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Performance of Competitive and Indirect Enzyme-Linked Immunosorbent Assays, Gel Immunoprecipitation with Native Hapten Polysaccharide, and Standard Serological Tests in Diagnosis of Sheep Brucellosis

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Competitive and standard enzyme-linked immunosorbent assays (ELISAs), rose bengal (RB), complement fixation, and agar gel immunoprecipitation with native hapten (AGID-NH) were compared by using sera from *Brucella*-free, *Brucella melitensis*-infected, and *B. melitensis* Rev1-vaccinated sheep. The most sensitive tests were indirect ELISA and RB, and the most specific tests were AGID-NH and competitive ELISA. We show that RB followed by AGID-NH is a simple and effective system for diagnosing sheep brucellosis.

Sheep brucellosis is a zoonotic disease that causes human suffering and great economic losses. When implemented, the control of this disease is usually based on vaccination, serological testing, and culling. Until now, the best vaccine available has been the smooth (S) *Brucella melitensis* Rev1 strain. Although this strain is useful, it does not afford 100% protection, and it induces a strong antibody response to the S lipopolysaccharide (S-LPS), particularly when used in adult sheep. Since S-LPS is the most relevant antigen in conventional serological tests such as the rose bengal (RB) and the complement fixation (CF) assays (3), it is not surprising that Rev1 vaccination interferes with serological diagnosis. Vaccination of young sheep (rather than adult sheep) by the conjunctival route (rather than subcutaneously) reduces the antibody response without significantly affecting the protection levels; even so, conventional serological diagnosis requires the use of screening and confirmatory tests such as RB and CF, respectively. However, the use of these two tests does not result in 100% sensitivity and specificity (6, 7, 17).

To reduce these restrictions in the use of the vaccine and to facilitate serological diagnosis, alternative assays have been investigated, including tests that detect antibodies to proteins (8, 9, 16, 18, 25) and to the S-LPS-related native hapten (NH) polysaccharide (5, 12, 13, 17). Moreover, indirect enzyme-linked immunosorbent assays (iELISAs) with S-LPS have been investigated, but when adjusted to optimal sensitivity, they lack specificity for sera from vaccinated sheep (7, 17). Similar problems are encountered in the diagnosis of cattle brucellosis when vaccination with *Brucella abortus* 19 is implemented, so to improve the specificity of the iELISAs under these conditions, a competitive ELISA (cELISA) was developed (14, 15, 21, 22). This cELISA is based on the displacement of serum antibodies by a fixed concentration of a mouse monoclonal antibody (MAb) against the common (C/Y) epitope, which is the dominant epitope in the O polysaccharides of both *B. abortus* and *B. melitensis* and is the most relevant in serological diagnosis. Since the cELISA does not involve the use of a specific conjugate anti-animal species immunoglobulin, this assay can be easily adapted to detect *Brucella* infections in different animal species. The aim of our work was to compare this cELISA, the iELISA, an immunoprecipitation assay with NH, and the standard tests.

Blood sera were obtained from sheep naturally infected with *B. melitensis* (29 with biotype 1 and 26 with biotype 3, as demonstrated by bacteriological cultures of necropsy samples [7, 19]) and from 60 sheep belonging to *Brucella*-free flocks. Sera were tested by the cELISA system supplied by the Joint FAO/IAEA Division of the International Atomic Energy Agency (Vienna, Austria), which was shipped as a kit with the necessary protocols and computation analysis procedures. The kit contained 96-well polystyrene plates, standardized *B. abortus* biotype 1 S-LPS phenol-water extract (24), mouse MAb M84 of C/Y specificity, horseradish peroxidase-conjugated goat anti-mouse immunoglobulin G (heavy plus light chain specificity), 2,2′-azinobis(3-ethylbenzthiazolinesulfonic acid) (ABTS) diammonium salt substrate, buffer substances, and negative and positive (strongly, intermediately, and weakly) bovine sera as controls (15). To adapt the cELISA for testing sheep sera, these controls were replaced by pools of sera from *Brucella*-free or *B. melitensis*-infected sheep (see above), and the dilutions of the positive pools were adjusted to yield optical densities equivalent to the strongly, intermediately, and weakly positive bovine controls. The assay was carried out as described in previous works (14, 15, 22), and 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 triplicate test with the MAb alone] × 100}. The iELISA was performed with a crude *B. melitensis* S-LPS preparation (1, 4, 11, 17) and peroxidase-conjugated protein G, and the results were expressed as the percentage of the optical density with respect to a strongly positive control serum (1, 13, 17). The agar gel immunodiffusion test for detecting NH-precipitating antibodies (AGID-NH) was performed with 1% Noble agar (Difco Laboratories, Detroit, Mich.) gels in 10% NaCl–0.1 M NaOH-H_3BO_4 (pH 8.3) with 20 μl of serum and the antigen wells set 3 mm apart. The antigen was an NH-rich *B. melitensis* 16M hot-water extract in which the NH precipitation band is characteristic (4, 10, 11, 20). The CF (3) and RB

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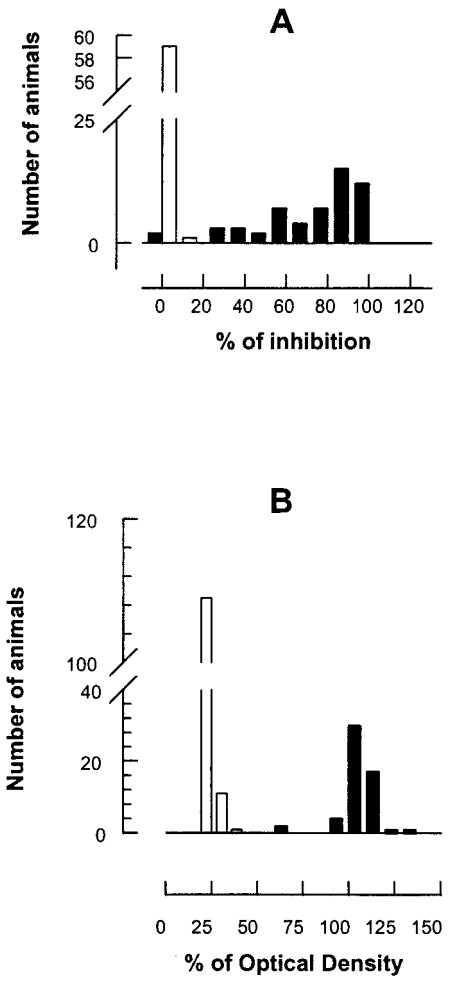

FIG. 1. Distribution of the sera of *B. melitensis*-infected (black bars) and *Brucella*-free (open bars) sheep according to the results of the cELISA (A) and the iELISA (B).

tests were also used, the latter with a 3:1 proportion of serum to antigen for optimal sensitivity (6).

Receiver-operating characteristic analyses (SAS statistical package version 6 [SAS Institute Inc.]) of the results obtained by the iELISAs and cELISAs of the sera from the *B. melitensis*-infected and *Brucella*-free sheep showed that both assays yielded optimal sensitivity and specificity with the 1:50 serum dilution. Moreover, the results of each type of ELISA show that the distributions of the sera from sheep infected with *B. melitensis* biotype 1 and biotype 3 were similar (not shown), thus confirming that the S-LPS ELISAs for animal brucellosis perform similarly regardless of the antigen source (i.e., *B. abortus* or *B. melitensis*) and regardless of the infecting *Brucella* species or biotype (1). For the 1:50 serum dilution, the distribution of the absorbance values of the cELISAs (Fig. 1A) showed that binding of MAb M84 was less than 20% inhibited by the 60 sera from *Brucella*-free sheep and that all but 2 sera from *B. melitensis*-infected sheep inhibited MAb M84 binding by more than 20%. Thus, with the 20% cutoff, the sensitivity (percentage of truly infected animals identified as positive) and specificity (percentage of truly uninfected animals identified as negative) of the cELISA were 96 and 100%, respectively. With these sera, the iELISA (Fig. 1B) completely discriminated the sera from the *Brucella*-free and *B. melitensis*-infected populations (100% sensitivity and specificity), even though the difference in sensitivity from cELISA was not statistically significant ($P = 0.15$, two-tailed Student's t test). As demonstrated for the iELISA, the RB test showed 100% sensitivity, and both tests were more sensitive ($P < 0.05$) than the CF (92%) and AGID-NH (90%) tests. The sensitivities of the CF and AGID-NH tests were similar to each other ($P = 0.73$) and were not significantly different from that of the cELISA ($P = 0.40$ and $P = 0.24$, respectively). The AGID-NH, RB, and CF tests showed 100% specificities for sera from *Brucella*-free animals.

The specificities of these tests in the context of a vaccination program were studied by using sera of two groups of lambs plus two groups of adult sheep (all from *Brucella*-free flocks) that had been vaccinated either subcutaneously or conjunctivally with 10^9 CFU of *B. melitensis* Rev1 and maintained in a *Brucella*-free environment. For the purpose of this study, the animals were bled at different time intervals (Table 1), although only the last bleedings represented a situation similar to that found in ordinary eradication programs. The specificities of the tests (i.e., the percentage of vaccinated animals that tested negative and, therefore, would not be misdiagnosed as infected) varied depending upon the age of the animal and the route of vaccination (Table 1). As expected (17), the specificities of all tests were generally higher when the sera of conjunctivally vaccinated sheep and the sera of lambs were tested. The results also showed that the specificity of the cELISA was constantly higher than that of the iELISA, with very marked differences in the group of subcutaneously vaccinated lambs and, no matter which route of vaccination, in the vaccinated adult sheep. The specificity values in this last group were independent from the vaccination route. The cELISA and AGID-NH test were more specific. Although the differences were not statistically significant, the AGID-NH test seemed to perform better than cELISA only when the sera of adult sheep bled 5 months after subcutaneous vaccination were tested. The tests had similar specificities. The CF test, which is the standard confirmatory test (3, 6), showed higher specificity than the cELISA in only two of the bleedings of the conjunctivally vaccinated lambs.

It is noteworthy that a relatively simple test such as the AGID-NH test was as specific as the sophisticated cELISA. As demonstrated by the contrasting results of iELISA and cELISA, the diagnostic specificity of the latter is due to the elimination of low-affinity antibodies (dominant in the sera of vaccinated animals) by the competing anti-C/Y MAb. Antibody affinity, rather than epitopic differences between S-LPS and NH, is also likely to account in part for the performance of the AGID-NH test (2).

Immunochemical studies have shown that the NH and the O polysaccharide of the S-LPS (which is the serologically relevant section of S-LPS) have similar structures and epitopic densities (4, 12). In fact, antibodies to the NH can be absorbed with S-LPS (2). However, precipitation tests with S-LPS do not show the sensitivity and specificity of similar tests performed with NH (10, 11, 20), although NH and S-LPS yield similar results in both iELISA (1, 2, 13) and passive hemagglutination (2). To explain these apparently contradictory observations, we have proposed (2) that the higher specificities of the precipitation tests with NH result from two sets of factors. First, the dispersed state of the low-molecular-weight NH (4) in solution, as opposed to the highly aggregated S-LPSs, may be relevant in explaining their different behavior in precipitation tests. Second, if low-affinity antibodies are predominant after vaccination, the higher threshold affinity of precipitation tests com-

TABLE 1. Specificities of serological tests for brucellosis for sera from *B. melitensis* Rev1-vaccinated sheep[a]

Age (mo) at vaccination	Route	Bleeding time[b]	No. of sheep	% Specificity of:				
				RB test	CF	AGID-NH test	iELISA	cELISA
3	Subcutaneous	1	30	3.3	3.3	30.0	0	70.0
		4	28	14.3	46.4	96.43	10.7	85.7
		6	18	38.9	77.8	100	11.1	88.9
	Conjunctival	1	36	13.9	30.6	100	55.5	63.9
		2	20	25.0	90.0	100	25.0	85.0
		4	11	81.8	100	100	72.7	89.5
14	Subcutaneous	1	20	0	9.1	55.0	5.0	90.0
		5	47	14.9	44.7	85.1	10.6	87.2
	Conjunctival	1	28	0	0	92.8	28.6	85.7
		5	56	66.0	69.6	96.4	32.1	89.3

[a] Specificity is defined as the percentage of vaccinated animals that are negative in a given test.
[b] Number of months after vaccination.

pared to that of iELISAs (23) may explain why NH fails to react with sera from vaccinated animals in the former but not in the latter assay. Obviously, the comparison of the results of the i and cELISAs demonstrates that the sera of vaccinated sheep contain more antibodies of lower affinity than do sera from infected sheep, and this is consistent with the proposed hypothesis.

The results of this work have practical implications concerning the use of the tests evaluated. In the absence of vaccination, the iELISA and the much less sophisticated RB test (standardized and performed as described in reference 6) should be the tests of choice because of their very high sensitivities. When vaccination was implemented, no single test simultaneously afforded 100% sensitivity and specificity. Although the cELISA greatly improves the specificity of the iELISA, the data suggest that it is less sensitive. Therefore, the study of larger numbers of sera from bacteriologically positive animals is necessary before the use of the cELISA can be recommended as a single diagnostic test for *B. melitensis* sheep brucellosis. However, screening with either the iELISA or the RB assay followed by confirmation by means of either the cELISA or the AGID-NH test would afford the best combination of sensitivity and specificity. Both ELISAs are technically more demanding, and since they do not outperform the unsophisticated RB and AGID-NH tests, the latter seem to be the simplest choice for the diagnosis of sheep brucellosis when Rev1 vaccination is implemented.

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REFERENCES

1. **Alonso-Urmeneta, B., C. Marín, V. Aragón, J. M. Blasco, R. Díaz, and I. Moriyón.** 1998. Evaluation of lipopolysaccharides and polysaccharides of different epitopic structures in the indirect enzyme-linked immunosorbent assay for diagnosis of brucellosis in small ruminants and cattle. Clin. Diagn. Lab. Immunol. **5:**749–754.
2. **Alonso-Urmeneta, B., I. Moriyón, R. Díaz, and J. M. Blasco.** 1988. Enzyme-linked immunosorbent assay with *Brucella* native hapten polysaccharide and smooth lipopolysaccharide. J. Clin. Microbiol. **26:**2642–2646.
3. **Alton, G. G., L. M. Jones, R. D. Angus, and J. M. Verger.** 1988. Techniques for the brucellosis laboratory. Institut National de la Recherche Agronomique, Paris, France.
4. **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:**1070–1079.
5. **Blasco, J. M., R. Díaz, I. Moriyón, and M. D. Salvo.** 1984. Evaluation of a radial immunodiffusion test for diagnosing brucellosis in sheep and its possible value for differentiating infected from *Brucella melitensis* REV 1 vaccinated sheep. Dev. Biol. Stand. **56:**507–511.
6. **Blasco, J. M., B. Garin-Bastuji, C. M. Marín, G. Gerbier, J. Fanlo, M. P. Jiménez de Bagüés, and C. Cau.** 1994. Efficacy of different Rose Bengal and complement fixation antigens for the diagnosis of *Brucella melitensis* infection in sheep and goats. Vet. Rec. **134:**415–420.
7. **Blasco, J. M., C. Marín, M. Jiménez de Bagués, 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.
8. **Debbarh, H. S., A. Cloeckaert, M. S. Zygmunt, and G. Dubray.** 1995. Identification of seroreactive *Brucella melitensis* cytosoluble proteins which discriminate between antibodies elicited by infection and Rev.1 vaccination in sheep. Vet. Microbiol. **44:**37–48.
9. **Debbarh, H. S.-A., A. Cloeckaert, G. Bézard, G. Dubray, and M. S. Zygmunt.** 1996. Enzyme-linked immunosorbent assay with partially purified cytosoluble 28-kilodalton protein for serological differentiation between *Brucella melitensis*-infected and *B. melitensis* Rev.1-vaccinated sheep. Clin. Diagn. Lab. Immunol. **3:**305–308.
10. **Díaz, R., J. Toyos, M. D. Salvo, L. L. Fernández, B. Alonso-Urmementa, I. Moriyón, and I. Dorronsoro.** 1984. Studies on the polysaccharide B and native hapten of *Brucella* and *Yersinia enterocolitica* serotype 9. Dev. Biol. Stand. **56:**213–220.
11. **Díaz, R., J. Toyos, M. D. Salvo, and M. L. Pardo.** 1981. A simple method for the extraction of polysaccharide B from *Brucella* cells for use in the radial immunodiffusion test diagnosis of bovine brucellosis. Ann. Rech. Vet. **12:**35–39.
12. **Díaz-Aparicio, E., V. Aragón, C. Marín, B. Alonso, M. Font, E. Moreno, S. Pérez-Ortiz, J. M. Blasco, R. Díaz, and I. Moriyón.** 1993. Comparative analysis of *Brucella* serotype A and M and *Yersinia enterocolitica* O:9 polysaccharides for serological diagnosis of brucellosis in cattle, sheep, and goats. J. Clin. Microbiol. **31:**3136–3141.
13. **Díaz-Aparicio, E., C. Marín, B. Alonso-Urmeneta, V. Aragón, S. Pérez-Ortiz, M. Pardo, J. M. Blasco, R. Díaz, and I. Moriyón.** 1994. Evaluation of serological tests for diagnosis of *Brucella melitensis* infection of goats. J. Clin. Microbiol. **32:**1159–1165.
14. **Gall, D., and K. Nielsen.** 1994. Improvements to the competitive ELISA for detection of antibodies to *Brucella abortus* in cattle sera. J. Immunoass. **15:**277–291.
15. **Gall, D., A. Colling, O. Marino, E. Moreno, K. Nielsen, B. Perez, and L. Samartino.** 1998. Enzyme immunoassays for serological diagnosis of bovine brucellosis: a trial in Latin America. Clin. Diagn. Lab. Immunol. **5:**654–661.
16. **Hemmen, F., V. Weynants, T. Scarcez, J.-J. Letesson, and E. Saman.** 1995. Cloning and sequence analysis of a newly identified *Brucella abortus* gene and serological evaluation of the 17-kilodalton antigen that it encodes. Clin. Diagn. Lab. Immunol. **2:**263–267.

17. **Jiménez de Bagüés, M. P., C. M. Marín, J. M. Blasco, I. Moriyón, and C. Gamazo.** 1992. An ELISA with *Brucella* lipopolysaccharide antigen for the diagnosis of *B. melitensis* infection in sheep and for the evaluation of serological responses following subcutaneous or conjunctival *B. melitensis* strain Rev 1 vaccination. Vet. Microbiol. **30:**233–241.
18. **Letesson, J. J., A. Tibor, G. van Eynde, V. Wansard, V. Weynants, P. Denoel, and E. Saman.** 1997. Humoral immune responses of *Brucella*-infected cattle, sheep, and goats to eight purified recombinant *Brucella* proteins in an indirect enzyme-linked immunosorbent assay. Clin. Diagn. Lab. Immunol. **4:**556–564.
19. **Marín, C. M., M. P. Jiménez de Bagüés, M. Barberán, and J. M. Blasco.** 1996. Comparison of two selective media for the isolation of *Brucella melitensis* from naturally infected sheep and goats. Vet. Rec. **138:**409–411.
20. **Moreno, E., S. L. Speth, L. M. Jones, and D. T. Berman.** 1981. Immunochemical characterization of *Brucella* lipopolysaccharides and polysaccharides. Infect. Immun. **31:**214–222.
21. **Nielsen, K. H., L. Kelly, D. Gall, S. Balsevicius, J. Bosse, P. Nicoletti, and W. Kelly.** 1996. Comparison of enzyme immunoassays for the diagnosis of bovine brucellosis. Prev. Vet. Med. **26:**7–32.
22. **Nielsen, K. H., L. Kelly, D. Gall, P. Nicoletti, and W. Kelly.** 1995. Improved competitive enzyme immunoassay for the diagnosis of bovine brucellosis. Vet. Immunol. Immunopathol. **46:**285–291.
23. **Peterfy, F., P. Kuusela, and O. Makelä.** 1998. Affinity requirements for antibody assays mapped by monoclonal antibodies. J. Immunol. **130:**1809–1813.
24. **Rojas, N., E. Freer, A. Weintraub, M. Ramirez, S. Lind, and E. Moreno.** 1994. Immunochemical identification of *Brucella abortus* lipopolysaccharide epitopes. Clin. Diagn. Lab. Immunol. **1:**206–213.
25. **Zygmunt, M. S., H. S. Debbarh, A. Cloeckaert, and G. Dubray.** 1994. Antibody response to *Brucella melitensis* outer membrane antigens in naturally infected and Rev1 vaccinated sheep. Vet. Microbiol. **39:**33–46.