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

P. M. Muñoz,1 C. M. Marín,1 D. Monreal,2 D. González,2 B. Garín-Bastuji,3 R. Díaz,2 R. C. Mainar-Jaime,4 I. Moriyón,2 and J. M. Blasco1*

Centro de Investigación y Tecnología Agroalimentaria, Gobierno de Aragón, Zaragoza,1 and Departamento de Microbiología, Universidad de Navarra, Pamplona,2 Spain; OIE/FAO Reference Laboratory for Brucellosis, Agence Française de Sécurité Sanitaire des Aliments, Maisons-Alfort, France3; and Department of Veterinary Microbiology, WCVM, University of Saskatchewan, Saskatoon, Canada4

Received 20 August 2004/Returned for modification 9 September 2004/Accepted 15 October 2004

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 S-type lipopolysaccharide, and 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 chaotrophic 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 S-LPSs. A competitive ELISA with Brucella S-LPS and MB84 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 or native 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 maltophilia, 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 S-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...
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-persamoyl in α-(1-2) linkages that is indistinguishable from the O-chain of B. abortus subtype 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 persamoyl 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 Yersinia) in ELISA by means of MAb of C/Y specificity (43, 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 5 days on 3% horse blood agar (BBL Microbiology Systems, Cockeysville, MD). 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, 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 Brucella-free herds (see below) and three isolates of Y. enterocolitica recovered from FPSR herds 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 (in some cases and then every 3 weeks for 3 months), and 20 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 during the 5 previous years were used as the third 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 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 run 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 cytopsotic 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
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 \textit{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 \textit{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 \[
\frac{\text{mean absorbance value of the duplicate test sample}}{\text{mean absorbance value of the duplicate test sample with the MAb alone}} \times 100.
\]

(iv) Double gel immunodiffusion. Double gel immunodiffusion was performed in 1% Noble agar (Difco Laboratories, Detroit, Mich.) with 10% NaCl-0.1 M NaOH-H3BO4 (pH 8.3) with 20 μl of serum and antigen in wells set 3 mm apart (39). In this test, the antigen used (\textit{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 1. Denomination, source, and main characteristics of the antigens used in the different diagnostic tests

| Antigen     | Source                        | Characteristics                                                                 | Test                           | Reference |
|-------------|-------------------------------|---------------------------------------------------------------------------------|--------------------------------|-----------|
| Crude S-LPS | \textit{B. melitensis} 16M   | 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. Traces of NH and group 3 Omps present. | Double gel immunodiffusion, indirect and chaotropic ELISA | 1         |
| S-LPS       | \textit{B. abortus} S19      | 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. | Competitive ELISA              | 1         |
|             | \textit{B. suis} Thomsen     | S-LPS (lipid A and core epitopes). Perosamine O-polysaccharide of structure presumed to be similar to that of \textit{B. abortus} but with markedly reduced reactivity with MAb 12G12; this MAb reacts with other \textit{Brucella} S-LPSs but not with \textit{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          | \textit{B. abortus} S19      | O-polysaccharide of \textit{B. abortus} bv. 1 (see above) plus core epitopes.  | Indirect ELISA                | 25        |
| NH          | \textit{B. melitensis} 16M   | 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. Moriño (unpublished results) |
| R-LPS       | \textit{B. abortus} per (R mutant) | R-LPS (lipid A and core epitopes). | Indirect ELISA               | 44        |
| R-LPS-Omps  | \textit{B. ovis} Reo198 (R mutant) | R-LPS (lipid A and core epitopes) and group 3 Omps. | Indirect ELISA               | 54        |
| BP26        | \textit{B. abortus} S19      | Recombinant BP26 (\textit{Brucella} periplasmic protein). | Indirect ELISA               | 6, 13, 57 |
| Cytosolic fraction | \textit{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) diaminonitro 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 \textit{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 \textit{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-H3BO4 (pH 8.3) with 20 μl of serum and antigen in wells set 3 mm apart (39). In this test, the antigen used (\textit{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.
| Test          | Antigen          | % Sensitivity (95% CI) and no. of sera tested for cattle infected with: | Cutoffs | % Specificity (95% CI) and no. of sera for Brucella-free cattle: |                  |
|--------------|------------------|------------------------------------------------------------------------|---------|---------------------------------------------------------------|------------------|
| RBT          | *B. abortus* whole cells | 100 (97.1–100), 114                                                    | 100 (96.7–100), 112 | Not applicable                                               | 86.4 (79.1–91.9), 125 |
| CFT          | *B. abortus* whole cells | 100 (97.1–100), 114                                                    | 20      | 100 (96.7–100), 112                                           | 94.4 (88.8–97.7), 125 |
| Indirect ELISA | *B. melitensis* crude S-LPS | 100 (96.8–100), 114                                                    | >31.12  | 100 (96.7–100), 112                                           | 42.9 (24.5–62.8), 28 |
|              | *B. melitensis* NH | 100 (96.8–100), 114                                                    | >35.29  | 100 (96.7–100), 112                                           | 42.9 (24.5–62.8), 28 |
|              | *B. abortus* PS | 100 (96.8–100), 114                                                    | >23.07  | 100 (96.7–100), 112                                           | 7.1 (1.1–23.5), 28  |
|              | *B. suis* S-LPS | 100 (96.8–100), 114                                                    | >15.87  | 100 (96.7–100), 112                                           | 0 (0.0–12.5), 28   |
|              | *E. hermannii* S-LPS | 95.3 (84.2–99.3), 43                                                  | >75.04  | 100 (91.3–100), 41                                           | 29.6 (13.8–50.2), 125 |
| Chaotropic ELISA | *B. melitensis* crude S-LPS | 100 (96.8–100), 114                                                    | >12.62  | 100 (96.7–100), 112                                           | 64.3 (44.1–81.3), 28 |
|              | *B. melitensis* NH | 97.4 (92.5–99.4), 114                                                   | >25.79  | 100 (96.7–100), 112                                           | 97.6 (93.1–99.5), 125 |
|              | *B. abortus* PS | 89.5 (82.3–94.4), 114                                                   | >21.22  | 100 (96.7–100), 112                                           | 100 (97.1–100), 125 |
|              | *B. melitensis* crude S-LPS | 90.5 (83.2–95.3), 105                                                  | >35.73  | 100 (95.9–100), 90                                           | 85.7 (67.3–95.9), 28 |
| Competitive ELISA | *B. abortus* S-LPS | 85.1 (77.2–91.1), 114                                                   | >25.79  | 100 (96.7–100), 112                                           | 100 (97.1–100), 125 |
|              | *B. melitensis* NH | 92.9 (85.1–97.3), 84                                                   | >35.73  | 100 (96.7–100), 112                                           | 88.8 (81.9–93.7), 125 |

---

* 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.
Confidence intervals (CI) were calculated (MedCalc 7.2.0.0). Specificities were (percentage of sera from culture-positive animals scoring positive), the specificity 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, NP, 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

| Test | Antigen | Sensitivity (%) and no. of sera tested for cattle infected with: |
|------|---------|---------------------------------------------------------------|
| Indirect ELISA | OmpF | 100 (65.9–91.6), 42 |
| | R-LPS | 91.8 (80.4–97.7), 49 |
| | B. abortus | 91.2 (83.4–94.6), 49 |
| | B. melitensis | 88.6 (78.1–93.8), 91 |
| | | **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.** |
| **TABLE 2.** Cutoffs for ELISA were selected to result in 100% specificity with the population of sera from Brucella-free animals not exposed to Y. enterocolitica O:9. | **Protein-RID** | **Cutoff ELISA** | **Test** |
| | | | OmpF | R-LPS | OmpF | R-LPS |
| | | | (a) | (b) | (c) | (d) |
| **Cutoffs** | **Protein-RID** | **Cutoff ELISA** | **Test** | **Antigen** | **S-LPS** | **R-LPS** | **B. abortus** | **B. melitensis** |
| | | | | Experimental cattle | From FPSR herds | | | |
| | | | | Not exposed to Y. enterocolitica O:9 | | | | |
| | | | | | | | | | |
| | | | | | | | | | |
| | | | | | | | | | |
| | | | | | | | | | |
| | | | | | | | | | |
| | | | | | | | | | |
| | | | | | | | | | |
| | | | | | | | | | |
| | | | | | | | | | |
| | | | | | | | | | |
| | | | | | | | | | |
| | | | | | | | | | |
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 NH-RID test had a sensitivity higher than those of 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 possibility that LPS epitopes other than those in the O-chain of the S-LPS could discriminate brucellosis from *Y. enterocolitica* O:9 was tested 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

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.
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.

| Antigen | Results for Brucella-free populations with characteristic indicated | 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) | 0.999 (0.98–1) |
| Y. enterocolitica O:9 | 79.57 100 (98–100) | 1 (0.988–1) | 0.988 (0.97–1) |
| Experimentally infected with Y. enterocolitica O:9 | 93.1 98.9 (96.2–99.8) | 0.974 (0.96–1) | 0.988 (0.97–1) |
| Not exposed to Y. enterocolitica O:9 | 35.29 100 (98–100) | 0.999 (0.98–1) |
| B. melitensis NH | 90.6 97.9 (94.7–99.4) | 0.999 (0.987–1) |
| Member of FPSR herds | 102.5 96.3 (92.5–98.5) | 0.974 (0.96–1) |
| Chaotropic ELISA  
B. melitensis crude S-LPS 1 (0.988–1) | 12.62 100 (98–100) | 1 (0.981–1) |
| 2 M KSCN | 19.35 99.5 (97.1–99.9) | 0.999 (0.981–1) |
| B. melitensis crude S-LPS 0.994 (0.977–0.999) | 21.15 99.5 (97.1–99.9) | 0.999 (0.981–1) |
| 3 M KSCN | 25.79 97.9 (94.7–99.4) | 0.999 (0.981–1) |
| Chaotropic ELISA  
B. abortus per R-LPS 0.997 (0.983–0.999) | 38.47 100 (98–100) | 0.999 (0.987–1) |
| 2 M KSCN | 19.65 98.9 (96.2–99.8) | 0.999 (0.987–1) |
| B. abortus per R-LPS 0.994 (0.977–0.999) | 52.5 85.7 (79.9–90.4) | 0.999 (0.987–1) |
| 3 M KSCN | 21.2 99.5 (97.1–99.9) | 0.999 (0.981–1) |
| Chaotropic ELISA  
B. abortus BP26 0.973 (0.949–0.988) | >31.12 100 (98–100) | 0.999 (0.981–1) |
| 2 M KSCN | >31.12 100 (98–100) | 0.999 (0.981–1) |
| B. abortus BP26 0.973 (0.949–0.988) | >31.12 100 (98–100) | 0.999 (0.981–1) |
| 3 M KSCN | >31.12 100 (98–100) | 0.999 (0.981–1) |
| Chaotropic ELISA  
B. melitensis 115 cytosol 0.999 (0.985–1) | >93.1 100 (98–100) | 0.999 (0.981–1) |
| 2 M KSCN | >93.1 100 (98–100) | 0.999 (0.981–1) |
| B. melitensis 115 cytosol 0.999 (0.985–1) | >93.1 100 (98–100) | 0.999 (0.981–1) |
| 3 M KSCN | >93.1 100 (98–100) | 0.999 (0.981–1) |
| Chaotropic ELISA  
B. abortus S-LPS 0.999 (0.981–1) | >93.1 100 (98–100) | 0.999 (0.981–1) |
| 2 M KSCN | >93.1 100 (98–100) | 0.999 (0.981–1) |
| B. abortus S-LPS 0.999 (0.981–1) | >93.1 100 (98–100) | 0.999 (0.981–1) |
| 3 M KSCN | >93.1 100 (98–100) | 0.999 (0.981–1) |
| Chaotropic ELISA  
B. abortus S-LPS 0.999 (0.981–1) | >93.1 100 (98–100) | 0.999 (0.981–1) |
| 2 M KSCN | >93.1 100 (98–100) | 0.999 (0.981–1) |
| B. abortus S-LPS 0.999 (0.981–1) | >93.1 100 (98–100) | 0.999 (0.981–1) |
| 3 M KSCN | >93.1 100 (98–100) | 0.999 (0.981–1) |
| Chaotropic ELISA  
B. melitensis crude S-LPS 1 (0.988–1) | >93.1 100 (98–100) | 0.999 (0.981–1) |
| 2 M KSCN | >93.1 100 (98–100) | 0.999 (0.981–1) |
| B. melitensis crude S-LPS 1 (0.988–1) | >93.1 100 (98–100) | 0.999 (0.981–1) |
| 3 M KSCN | >93.1 100 (98–100) | 0.999 (0.981–1) |
| Chaotropic ELISA  
B. abortus S-LPS 0.999 (0.981–1) | >93.1 100 (98–100) | 0.999 (0.981–1) |
| 2 M KSCN | >93.1 100 (98–100) | 0.999 (0.981–1) |
| B. abortus S-LPS 0.999 (0.981–1) | >93.1 100 (98–100) | 0.999 (0.981–1) |
| 3 M KSCN | >93.1 100 (98–100) | 0.999 (0.981–1) |

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.

ACKNOWLEDGMENTS

P. M. Muñoz and D. Monreal are recipients of grants from the Ministerio de Ciencia y Tecnología de Spain (FP6/2000–2005) and from the University of Navarra, respectively. Research at the laboratories of the authors is supported by the European Commission (research contract QLK2-CT-2002-00918) and by the Ministerio de Ciencia y Tecnología de Spain (proyecto AGL2000-0305-C02-01 and -02), by the Ministère de l’Agriculture, de la Pêche et des Affaires Rurales of France, and Redes Temáticas de Investigación Cooperativa del FIS G03/024.

We thank M. J. de Miguel (CITA), M. Pardo (Universidad de Navarra), and C. Cau (AFSSA) for excellent technical assistance. E. hermannii S-LPS and recombinant BP26 were the generous gifts of M. B. Perry and O. Rosetti, respectively.

REFERENCES

1. Alonso-Urmeneta, B., C. M. Marin, V. Aragón, J. M. Blasco, R. Díaz, and I. Moriyón. 1998. Evaluation of lipopolysaccharides and polysaccharides of different epithelial strains of Brucella abortus in cattle and their response against lipopolysaccharide and cytoplasmic proteins of Brucella melitensis. J. Clin. Microbiol. 36:749–754.
2. Alonso-Urmeneta, B., I. Moriyón, R. Díaz, and J. M. Blasco. 1988. Enzyme-linked immunosorbent assay in Brucella abortus infected cattle using crude polysaccharide. J. Clin. Microbiol. 26:2642–2646.
3. Altman, D. G. 1991. Practical statistics for medical research, p. 229–276. Chapman & Hall, London, United Kingdom.
4. Allen, G. G., L. M. Jones, J. W. Cherwonogrodzky, D. Angus, and J. M. Verger. 1988. Techniques for the brucellosis laboratory. Institut National de la Recherche Agronomique, Paris, France.
5. Anonymous. 2004. Bovine brucellosis. In OIE manual of diagnostic tests and vaccines for terrestrial animals, 5th ed. p. 409–438. Office International des Epizooties, Paris, France.
6. Aragón, V., R. Díaz, E. Moreno, and I. Moriyón. 1996. Characterization of Brucella abortus and Brucella melitensis native haptens as outer membrane O-type polysaccharides independent from the smooth lipopolysaccharide. J. Bacteriol. 178:1073–1079.
7. Arese, A., S. Cravero, M. Boschiroli, E. Golda, L. F. Abdón, C. Velkovitsky, R. Kittelberger, and C. A. Fossati. 1996. Humoral immune response against lipopolysaccharide and cytoplasmic proteins of Brucella abortus in cattle vaccinated with B. abortus S19 or experimentally infected with Yersinia enterocolitica serotype O:9. Clin. Diagn. Lab. Immunol. 3:472–476.
8. Blasco, J. M., C. M. Marin, M. P. Jiménez de Bagüés, M. Barberán, A. Hernández, L. Molina, J. Velasco, R. Díaz, and I. Moriyón. 1994. Evaluation of allergic and serological tests for diagnosing Brucella melitensis infection in sheep. J. Clin. Microbiol. 32:1835–1840.
9. Cherwonogrodzky, J. W., and K. H. Nielsen. 1988. Brucella abortus 1119–3 O-chain polysaccharide to differentiate sera from B. abortus S19-vaccinated and field-strain-infected cattle by agar gel immunodiffusion. J. Clin. Microbiol. 26:1120–1123.
10. Chukwu, C. C. 1985. Differentiation of Brucella abortus and Yersinia enterocolitica serotype 09 infections: use of lymphocyte transformation test. Int. J. Zoonoses 12:126–135.
11. Chukwu, C. C. 1985. Evaluation of brucellin skin test for bovine brucellosis. Int. J. Zoonoses 12:6–13.
12. Chukwu, C. C. 1987. Differentiation of Brucella abortus and Yersinia enterocolitica serotype 09 infections in cattle: the use of specific lymphocyte transformation and brucellin skin tests. Vet. Q. 9:134–142.
13. Cloeckaert, A., S. Baucheron, N. Vizcaíno, and M. S. Zygmunt. 2001. Use of recombinant BP26 protein in serological diagnosis of Brucella melitensis infection in sheep. Clin. Diagn. Lab. Immunol. 8:772–775.
14. Cloeckaert, A., P. Kerkhofs, and J. N. Limet. 1992. Antibody response to Brucella outer membrane proteins in bovine brucellosis: immunoblot analysis and competitive enzyme-linked immunosorbent assay using monoclonal antibodies. J. Clin. Microbiol. 30:3168–3174.
15. Cloeckaert, A., J. M. Verger, M. Grayon, and N. Vizcaíno. 1996. Molecular and immunological characterization of the major outer membrane proteins of Brucella, FEMS Microbiol. Lett. 145:1–8.
16. Cloeckaert, A., V. E. Weynants, J. Godfroid, J. M. Verger, M. Grayon, and M. S. Zygmunt. 1998. O-Poly saccharide epitope heterogeneity at the surface of Brucella spp. studied by enzyme-linked immunosorbent assay and flow cytometry. Clin. Diagn. Lab. Immunol. 5:465–470.
17. Diaz, R. 1974. Valor de la prueba del rosa de bengala y la demostración de anticuerpos anti-proteína de Brucella en el diagnóstico serológico de brucelosis y yersiniosis. Med. Clin. Barc. 63:463–466.
brane permeability and cationic peptide resistance. Infect. Immun. 68:3210–3218.

62. Velasco, J., R. Diaz, M. J. Grilló, M. Barberán, C. M. Marin, J. M. Blasco, and I. Moriyón. 1997. Antibody and delayed-type hypersensitivity responses to Ochrobactrum anthropi cytosolic and outer membrane antigens in infections by smooth and rough Brucella spp. Clin. Diagn. Lab. Immunol. 4:279–284.

63. Verger, J. M., B. Garin-Bastuji, M. Grayon, and A. M. Mahé. 1989. La brucellose bovine à Brucella melitensis en France. Ann. Rech. Vet. 20:93–102.

64. Verger, J. M. 1985. B. melitensis infection of cattle, p. 197–203. In J. M. Verger and M. Plommet (ed.), Brucella melitensis. Martinus Nijhoff, Dordrecht, The Netherlands.

65. Weynants, V. E., D. Gilson, A. Cloeckaert, P. A. Denoel, A. Tibor, P. Thiange, J. N. Limet, and J. J. Letesson. 1996. Characterization of a monoclonal antibody specific for Brucella smooth lipopolysaccharide and development of a competitive enzyme-linked immunosorbent assay to improve the serological diagnosis of brucellosis. Clin. Diagn. Lab. Immunol. 3:309–314.

66. Weynants, V. E., D. Gilson, A. Cloeckaert, A. Tibor, P. A. Denoel, F. Godfroid, J. N. Limet, and J. J. Letesson. 1997. Characterization of smooth lipopolysaccharides and O polysaccharides of Brucella species by competition binding assays with monoclonal antibodies. Infect. Immun. 65:1939–1943.

67. Weynants, V. E., J. Godfroid, B. Limbourg, C. Saegerman, and J. J. Letesson. 1995. Specific bovine brucellosis diagnosis based on in vitro antigen-specific gamma interferon production. J. Clin. Microbiol. 33:706–712.

68. Weynants, V. E., A. Tibor, P. A. Denoel, C. Saegerman, J. Godfroid, P. Thiange, and J. J. Letesson. 1996. Infection of cattle with Yersinia enterocolitica O:9: a cause of the false positive serological reactions in bovine brucellosis diagnostic tests. Vet. Microbiol. 48:101–112.