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# Enzyme-Linked Immunosorbent Assay with Major Outer Membrane Proteins of *Brucella melitensis* to Measure Immune Response to *Brucella* Species

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**We developed an enzyme-linked immunosorbent assay (ELISA) system to measure human immunoglobulin G (IgG) and IgM response to the major outer membrane proteins of *Brucella melitensis*. The ELISA was more sensitive in detecting antibody than a standard microagglutination (MA) test with *B. abortus* antigen. Of 101 sera from persons with suspected brucellosis, 79 (78.2%) gave ELISA IgM titers greater than or equal to the *B. abortus* MA titer without 2-mercaptoethanol (2ME), which measures both IgM and IgG. Of the 101 sera, 97% gave ELISA IgG titers greater than or equal to the MA with 2ME titer. A total of 58 sera, drawn from 11 human patients from 1 to 29 weeks after onset of brucellosis, gave higher geometric mean titers for the ELISA IgG test than for the MA with 2ME test. These 58 sera also gave ELISA IgM geometric mean titers that were greater than or within one doubling dilution of the geometric mean titers of MA without 2ME. In addition to detecting antibody response to *B. abortus*, *B. melitensis*, and *B. suis*, the ELISA was sensitive to antibody response to human and canine infections with *B. canis*. The *B. canis* antibody response is not detected by the MA test with *B. abortus* antigen. The ELISA, with a standard preparation of major outer membrane proteins of *B. melitensis* as antigen, appears to be useful in measuring antibody response in humans to infections by all species of *Brucella* known to infect humans.**

Human brucellosis is usually diagnosed based on patient history, symptoms, and serologic tests (10, 14, 30). A number of serologic tests have been used including agglutination (1, 3, 18, 22, 24), complement fixation (13), radioimmunoassays (12, 21), immunofluorescent assays (10), and enzyme-linked immunosorbent assays (ELISAs) (5, 8, 11, 16, 17, 20, 26, 28).

Previous ELISAs for human antibody response to *Brucella* infections have used whole-cell antigen (*Brucella abortus*) (8, 28) or lipopolysaccharide (LPS) antigen (extract of *B. abortus* cells) (5, 11, 16, 17, 20). Lamb et al. (15) showed that use of LPS as the solid-phase antigen in *B. abortus* ELISAs could result in nonspecific binding of immunoglobulin M (IgM) to the LPS which could cause false-positive results. Tabatabai and Deyoe (32, 33) suggested that proteins could be of potential value as a vaccine and diagnostic reagent for *Brucella* infection. They developed an ELISA for bovine serum antibodies to *B. abortus* using salt-extractable protein antigens (33).

None of the researchers listed above reported results of their tests in detecting antibody response to *B. canis*, which may infect humans. For brucellosis in humans most serologic tests detect antibodies to the polysaccharides of *B. abortus* and also should detect antibodies to *B. melitensis* and *B. suis* because there is cross-reactivity between these species. Because *B. canis* does not share A and M antigens with these other species, other serologic tests are needed to detect antibodies to *B. canis* (18). Recently, agglutination tests for human sera have been developed to detect antibodies to *B. canis* (7, 23). Santos et al. (27) have shown a great deal of antigenic relatedness between the major outer membrane proteins (MOMPs) of *Brucella* species. Because the MOMPs have potential to be antigenically related in all species of *Brucella*, we developed an ELISA for antibodies to these proteins in an attempt to have one test system that could detect brucellosis caused by all species of *Brucella*. We tested human sera with anti-human IgG and anti-human IgM horseradish peroxidase conjugates. We compared these with results obtained with microagglutination (MA) tests with *B. abortus* cells as antigen with and without 2-mercaptoethanol (2-ME) (3). Because we were able to obtain only a small number of human sera from patients with suspected *B. canis* infection, we also tested a small number of sera from dogs suspected of having *B. canis* infections, with an anti-dog horseradish peroxidase conjugate and by a similar MA test with *B. canis* antigen.

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## MATERIALS AND METHODS

**Microorganisms and growth conditions.** A pure culture of *B. melitensis* M15 (type 1) was provided by J. Roux, Institut de Biologie, Montpellier, France. The culture (smooth strain) was first grown on tryptose agar (Difco Laboratories, Detroit, Mich.) (2, 9) in a 1-liter screw-cap culture bottle containing 100 ml of medium. The culture was incubated at 37°C for 72 h as seed. The seed culture was then suspended in a phosphate buffer (pH 7.2) and inoculated into the same tryptose agar medium as described above for the seed culture preparation. The production culture was incubated at 37°C for 5 days. For harvest, 15 ml of sterile phosphate

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TABLE 1. Comparison of regular MA titer to ELISA IgM titer for 101 serum specimens from humans suspected of having brucellosis

| MA titer | No. of sera | No. of specimens with the following ELISA IgM titers[a]: | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | >3 dilutions lower | 3 dilutions lower | 2 dilutions lower | 1 dilution lower | Same | 1 dilution higher | 2 dilutions higher | 3 dilutions higher | >3 dilutions higher |
| <20 | 7 | | | | | | | 4 | 1 | 2 |
| 20 | 4 | | | | | 1 | 3 | | | |
| 40 | 4 | | | | 1 | 2 | 1 | | | |
| 80 | 16 | | | | 3 | 5 | 3 | 1 | 2 | 2 |
| 160 | 14 | | 2 | 3 | 1 | 4 | 1 | 2 | | 1 |
| 320 | 19 | 3 | 1 | 3 | 2 | 3 | 3 | 1 | 2 | 1 |
| 640 | 10 | 3 | 3 | | 3 | | | 1 | | |
| 1,280 | 11 | 1 | | | 1 | 4 | 2 | 3 | | |
| 2,560 | 9 | | 1 | 1 | 1 | | 6 | | | |
| 5,120 | 6 | | | | 3 | 3 | | | | |
| >5,120 | 1 | | | | | 1 | | | | |

[a] Titer is the reciprocal of the highest dilution giving positive agglutination in the MA test or a positive OD in the ELISA. MA measures IgG- and IgM-agglutinating antibodies.

buffer (pH 7.2) was added to each bottle to wash off the cells from the agar surface. The pooled cell suspension was killed by 50% acetone at 4°C for 16 to 24 h.

**Isolation of *B. melitensis* cell envelope.** A modification of the procedure of Rosenbusch (25) was used for the cell envelope preparation. Acetone-killed *B. melitensis* was collected by centrifugation (5,000 × *g* for 30 min) and washed once with buffer A (20 mM Tris hydrochloride [pH 7.1] and 8 mM $MgSO_4$). Cells were suspended at 100 mg (wet weight)/ml of buffer A containing 20% sucrose and 1% Triton X-100 and stirred overnight at room temperature to solubilize the cytoplasmic membrane. The Triton X-100-treated cells, recovered by centrifugation, were suspended in buffer A. The suspended cells were broken by processing with glass beads in a Waring blender (American Scientific Products, McGaw Park, Ill.) for 30 s. After differential centrifugation (2,000 × *g* for 10 min) to remove the glass beads and unbroken cells, cell envelope material was collected by centrifugation at 30,000 × *g* for 2 h.

**Preparation of MOMPs by SDS extraction.** Cell envelope pellets were suspended in buffer A (100 mg/ml) and added dropwise to an equal volume of boiling 4% sodium dodecyl sulfate (SDS; the final SDS concentration was 2%). The solution was kept at 100°C for 5 min; the mixture was then cooled and kept gently stirred at room temperature for 2 h. The insoluble fraction was recovered by centrifugation at 100,000 × *g* for 1 h, washed exhaustively with distilled water, and then digested by lysozyme (2%; wt/wt) for 24 h at 37°C. The protein pellet was collected by centrifugation at 100,000 × *g* for 2 h. The membrane proteins were lyophilized and stored at −20°C. The yield of the MOMPs was about 11% of the dry weight of the cells.

**ELISA test system.** Plates (Immulon II; Dynatech Laboratories, Inc., Alexandria, Va.) were first incubated with 0.1 ml per well of 12.5 μg of MOMPs per ml diluted in carbonate-bicarbonate buffer at pH 9.6 (1.59 g of $Na_2CO_3$ and 2.93 g of $NaHCO_3$ per liter of $H_2O$) for 12 to 18 h at 37°C. Plates were then emptied, and 0.2 ml of carbonate-bicarbonate buffer (pH 9.6) containing 10% normal goat serum was added per well, and plates were incubated for 2 h at 37°C. Plates were then washed with phosphate-buffered saline (pH 7.4) with a plate washer (Skatron Microwash, II; Skatron Inc., Sterling, Va.). Next, 0.2 ml of sera, diluted 1:40, was added to the first well in each column. Sera were diluted in phosphate-buffered saline containing 0.1% Tween 20 and 10% normal goat serum. Serial twofold dilutions of sera were made in the plates so that each well contained 0.1 ml of diluted serum. Plates were incubated for 1 h at 37°C and washed as described above. Predetermined dilutions of the appropriate conjugate were added in 0.1-ml volumes per well, and plates were incubated for 1 h at 37°C. Conjugates used were goat anti-human IgM (mu chain specific; Cappel Laboratories, Cochranville, Pa.), goat anti-human IgG (heavy chain specific; Miles Laboratories, Naperville, Ill.), and goat anti-dog IgG (Cappel Laboratories). After incubation the plates were washed as described above, and 0.1 ml of substrate solution containing 0.2% *O*-phenylenediamine (OPD; New England Nuclear Corp., Boston, Mass.) as indicator and 0.015% $H_2O_2$ in a substrate buffer (pH 6.3) containing 17 mM citric acid in 65 mM phosphate and 0.01% merthiolate were added to each well. After incubation at 37°C for 30 min, 0.05 ml of 4.5 M $H_2SO_4$ was added per well to stop the reaction, and the absorbance was determined with an ELISA reader (Dynatech Laboratories).

**MA tests.** MA tests for *B. abortus*-agglutinating antibodies were run as described previously (3). These tests were run with 2ME (MA-2ME) and without 2ME (MA). MA tests for *Francisella tularensis*-agglutinating antibodies were also run as described previously (4), except that sera were also diluted in 2ME containing diluent. Tests for *B. canis*-agglutinating antibodies were likewise performed with *B. canis* antigen. *B. canis* antigen was grown and prepared as described previously (18).

**Human sera.** One hundred sera from normal humans were run in the ELISA system with both conjugates to determine an optical density (OD) for use as a positive cutoff value. These sera were obtained from the Centers for Disease Control serum bank and gave titers of <1:160 for brucellosis by MA testing. One hundred and one sera from humans suspected of having brucellosis caused by *B. abortus*, *B. suis*, or *B. melitensis* were run in the ELISA system with both goat anti-human IgG and goat anti-human IgM horseradish peroxidase conjugates. These same sera were also run in the MA test and the MA-2ME test with *B. abortus* as antigen. Three sera from patients with brucellosis with *B. canis* as the suspected etiologic agent were also run as described above and in MA tests with *B. canis* as antigen. Thirty sera from patients suspected of having tularemia were also run in the ELISA system and in MA tests with *B. abortus* and *F. tularensis* antigens. Sera were obtained from various collections at the Centers for Disease Control.

**Canine sera.** Twenty-seven sera from normal dogs were run with an anti-dog IgG conjugate to determine a positive cutoff value. Twelve sera from dogs with suspected

TABLE 2. Comparison of MA-2ME titer to ELISA IgG titer for 101 serum specimens from humans suspected of having brucellosis

| MA-2ME titer | No. of sera | No. of specimens with the following ELISA titers[a]: | | | | | |
|---|---|---|---|---|---|---|---|
| | | 1 dilution lower | Same | 1 dilution higher | 2 dilutions higher | 3 dilutions higher | >3 dilutions higher |
| <20 | 28 | | 1 | | 11 | 4 | 12 |
| 20 | 10 | | | 2 | 2 | 2 | 4 |
| 40 | 10 | | | 2 | 1 | 2 | 5 |
| 80 | 14 | 1 | | 1 | 3 | 3 | 6 |
| 160 | 20 | 1 | 1 | 2 | 7 | 1 | 8 |
| 320 | 10 | 1 | | 4 | 2 | 3 | |
| 640 | 7 | | 2 | | 1 | 4 | |
| 1,280 | 1 | | | | 1 | | |
| 2,560 | 1 | | 1 | | | | |

[a] Titer is the reciprocal of the highest dilution giving positive agglutination in the MA test or a positive OD in the ELISA.

brucellosis were run in the ELISA system and in two MA tests, one with *B. abortus* as antigen and the other with *B. canis* as antigen.

**Case time studies.** Sera from 11 patients with blood cultures positive for *Brucella* species were studied. The sera were from blood drawn 1 to 29 weeks after onset of the disease, generally at 1- to 2-week intervals. Fifty-eight sera were available representing one to eight specimens from each patient. The ELISA system with both anti-human IgG and anti-human IgM horseradish peroxidase conjugates was run on each specimen as well as MA and MA-2ME tests with *B. abortus* as antigen.

## RESULTS

**Human sera.** We used the mean OD reading plus 2 standard deviations at a 1:160 dilution of 100 normal human sera to determine the positive cutoff point for both IgG and IgM titers. With these values 95% of the normal IgG values and 98% of the normal IgM values would be negative at 1:160. In Table 1 is shown a comparison of the MA results with the ELISA IgM results for 101 suspect sera. Of 101 sera from persons with suspected brucellosis, 68 (67.3%) gave ELISA IgM titers greater than or equal to the *B. abortus* MA titer which measures both IgM and IgG. Of the 101 sera, 98 (97%) gave ELISA titers greater than or equal to the MA-2ME titer (Table 2). A total of 77 (76.2%) of the sera gave titers of 1:160 or greater in the IgG ELISA, whereas only 39 (38.6%) were 1:160 or greater in the MA-2ME test, and 70 (69.3%) were 1:160 or greater in the MA test which measures IgG and IgM titer. Sixty (59.4%) gave titers of 1:160 or greater in the IgM ELISA. Results for three sera from humans suspected of having brucellosis caused by *B. canis* are shown in Table 3. Titers for all three sera were <1:20 by the *B. abortus* MA test but gave MOMP ELISA results similar to the *B. canis* MA results.

TABLE 3. Comparison of titers from MOMP ELISA tests and MA tests from three human patients suspected of having brucellosis caused by *B. canis*

| Patient no. | Titer by the following tests[a]: | | | | | |
|---|---|---|---|---|---|---|
| | *B. canis* MA | | *B. abortus* MA | | ELISA | |
| | MA | MA-2ME | MA | MA-ME | IgM | IgG |
| 1 | 160 | 20 | <20 | <20 | 40 | 160 |
| 2 | 80 | <20 | <20 | <20 | 40 | <40 |
| 3 | ≥2,560 | ≥2,560 | <20 | <20 | 1,280 | 320 |

[a] Titer is the reciprocal of the highest dilution giving positive agglutination in the MA test or a positive OD in the ELISA.

We plotted $\log_2$ geometric mean titers versus weeks postonset for the 58 sera from 11 confirmed brucellosis cases for ELISA IgM and IgG antibody levels (Fig. 1) and MA and MA-2ME antibody levels (Fig. 2). The geometric mean MA-2ME titers were 1:80 or below throughout the period shown for these specimens. The regular MA titer dropped to below 1:160 after weeks 11 to 12. The ELISA geometric mean IgG titer declined from 1:320 at weeks 3 to 4 to 1:80 by weeks 23 to 24 but remained at 1:160 for the one available specimen as late as week 26. In some cases infection was caused by *B. abortus* and in some by *B. suis*; however, similar results were obtained regardless of the *Brucella* species causing the infection.

In Fig. 3 are shown geometric mean titers ±2 standard deviations for 27 sera from patients with titers ≥1:160 to *F. tularensis*. The sera were tested by *Brucella* IgM ELISA, IgG ELISA, MA, MA-2ME, and *F. tularensis* MA and MA-2ME. The *F. tularensis* MA geometric mean titer was much higher than the other titers, including the *Brucella* ELISA IgG and IgM titers. Of the 27 sera, 3 had *Brucella* titers that were >1:160 by MA. Of the 27 sera, 1 had an IgM ELISA titer of 1:160, and 2 had an IgG ELISA titer of >1:160. All except one *F. tularensis* MA individual titer was at least one doubling dilution higher than the *Brucella* IgM and IgG ELISA titers.

**Canine sera.** In Table 4 are given results of tests on sera from 12 dogs suspected of having *B. canis* infection. We used the mean plus 2 standard deviations from ELISA results run on 27 sera from normal dogs as the cutoff value to determine the ELISA titer. With this cutoff point, 96.3% (26 of 27) of the normal sera were negative at 1:160. All of the sera from dogs suspected of having *B. canis* infection had titers greater than or equal to the *B. canis* MA titer. All of these same sera gave negative *B. abortus* MA titers at a 1:20 dilution.

## DISCUSSION

The positive cutoff point used for human sera for this study was the mean plus 2 standard deviations of the OD obtained for 100 normal human sera diluted at 1:160. With this cutoff, 97% of the sera of patients tested had ELISA IgG titers greater than or equal to the MA-2ME titer which should measure agglutinating IgG. Not all IgG is agglutinating; these nonagglutinating antibodies would not be observed by a MA test (14). On first inspection the IgM ELISA appears less sensitive than the MA test (Table 1); however, the MA test measures both IgG and IgM, whereas the IgM ELISA measures only IgM. We used heavy-chain-specific anti-human IgG and IgM horseradish peroxidase conjugates.

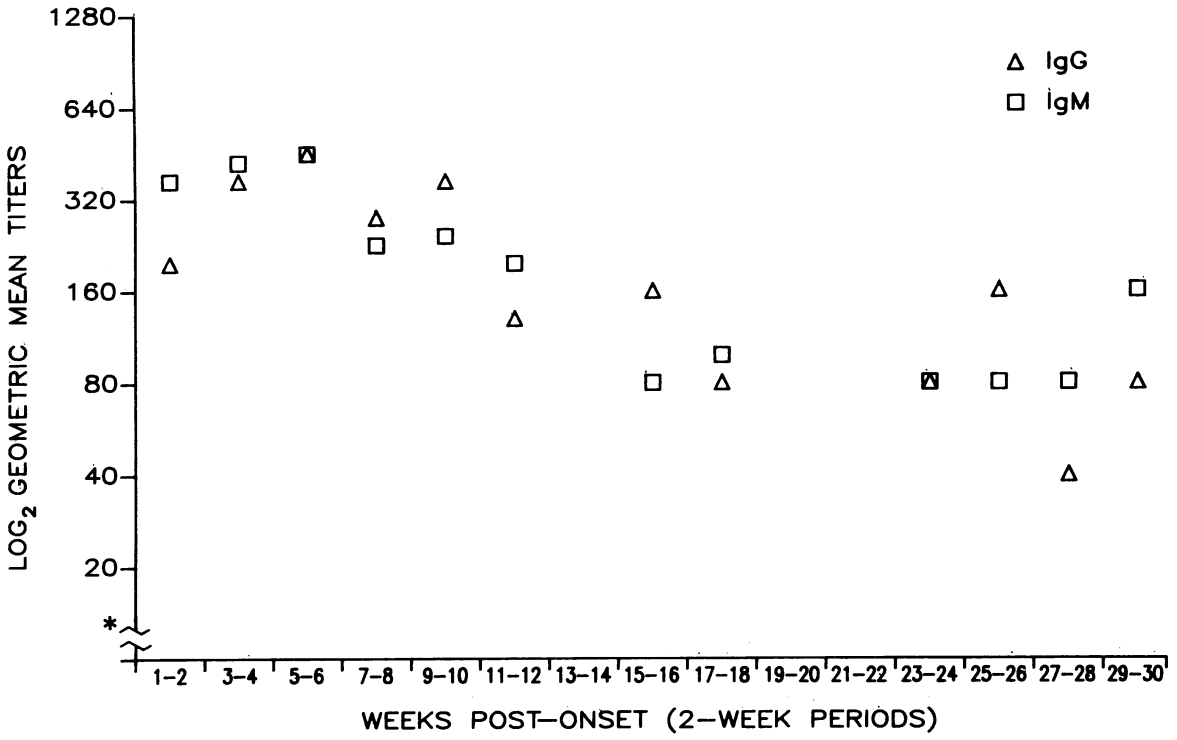


FIG. 1. Geometric mean of ELISA IgM and IgG titers for sequential specimens from 11 human patients with brucellosis. *, Titer <20.

In most recent studies with polysaccharide antigen high anti-*Brucella* IgM antibody levels are associated with recent exposure or acute infection, and high IgG levels are associated with multiple exposure or chronic or persistent infection (8, 11, 28). In our case studies (Fig. 1), however, we did not see this difference with the ELISA; both IgM and IgG levels were high in sera from patients with acute infection. In the MA tests (Fig. 2) the MA titers were higher in the earlier

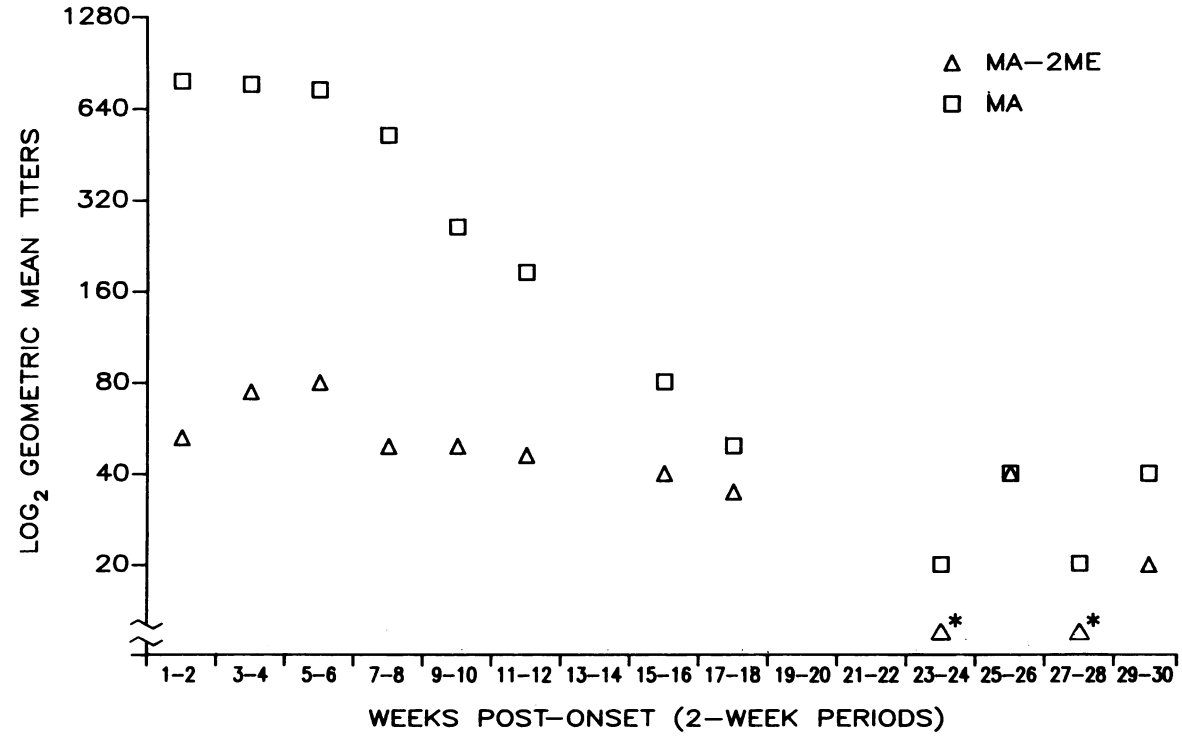


FIG. 2. Geometric mean of MA and MA-2ME titers for sequential specimens from 11 human patients with brucellosis. *, Titer <20.

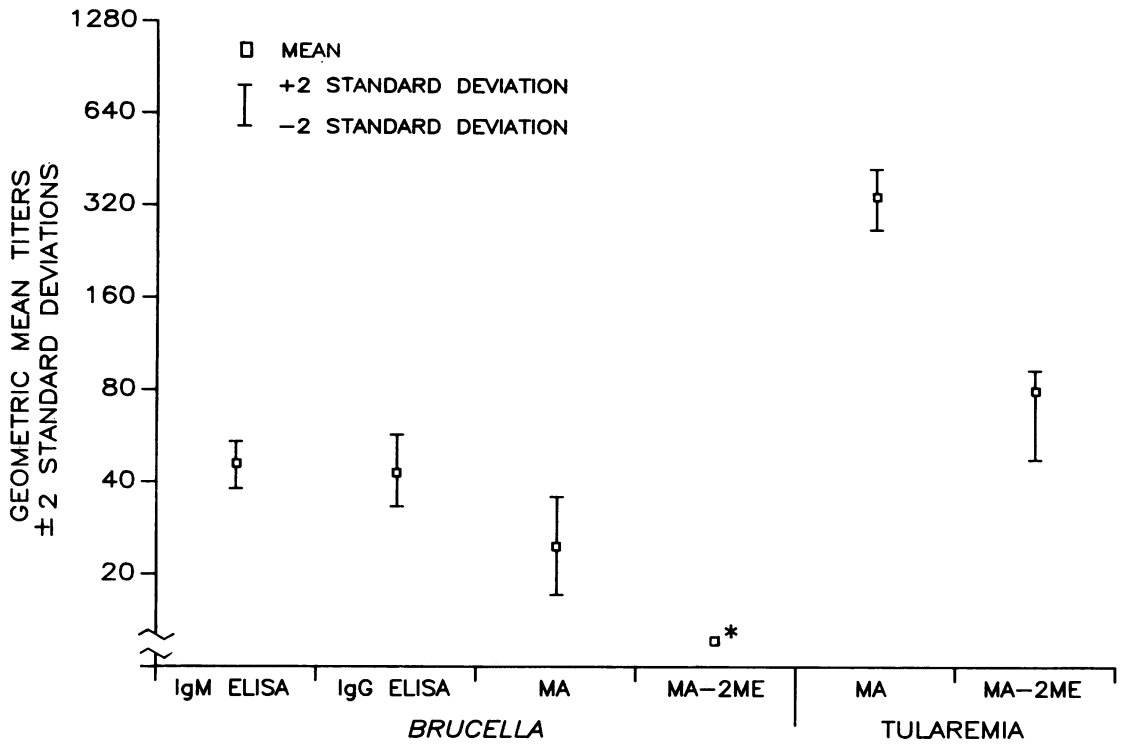


FIG. 3. Comparison of geometric mean titers of sera from patients with tularemia run by *Brucella* IgM ELISA, IgG ELISA, MA, MA-2ME, and tularemia MA and MA-2ME. *, Titer <20.

sera than were the MA-2ME titers. These differences could be due to different antibody response to the protein antigen or to the fact that the MA test is much better at detecting IgM, which is a more efficient agglutinating antibody.

Most human brucellosis is caused by *B. abortus*, *B. suis*, or *B. melitensis* (18). These species and *B. neotomae* contain two cell wall antigens referred to as A and M and occur in the smooth form in nature. *B. canis* and *B. ovis* occur in the rough form in nature and lack the A and M antigens. *B. ovis* has not been associated with disease in humans. Recent studies suggest that human brucellosis caused by *B. canis* may be more common than originally assumed (19, 22, 23, 31). The agglutination tests and other serologic tests with *B. abortus* as antigen commonly done to detect antibodies in humans with suspected brucellosis do not detect antibodies to *B. canis* because *B. canis* lacks the A and M antigens. It appears, however, that infection by *B. canis* does elicit immune response to the MOMPs and that this response can be measured by an ELISA based on the MOMPs of *B. melitensis*. Antibody response to the MOMPs of *B. melitensis* were seen in sera from human patients infected with *B. abortus*, *B. melitensis*, *B. suis*, and *B. canis* and in sera from dogs suspected of infection with *B. canis*. Recently, Verger et al. (34) showed that by DNA hybridization by the S1 nuclease method, all members of the genus *Brucella* that they tested (including representatives from all species and biogroups) formed a single DNA-DNA hybridization group. With this close genetic relatedness within the genus *Brucella* it would not be surprising for the various species to have a common or similar MOMP. The MOMP ELISA test appears to be sensitive to antibodies to *B. canis* as well as to *B. abortus*, *B. suis*, and *B. melitensis*. In Table 3 are shown results of tests on sera from three patients with brucellosis caused by *B. canis*. These sera had no measurable titer by the *B. abortus* MA test, but gave ELISA results similar to the *B. canis* MA results. Sera from 12 dogs with *B. canis* infections (Table 4) also gave ELISA titers that were equal to or greater than the *B. canis* MA titers. The positive cutoff point for the canine sera was higher than that for human sera, possibly due to low antibody levels in some of the supposedly normal dogs due to earlier infections because canine brucellosis is common in dogs (19, 31).

For the 58 sera from 11 human patients with brucellosis presented in Fig. 1 and 2, the ELISA titers tended to be higher than the MA titers. Because these were from patients with culturally confirmed brucellosis, this indicates that the ELISA is, indeed, a more sensitive test than the MA test for diagnosis of brucellosis. Further studies with sera from a larger number of culturally confirmed cases are needed to

TABLE 4. Comparison of *Brucella* serologic test results on sera from 12 dogs with suspected *B. canis* infection

| Specimen no. | MOMP ELISA titer[a] | *B. canis* MA titer[b] |
|---|---|---|
| 1 | 1,280 | 640 |
| 2 | 1,280 | 640 |
| 3 | 2,560 | 320 |
| 4 | ≥5,120 | 1,280 |
| 5 | 160 | 160 |
| 6 | 640 | 320 |
| 7 | 1,280 | 640 |
| 8 | ≥5,120 | ≥2,560 |
| 9 | 2,560 | 640 |
| 10 | ≥5,120 | 1,280 |
| 11 | ≥5,120 | ≥2,560 |
| 12 | 2,560 | ≥2,560 |

[a] Titer is the reciprocal of the highest dilution giving positive agglutination in the MA test or a positive OD in the ELISA.
[b] MA titers with *B. abortus* antigen were all <20.

establish the sensitivity and clinical significance of the IgM and IgG titers in the ELISA tests.

There are known cross-reactions between *B. abortus* and *Vibrio cholerae*, *F. tularensis*, *Yersinia enterocolitica* serogroup O:9, *Salmonella* group N serotypes, and *Escherichia coli* O:116 and O:157 (6, 18, 29). We had available to us 27 sera from patients with tularemia which gave *F. tularensis* MA titers that were ≥1:160. Earlier studies have indicated that 18 to 40% of patients with naturally acquired tularemia infections have agglutinating antibodies to *B. abortus*. The antibody level was always less than that to the homologous antigen (29). When we compared our *Brucella* ELISA results with the *F. tularensis* MA results, 23 of 27 (85.18%) had *Brucella* ELISA titers lower than the *F. tularensis* MA titers; 4 of the sera had ELISA titers equal to the MA titers. When we compared the *F. tularensis* MA titer with the *Brucella* MA titer, 26 of 27 (96.3%) had *Brucella* MA titers lower than the *F. tularensis* MA titers; one had a *Brucella* MA titer equal to the *F. tularensis* MA titer. It is not surprising that the *Brucella* ELISA titers would be somewhat higher than the *Brucella* MA titers because the ELISA appears to be a more sensitive test. We cannot rule out the possibility that the patients who had higher titers to both antigens were infected with both organisms concurrently or at some time. MOMP ELISA for antibodies to *Brucella* does not avoid the problem of cross-reactivity with antibodies to *F. tularensis*. Clinical history must be considered when serologic tests are used to diagnose brucellosis. It is possible that small amounts of cross-reacting polysaccharide are present in the MOMP preparation. However, analysis of the MOMP has shown that it is ≥90% protein and contains <1% carbohydrate and <1% LPS. SDS-polyacrylamide gel electrophoresis analysis of the MOMP showed at least six bands with the major band around 31 kilodaltons (data not shown). Protein epitopes might be resolved by enzyme-linked immunoelectrotransfer blots of SDS-polyacrylamide gel electrophoresis gels. Enzyme-linked immunoelectrotransfer blots of MOMPs could show antibody response to individual protein antigens. The MOMP ELISA has the potential to achieve greater specificity for *Brucella* antibodies through the use of more highly purified MOMPs. If it could be shown that only a portion of the proteins crossed with heterologous antigens it may be possible to eliminate the crossing proteins from the capture antigen while retaining full sensitivity for the homologous antibodies.

We were able to keep plates that had wells activated with MOMPs and were blocked and stored under phosphate-buffered saline containing 0.1% Tween 20 and 10% normal goat serum for at least 1 month at 4°C without detectable loss of activity. These plates gave comparable results to plates that had antigen attached overnight and were blocked and run on the next day (data not shown). Thus the convenience of the test is established.

The ELISA presented here with MOMPs extracted from *B. melitensis* as capture antigen was found to be a sensitive test for detecting antibodies to *B. abortus*, *B. suis*, *B. melitensis*, and *B. canis*. The ELISA was shown to detect antibodies to *B. canis* in both humans and dogs and was more sensitive than the MA tests for human sera when antibodies to *B. abortus*, *B. suis*, and *B. melitensis* were measured.

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