

Diagnostic Usefulness of Antibodies against Ribosome Recycling Factor from *Brucella melitensis* in Human or Canine Brucellosis

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The diagnostic usefulness of an enzyme-linked immunosorbent assay (ELISA) using a purified recombinant ribosome recycling factor from *Brucella melitensis* (CP24 antigen) was tested in human and canine infections caused by smooth and rough *Brucella* species, respectively. Anti-CP24 antibodies were detected in 9 (43%) of 21 consecutive cases of canine brucellosis and in 8 (53%) of 15 dogs followed for 60 days after the diagnosis of acute brucellosis. Among eight patients with acute brucellosis, anti-CP24 antibodies were detected in four in the 10 weeks following diagnosis, but the remaining four were negative during the whole follow-up (22 weeks). The frequency of anti-CP24 antibodies was also low among 24 patients with subacute brucellosis and 23 patients with chronic illness (29 and 26%, respectively). While all patients positive for anti-CP24 antibodies were also positive for antibodies to total cytoplasmic proteins of *Brucella* (CP), five were negative for antibodies to another cytoplasmic protein, the *Brucella* lumazine synthase (BLS). When a larger sample of 35 human sera negative for anti-BLS antibodies was assayed, 85.7% were positive for anti-CP24 antibodies, suggesting that the combined measurement of both reactivities could yield a higher sensitivity than any test alone. To test this hypothesis, an ELISA combining both antigens was designed. The percentage of positive results among chronic cases was higher for this assay than for the individual measurement of anti-CP24 or anti-BLS antibodies (83 versus 26 and 65%, respectively) and was closer to the value obtained for anti-CP antibodies (91%). The frequency of anti-CP24 antibodies is low in both canine and human brucellosis. In the latter case, however, an ELISA combining CP24 and BLS is more sensitive than assays measuring anti-CP24 or anti-BLS antibodies separately and almost as sensitive as the ELISA using CP.

Brucellosis is a zoonosis with a worldwide distribution which affects cattle, sheep, goats, pigs, and dogs and is transmitted to human beings in several ways. Because of the low sensitivity of bacteriological methods, serological tests are generally preferred to establish the diagnosis in both humans and animals. According to the nature of their lipopolysaccharide (LPS) molecules, *Brucella* species are classified as rough or smooth. An important drawback of conventional serological techniques (i.e., agglutination tests) is that the bacterial suspension used to diagnose infections caused by smooth species (*Brucella abortus*, *Brucella melitensis*, and *Brucella suis*) are not useful for diagnosing infections caused by rough species (*Brucella ovnis* and *Brucella canis*), and vice versa. Therefore, antigens shared by all *Brucella* species could be of special interest for diagnostic purposes. Many such antigens have been described in recent years. For example, we have shown that the *Brucella* lumazine synthase (BLS), an 18-kDa cytoplasmic protein present in all *Brucella* species, is useful in the diagnosis of human and canine brucellosis (3, 9). Another antigen found in all *Brucella* species is a homologue of the ribosome recycling factor (RRF) from *Escherichia coli* and other species. This *Brucella* protein, known as CP24, is detected mainly in the cytoplasmic fraction

of smooth and rough brucellae (5). It has been shown that CP24 is recognized by sera from sheep experimentally infected with *B. melitensis* but not by sera from animals vaccinated with the *B. melitensis* attenuated strain Rev-1 (6). In addition, the CP24 protein expressed in recombinant form in *E. coli* was recognized by sera from *Brucella*-infected sheep (12). However, the diagnostic usefulness of recombinant CP24 (rCP24) has not been assayed by enzyme-linked immunosorbent assay (ELISA) and has not been tested in infections caused by other *Brucella* species or in hosts other than sheep. We have recently described the preparation of purified rCP24 and have shown that this protein elicits a vigorous antibody response in immunized mice (4a). In the study presented here, we have investigated the diagnostic usefulness of an ELISA using purified CP24 in human and canine infections caused by smooth and rough *Brucella* species, respectively.

MATERIALS AND METHODS

Serum samples. (i) **Human sera.** A total of 83 sera from patients at different stages of brucellosis were assayed. The initial cohort included 55 patients (71 samples) and was defined on clinical grounds. According to classical criteria (10), patients were classified as having acute brucellosis when the duration of symptoms was up to 8 weeks ($n = 8$), as having subacute illness when symptoms were present for 9 to 52 weeks ($n = 24$), and as having chronic brucellosis when symptoms had lasted for more than 1 year ($n = 23$). All acute patients were infected by *B. melitensis* and at diagnosis had a duration of illness of up to 40 days. Anti-CP24 antibodies were assayed in samples obtained at diagnosis and 10 and 22 weeks later. At diagnosis, these patients were positive by standard tube agglutination (STA), immunoglobulin M (IgM) antibodies to LPS by ELISA, and IgM and/or IgG antibodies to total cytoplasmic proteins of *Brucella* (CP).

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For subacute cases, the time since onset of symptoms ranged from 3 to 12 months (mean \pm standard deviation [SD], 7.4 ± 3.1 months). At the time of blood sampling, all these patients were positive by STA and anti-LPS IgG. Anti-CP IgG was positive in 23 cases, anti-LPS IgM was positive in 21 cases, and anti-BLS IgG was positive in 20 cases. Blood cultures were performed in 17 subacute cases, and *Brucella* was isolated in 9. For chronic cases, time since initial symptoms ranged from 30 to 180 months (mean \pm SD, 85.4 ± 51.3 months). At the time of blood sampling, all these patients were positive by STA and for anti-LPS IgG. Anti-CP IgG was detected in 21 cases, anti-BLS IgG was detected in 15 cases, and anti-LPS IgM was detected in 11 cases. After the results of anti-CP24 and anti-BLS were compared (see Results), a subsequent sample of 35 sera negative for anti-BLS was chosen. All these sera were positive for anti-LPS IgG and also for anti-CP IgG. Fifty serum samples from healthy donors were used to establish the cutoff for ELISAs.

(ii) **Canine sera.** A total of 69 serum samples from dogs infected with *B. canis* were assayed. One group comprised 21 sera from consecutive cases of canine brucellosis, most of which were positive for *B. canis* by blood culture. In addition, serial samples from diagnosis to 60 days postdiagnosis were obtained from 15 dogs involved in an outbreak of canine brucellosis. All 36 dogs had high titers of antibodies to *B. canis* strain M⁻ by agglutination. Serum samples from 30 healthy dogs were assayed under the same conditions to establish the cutoff for the assay.

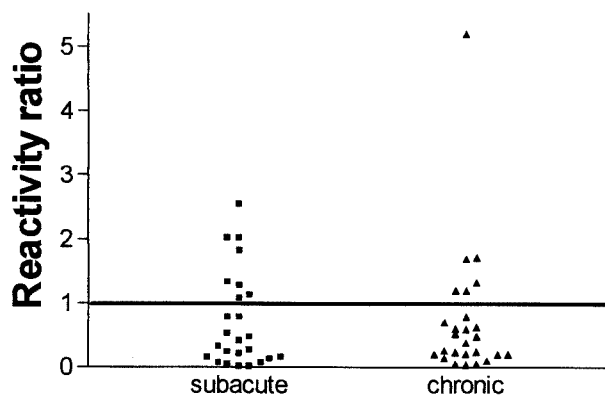
rCP24. The open reading frame of CP24 was cloned in the Pet17b vector (Novagen, Madison, Wis.) as described previously (4a). Briefly, the sequence information previously reported by Vizcaíno et al. (12) was used to design specific primers for CP24. *B. melitensis* genomic DNA was purified and used as a template for PCR with *Pfu* DNA polymerase (Stratagene, La Jolla, Calif.). After ligation, the mix was used to transform *E. coli* DH5 α competent cells (Invitrogen, Carlsbad, Calif.), and miniprep plasmid DNA was purified from overnight cultures. The plasmid DNA of a clone containing the insert in the right orientation was used to transform *E. coli* strain BL21(DE3) competent cells (Stratagene). rCP24 was successfully expressed after induction with IPTG (isopropyl- β -D-thiogalactopyranoside; 1 mM) in the soluble fraction of *E. coli* cells. This preparation was purified using a Mono Q column in a fast protein liquid chromatography apparatus (Pharmacia, Uppsala, Sweden). rCP24 was adsorbed with Sepharose-polyomyxin B to eliminate LPS contamination. This preparation contained less than 0.05 endotoxin units per mg of protein, as assessed by a *Limulus* amoebocyte lysate analysis kit (Sigma, St Louis, Mo.). The identity of rCP24 was confirmed by Western blotting revealed with the B12A15 anti-CP24 monoclonal antibody (MAb).

ELISA with CP24. Maxisorp polystyrene plates (Nunc, Roskilde, Denmark) were sensitized with 0.5 μ g of CP24 per well diluted in phosphate-buffered saline (PBS). The plates were blocked with 200 μ l of PBS containing 3% skim milk/well. After a wash with PBS containing 0.05% Tween 20, sera diluted in PBS containing 0.05% Tween 20 and 1% skim milk were dispensed. Specific antibodies were detected with horseradish peroxidase-conjugated antibodies to human IgG (Jackson, West Grove, Pa.) or dog immunoglobulins (Sigma). The reaction was developed by adding *ortho*-phenylenediamine (2 μ g μ l⁻¹ in 0.1 M citrate-phosphate buffer containing 0.03% H₂O₂) and was stopped with 4 N H₂SO₄. To establish the cutoff value of the assays, serum samples from uninfected controls were tested under the same conditions indicated above. The cutoff value for each ELISA system was calculated as the mean specific optical density (OD) of control sera plus 3 SDs.

Other serological methods. Agglutination tests for human brucellosis (slide agglutination, STA, and 2-mercaptoethanol) were performed according to the methods of Alton et al. (1). The rapid slide agglutination test for canine brucellosis was performed using the *B. canis* M⁻ strain as described by Carmichael and Joubert (4), and the reaction was rated from 1+ to 4+ according to the observer's experience. Antibodies to CP, BLS, and LPS in human samples and to BLS in canine samples were measured by ELISA as described previously (2, 3, 9).

RESULTS

Human sera. Anti-CP24 antibodies were first assayed in serial samples from patients with acute brucellosis (duration of illness, up to 40 days). At diagnosis, these antibodies were detected in only two of the eight patients, whose durations of illness were 30 and 15 days. The ratios between the OD of each sample and the cutoff for the assay (reactivity ratios) were 4.56 and 1.21, respectively. Both patients had low titers of anti-CP IgG (100 and 200, respectively), and the second patient was



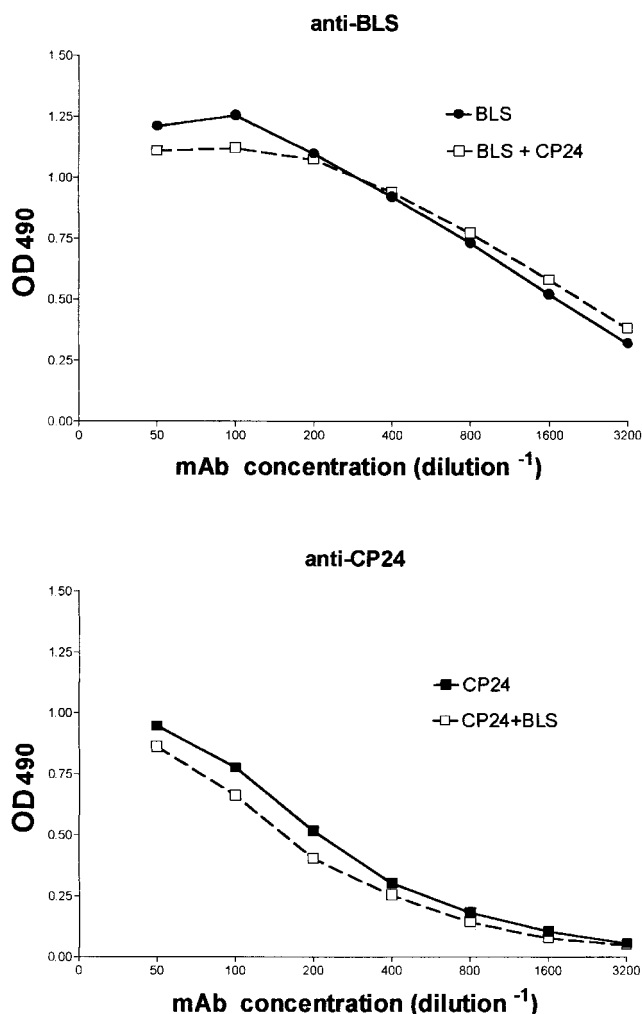


FIG. 2. Reactivities of specific MAbs against CP24 or BLS by ELISA using wells coated with the homologous antigen or with both antigens.

in the combined ELISA (after the determination of the cutoff specific for this test), 23 (66%) yielded positive results. Six sera positive for anti-BLS antibodies, included as controls, were also positive in the combined assay. The serum samples from subacute and chronic cases that had been assayed for anti-CP24 antibodies were also tested in the combined ELISA. As shown in Table 1, the percentage of positive results was higher for the combined test than for any of the independent assays (CP24 or BLS), especially in the chronic cases.

TABLE 1. Diagnostic performance of combined ELISA with CP24 and BLS in patients with subacute or chronic brucellosis

Cases	% Samples positive for antibodies to:			CP
	BLS	CP24	BLS + CP24 ^a	
Subacute (n = 24)	83	29	87.5	96
Chronic (n = 23)	65	26	83	91

^a Combined ELISA with both antigens in the same well.

Canine sera. Only 9 (43%) of the 21 serum samples from consecutive cases of canine brucellosis were positive by ELISA against CP24, with reactivity ratios that varied from 1.13 to 5.27 (mean, 2.26). No differences were found between CP24-positive and -negative samples regarding their reactivities against the BLS protein (mean reactivity ratios, 4.50 and 4.95, respectively). All but two dogs lacking anti-CP24 antibodies had *B. canis* M⁻ agglutination rated 3+ or 4+. Among the 15 dogs involved in an outbreak of brucellosis and followed for 60 days after diagnosis, 7 (47%) were negative for anti-CP24 antibodies during the whole follow-up, 4 were always positive, 3 were positive from day 0 to day 15, and 1 dog initially negative became positive at 60 days. In all these cases, anti-BLS antibodies were first detected at diagnosis or at day 15. As with the consecutive cases, no correlation was found between the presence of anti-CP24 antibodies and the reactivity ratio of anti-BLS antibodies.

DISCUSSION

In this study, antibodies to the CP24 cytoplasmic antigen of *Brucella* were assessed in two different host species infected with rough or smooth *Brucella* strains and having a wide range of duration of illness. In spite of this variety of status, a common factor was evident: CP24 is recognized by a low percentage of the individuals that have had contact with *Brucella*. This finding is surprising in view of the good immunogenicity of rCP24 in mice (4a). One possible explanation for the low prevalence of anti-CP24 antibodies in naturally infected hosts is that the protein may have a low expression in *Brucella* cells. In any case, this expression level seems to affect the immune responses to smooth and rough strains of *Brucella* similarly, since anti-CP24 antibodies were as prevalent in human brucellosis as in canine brucellosis. Alternatively, CP24 may be significantly expressed or recognized only during some stages of the infection. This possibility seems unlikely, however, since the frequencies of anti-CP24 antibodies were similar in patients with acute, subacute, and chronic brucellosis. Moreover, most patients who tested negative during the acute phase of the disease did not develop anti-CP24 antibodies during the subacute phase. Conceivably, the anti-CP24 response may depend on multiple factors, including a favorable match between the major histocompatibility complex of the host and the antigenic epitopes of the protein. The absence of a uniform response to CP24 among infected humans and dogs is in line with the results obtained by Debarh et al. in infected sheep (6). By immunoblotting with a cytosoluble fraction of *B. melitensis* H38, less than 50% of the sera from ewes naturally infected with *B. melitensis* reacted with the CP24 protein. In contrast, this antigen was recognized by all the ewes experimentally infected with *B. melitensis* H38. These results would tend to favor the hypothesis of a low level of expression of CP24 in *Brucella* cells, which would be offset by the large inoculum usually employed in experimental infections.

In spite of the low prevalence of anti-CP24 antibodies in infected hosts, this response was detected in almost all the patients who had antibodies to an extract of CP but lacked antibodies to a particular component of CP (BLS). The reasons for this combination of results are unknown. Notably, the simultaneous measurement of anti-CP24 and anti-BLS anti-

bodies yielded positive results for 91% of the patients positive for anti-CP antibodies. This suggests that an ELISA combining a discrete array of recombinant cytoplasmic proteins of *Brucella* could be more sensitive than those based on a unique cytoplasmic antigen and could approach the sensitivity of the assay performed with the CP antigen. The latter antigen is obtained from *Brucella* cells by sequential disruption, ultracentrifugation, and LPS removal by immunoaffinity. As with other complex antigens obtained from whole bacteria by extractive methods, such as brucellin and hot saline (8, 11), the properties of CP may vary according to the strain used, the culture conditions, and the efficiency of the extraction. In the case of brucellin, for example, the variation in the protein content of the batches has been suggested as a possible cause of heterogeneity in the results of diagnostic tests (7). The development of an ELISA combining several recombinant proteins would not only simplify the preparation of the antigens but would also allow a better standardization of the assay. Our results suggest that this combined ELISA is feasible and would be almost as sensitive as the assay based on native antigens.

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REFERENCES

1. 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.
2. Baldi, P. C., S. E. Miguel, C. A. Fossati, and J. C. Wallach. 1996. Serological follow-up of human brucellosis by measuring IgG antibodies directed to LPS and cytoplasmic proteins of *Brucella*. Clin. Infect. Dis. 22:446-455.
3. Baldi, P. C., M. M. Wanke, M. E. Loza, N. Monachesi, and C. A. Fossati. 1997. Diagnosis of canine brucellosis by detection of IgG antibodies against an 18 kDa cytoplasmic protein of *Brucella* spp. Vet Microbiol. 57:273-281.
4. Carmichael, L. E., and J. C. Joubert. 1987. A rapid slide agglutination test for the serodiagnosis of *Brucella canis* infection that employs a variant (M-) organism as antigen. Cornell Vet. 77:3-12.
- 4a. Cassataro, J., C. A. Velikovskiy, G. H. Giambartolomei, S. Estein, L. Bruno, A. Cloeckert, R. Bowden, M. Spitz, and C. A. Fossati. Immunogenicity of the *Brucella melitensis* recombinant ribosome recycling factor-homologous protein and its cDNA. Vaccine, in press.
5. Cloeckert, A., H. S. Debbbarh, M. S. Zygmunt, and G. Dubray. 1996. Production and characterisation of monoclonal antibodies to *Brucella melitensis* cytosoluble proteins that are able to differentiate antibody responses of infected sheep from Rev.1 vaccinated sheep. J. Med. Microbiol. 45:206-213.
6. Debbbarh, H. S., A. Cloeckert, M. S. Zygmunt, and G. Dubray. 1995. Identification of sero-reactive *Brucella melitensis* cytosoluble proteins which discriminate between antibodies elicited by infection and Rev.1 vaccination in sheep. Vet. Microbiol. 44:37-48.
7. Denoel, P. A., T. K.-O. Vo, V. E. Weynants, A. Tibor, D. Gilson, M. S. Zygmunt, J. N. Limet and J.-J. Letesson. 1997. Identification of the major T-cell antigens present in the *Brucella melitensis* B115 protein preparation, Brucellergene OCB. J. Med. Microbiol. 46:801-806.
8. Gamazo, C., A. J. Winter, I. Moriyón, J. I. Riezu-Boj, J. M. Blasco, and R. Diaz. 1989. Comparative analyses of proteins extracted by hot saline or released spontaneously into outer membrane blebs from field strains of *Brucella ovis* and *Brucella melitensis*. Infect. Immun. 57:1419-1426.
9. Goldbaum, F. A., J. Leoni, J. C. Wallach, and C. A. Fossati. 1993. Characterization of an 18-kilodalton *Brucella* cytoplasmic protein which appears to be a serological marker of active infection of both human and bovine brucellosis. J. Clin. Microbiol. 31:2141-2145.
10. Gotuzzo, E., and C. Cellillo. 1992. *Brucella*, p. 1513-1521. In S. L. Gorbach, J. G. Bartlett, and N. R. Blacklow (ed.), Infectious diseases. W. B. Saunders Company, Philadelphia, Pa.
11. Jones, L. M., R. Diaz, and A. G. Taylor. 1973. Characterization of allergens prepared from smooth and rough strains of *Brucella melitensis*. Br. J. Exp. Pathol. 54:492-508.
12. Vizcaíno, N., A. Cloeckert, G. Dubray, and M. S. Zygmunt. 1996. Cloning, nucleotide sequence, and expression of the gene coding for a ribosome releasing factor-homologous protein of *Brucella melitensis*. Infect. Immun. 64:4834-4837.