC analyses aimed at determining the overall performance (AUC values) of the different ELISA. For the *Brucella*-free cattle not exposed to *Y. enterocolitica* O:9, AUC values were similar in most tests. However, in the *Brucella*-free cattle infected with *Y. enterocolitica* O:9 or in members of FPSR flocks, the AUC values were significantly higher for the crude S-LPS, NH, *B. suis* S-LPS, and 1 or 2 M KSCN indirect ELISA. Accordingly, when the diagnostic cutoffs were optimized, it was possible to obtain 100% specificity values without significantly affecting the sensitivities (Table 4). When adjusted in this manner, the sensitivities of the best tests in the presence of diagnostic interferences due to *Y. enterocolitica* O:9 were equivalent to those of the NH-RID and counterimmunoelectrophoresis precipitation tests, which also showed 100% specificities.

DISCUSSION

The standardization and, to a great extent, the final performance of serological tests rely on the definitions of their sensitivity and specificity with sets of sera representative of the context in which they are to be applied. In this work, the positive control sera were selected on the basis of a positive serological result in the standard RBT and CFT tests, and brucellosis was confirmed bacteriologically to exclude the possibility of FPSR. Although this collection of sera is not truly representative of a *Brucella*-infected cattle population (some infected animals may be negative by either RBT or CFT or both), it is representative of a context where a diagnosis relying on standard tests has to take into account the FPSR problem. Accordingly, the sensitivities reported here for the various tests are relative to the results of RBT and CFT, and when they reach 100% the possibility that they would result in better sensitivity than the RBT and CFT under other circumstances cannot be excluded (47). Obviously, this bias has the same effect on all tests studied, and since it does not affect the specificity (the *Brucella*-free populations were selected on a different basis), our results are meaningful in comparative terms. Specificities were first estimated by using *Brucella*-free animals that had had no contact with *Y. enterocolitica* O:9. Moreover, since this negative control is not representative of brucellosis-free areas affected by FPSR, we also tested *Brucella*-free animals from flocks affected by FPSR or experimentally inoculated with *Y. enterocolitica* O:9.

ROC analysis allows both to adapt quantitative tests to a particular diagnostic objective through the selection of a specific cutoff value and to carry out statistical comparisons on test performance (31). One possibility is to select cutoffs providing the highest combined specificities and sensitivities; a performance index equal to the maximal sum of the percentages of sensitivity and specificity is sometimes used for comparisons. However, when brucellosis prevalence is zero or very low but FPSR occur, cutoff values maximizing specificity should be favored over those minimizing the number of misclassifications. Not surprisingly, our results show that diagnostic cutoffs had to be significantly increased to maximize specificity when FPSR occur. It is important to stress that comparisons based solely on AUC calculations may result in important misestimations if the existence of different epidemiological scenarios is neglected. As an example, the indirect ELISA with R-LPS-Omps complexes, *E. hermannii* S-LPS, and *B. abortus* PS resulted in a high overall test performance (AUC value) in some of the three *Brucella*-free scenarios but their overall diagnostic sensitivity was poor in the FPSR context.

The use of purified perosamine polysaccharides, such as NH or PS in indirect ELISA, did not outperform the relatively simple-to-obtain crude S-LPS, confirming previous reports (1, 2). It is noteworthy that this simple indirect ELISA performed better than chaotropic or competitive ELISA, as illustrated by the wider gap between the sera of *Brucella*-infected and *Brucella*-free animals (Fig. 1). These results show that antibodies to C/Y are diagnostically significant (and not merely cross-reacting) and, since the anti-C/Y MAb did not fully displace all *Y. enterocolitica* O:9 cross-reacting antibodies, stress the significance of the concept that A, M, C, and C/Y are overlapping epitopes (66). They also show that although the cross-reacting antibodies are mostly of low avidity, differences in this property are not wide enough to develop an ELISA simultaneously providing 100% specificity and sensitivity. In summary, consistent with previous works (46, 59, 65), our results do not support the conclusion that the competitive ELISA is a satisfactory test for differentiating *B. abortus* and *Y. enterocolitica* O:9 infections (48). Moreover, since this test is outperformed by others even in the absence of the FPSR problem, the competitive ELISA should not be used as it is currently for the international cattle trade (4) and other purposes requiring high sensitivity and specificity.

B. suis Thomsen S-LPS does not react with MAb 12G12, which is specific for the C epitope, does not react with *Y. enterocolitica* O:9 S-LPS, and improves ELISA specificity with sera from *Y. enterocolitica* O:9-infected animals when used as a competitive reagent (16, 65). However, this S-LPS contains the C/Y epitope and is similar, in this regard, to *Y. enterocolitica* O:9 S-LPS. This epitopic structure probably accounts for its low specificity in the FPSR context, but it is noteworthy that the apparent absence of the C epitopes in *B. suis* Thomsen S-LPS did not result in a reduced sensitivity, even in the *B. melitensis*-infected cattle. This finding suggests that the absence of C epitopes in this S-LPS becomes obscured in the indirect ELISA by the overlapping nature of the O-chain epitopes and that anti-C/Y antibodies are more significant in the diagnosis. With regard to the S-LPS from *E. hermannii* NRCC 4298, its O-polysaccharide reacts strongly with MAb of anti-M specificity (49) and its structure departs more from those of *B. abortus* bv. 1 and *Y. enterocolitica* O:9 O-polysaccharides than from that of *B. melitensis* (Table 1). Because of this fact, the *E. hermannii* S-LPS was tested and, consistent with the dominance of α-(1-3)-linked perosamine, it was somewhat more sensitive in detecting *B. melitensis*- than *B. abortus*-infected cattle and maintained an acceptable diagnostic sensitivity (Table 2). However, its specificity was poor when the *Brucella*-free animals experimentally infected with *Y. enterocolitica* O:9 or members of FPSR flocks were tested. The lack of specificity of this antigen is in all likelihood attributable to antibodies to the core and lipid A of enterobacteria in the sera of cattle.

Two overlapping epitopes have been defined in the core oligosaccharide of *Brucella* LPS, and several in the lipid A (55),

and they react with antibodies of *Brucella*-infected bovines (2, 56). However, to the best of our knowledge, this is the first evaluation of its diagnostic value. The performance of the indirect ELISA with the *B. abortus* R-LPS containing a complete core (44) or the *B. ovis* R-LPS-Omp3 complex was not satisfactory. As suggested before for the diagnosis of *B. ovis* in rams by indirect ELISA with R-LPS (62), taxonomically related bacteria present in the environment may account for these observations.

Immunoprecipitation tests with NH or polysaccharide B are sensitive and specific in discriminating infected animals from cattle vaccinated with *B. abortus* S19 (19, 22–24, 34, 38, 51) but have never been tested in the context of FPSR. NH precipitation tests were 100% specific in the three *Brucella*-free populations studied and resulted in a reasonable sensitivity level. Except for the degree of formylation, the O-chains of S-LPS and NH have closely related structures. However, the sensitivities of precipitation tests are higher with NH than with S-LPS (1, 19, 22). This result has been attributed to the high epitopic density of NH and to its disperse state in solution (which differs from the bulky micelle-like state of S-LPS), two properties which should favor its immunoprecipitation when only relatively high titers of antibody are present (1, 2, 39). On the other hand, the high specificity of NH-RID has been attributed to the comparatively high threshold antibody avidity required for a positive immunoprecipitation compared to other tests (39). The same explanation may account for the high specificity of NH precipitation tests in discriminating cattle experimentally infected with *Y. enterocolitica* O:9 or members of FPSR herds. As discussed above, there is a substantial difference in avidities (and titers) between the antibodies of *Brucella*-infected cows and those induced by *Y. enterocolitica* O:9.

Although of lower intensity and frequency than that induced against the S-LPS, the immunoresponse to *Brucella* proteins is highly specific for brucellosis (6, 7, 15, 35, 36, 60). Accordingly, double gel immunodiffusion and RID with the mixture of proteins contained in cytosolic fractions (Table 1) were 100% specific with the sera from animals infected by *Y. enterocolitica* O:9 or belonging to FPSR herds. Moreover, both tests had acceptable sensitivity. Previous works have suggested that in brucellosis, a multiple protein test may result in higher sensitivity than tests using purified proteins (8, 10, 11, 14, 35, 37). That hypothesis is confirmed in the present study by the contrasting results of immunoprecipitation tests and ELISA with BP26 (57). ELISA with the same cytosolic fraction, however, was not satisfactory, in all likelihood because of the problems related to the use of complex protein mixtures necessarily differing in concentration and adsorption ability.

The skin test with *Brucella* soluble proteins is currently recommended to examine herds suspected of FPSR (4, 30, 52) but shows relatively low sensitivity when compared to the most sensitive serological tests, such as the indirect ELISA; therefore, it is meaningful only when interpreted on a herd basis. Also, it requires a more complex method and is more expensive to implement than simply retesting serum samples suspected of being FPSR. Immunoprecipitation tests with NH or cytosolic proteins, used alone or simultaneously to increase sensitivities, may represent simple, economical, and practical diagnostic tools at the herd level in countries affected by the FPSR problem.

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