Evaluation of the Recombinant 10-Kilodalton Immunodominant Region of the BP26 Protein of \textit{Brucella abortus} for Specific Diagnosis of Bovine Brucellosis

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Brucellosis is a disease with worldwide distribution affecting animals and human beings. \textit{Brucella abortus} is the causative agent of bovine brucellosis. The cross-reactions of currently available diagnostic procedures for \textit{B. abortus} infection result in false-positive reactions, which make the procedures unreliable. These tests are also unable to differentiate \textit{Brucella}-infected and -vaccinated animals. The present work is focused on the use of a nonlipopolysaccharide (LPS) diagnostic antigen, a recombinant 10-kDa (r10-kDa) protein of \textit{B. abortus}, for specific diagnosis of brucellosis. The purified recombinant protein was used as a diagnostic antigen in plate enzyme-linked immunosorbent assay (p-ELISA) format to screen 408 bovine serum samples (70 presumptively negative, 308 random, and 30 vaccinated), and the results were compared with those of the Rose Bengal plate agglutination test (RBPT) and the standard tube agglutination test (STAT). Statistical analysis in presumptive negative samples revealed 100 and 98.41% specificity of p-ELISA with RBPT and STAT, and an agreement of 91.43% with the tests using Cohen’s kappa statistics. In random samples, the agreement of p-ELISA was 77.92% and 80.52% with RBPT and STAT, respectively. p-ELISA investigation of vaccinated samples reported no false-positive results, whereas RBPT and STAT reported 30% and 96.6% false-positive results, respectively. The data suggest that p-ELISA with r10-kDa protein may be a useful method for diagnosis of bovine brucellosis. Furthermore, p-ELISA may also be used as a tool for differentiating \textit{Brucella}-vaccinated and naturally infected animals.

Brucellosis, a contagious disease primarily affecting animals, is caused by members of the genus \textit{Brucella}, namely, \textit{Brucella abortus} (cattle, bison, and buffalo), \textit{B. melitensis} (goats and sheep), \textit{B. suis} (swine), \textit{B. canis} (dogs), \textit{B. ovis} (sheep), and \textit{B. neotomae} (rodents). Bovine brucellosis is caused by biovars of \textit{B. abortus}. The disease is asymptomatic in animals that have not conceived. Following infection with \textit{B. abortus}, pregnant adult females develop placentitis, resulting in abortion between the fifth and ninth months of pregnancy and infertility. Adult male cattle may develop orchitis, leading to infertility. Brucellosis has zoonotic potential in terms of its transmissibility to human beings attending infected livestock, showing symptoms in the form of low-grade undulant fever, night sweats, early fatigue, joint pain leading to spondylitis, and orchitis, and it is commonly known as “undulant fever,” “Mediterranean fever,” or “Malta fever” (7). The disease remains a public health problem and results in severe economic losses in terms of loss of fetuses and loss of animal products. In India, bovine brucellosis is widespread and appears to be on the increase in recent times, perhaps due to increased trade, free grazing, and the preponderance of natural bull service and rapid movement of livestock (17).

Due to its heterogeneous and poorly specific symptoms, diagnosis of brucellosis always requires laboratory confirmation, either by demonstration of specific antibodies or by isolation of the pathogen. The diagnosis of brucellosis based exclusively on \textit{Brucella} isolation presents many limitations. Culture results depend on various factors, including the \textit{Brucella} species, the duration of disease, the isolation method, and whether there has been any previous treatment with antibiotics. Slow growth of \textit{Brucella} primary cultures (up to 7 days), the risk involved in their handling, and poor sensitivity make culture unattractive (27, 28). Therefore, laboratory diagnosis of brucellosis very often relies on detecting specific serum antibodies (29). The most commonly used serological tests are the Rose Bengal plate agglutination test (RBPT), the standard tube agglutination test (STAT), and the complement fixation test (CFT). These tests principally measure antibodies against the immunodominant smooth lipopolysaccharides (S-LPS) (3, 5). The tests are associated with false-positive results due to serological cross-reactions with other Gram-negative bacteria, namely, \textit{Versinia enterococitica} O:9, \textit{Salmonella enterica} serovar Urbana group N, \textit{Francisella tularensis}, \textit{Escherichia coli} O:157, and \textit{Vibrio cholerae} (8, 16, 20). Because of antibodies against lipopolysaccharides in animals vaccinated with the \textit{Brucella} sp. attenuated S19 strain, the above-mentioned tests are also not reliable in differentiating vaccinated animals from infected ones. PCR and in \textit{situ} hybridization have also been used to diagnose brucellosis (9, 26). However, these approaches are poorly suited for use in general diagnostic laboratories.

The identification of specific antigens of \textit{Brucella} species is therefore a matter of great interest in the development of a specific serological test. BP26, a \textit{Brucella} genus-specific protein, has been identified independently by three research

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groups as a potential diagnostic antigen for brucellosis (6, 10, 18). Seco-Mediavilla et al. carried out epitope mapping of the BP26 protein of *B. melitensis* with monoclonal antibody, and an immunodominant region of the protein (from amino acids [aa] 55 to 152) reacted with sera from 13 *B. melitensis-* or *B. ovis-*infected sheep while no reaction was seen with nine brucellosis-negative sheep sera (21). To exploit the potential of this immunodominant region of BP26 for diagnosis of bovine brucellosis, we cloned and expressed the gene fragment and developed a plate enzyme-linked immunosorbent assay (p-ELISA) using the recombinant protein. The results of our study show the usefulness of purified recombinant 10-kDa (r10-kDa) protein as a specific diagnostic reagent for bovine brucellosis. Further, 10-kDa protein-based p-ELISA is also able to differentiate between *Brucella* infection and vaccination.

**MATERIALS AND METHODS**

**Reagents.** *B. abortus* strain S99 and RBPT and STAT reagents were obtained from the Indian Veterinary Research Institute (IVRI), Izatnagar, India. The DNA purification kit (Wizard genomic DNA purification kit) and the PCR product purification kit (Wizard SV gel and PCR cleanup system) were procured from Promega, Madison, WI. PCR was performed using an i-Cycler (Bio-Rad). The pQE-30 UA expression vector (Amp r) was harbored by the lacZ repressor-encoding plasmid pREP4 (Kan r) for recombinant protein expression. Ni-NTAagarose (NTA) agarose resin, and anti-His-horseradish peroxidase (HRP) conjugate were procured from Qiagen, Germany. Nitrocellulose membranes for Western blotting were procured from Millipore. Anti-cow IgG-HRP enzyme conjugate was purchased from Dakocytomation, Denmark. Agarose, isopropyl-thio-β-D-galactopyranoside (IPTG), 3,3'-diaminobenzidine tetrahydrochloride (DAB), o-phenylenediamine (OPD), and other chemicals were purchased from Sigma Chemicals Co. Medium components for bacterial growth were purchased from Difco and Hi-Media Laboratories, India.

**Serum samples.** Bovine serum samples (n = 408) were obtained from the Regional Disease Diagnostic Centre (RDDC), Udaipur, India. These sera included 70 samples from apparently healthy herds with no history of brucellosis (group I, presumptively negative), 308 random samples from different unorganized herds with a history of brucellosis (group II, random), and 30 serum samples from calves vaccinated with strain S19 of *B. abortus* (group III, vaccinated). Group III serum samples were collected between 25 and 35 days after vaccination with strain S19. Two serum pools of positive and negative serum samples (30 sera in each pool) were separately prepared for use as internal controls and for determining the cutoff in ELISA. Positive samples were from the herds with a history of brucellosis and were confirmed as positive by RBPT and STAT. Negative samples (n = 30) that were confirmed as negative by RBPT and STAT were picked from group I sera.

**Cloning and expression of the 10-kDa recombinant protein.** *B. abortus* S99 genomic DNA was isolated using a DNA purification kit (Promega) according to the manufacturer’s instructions. A DNA fragment of 282 bp was amplified by the following set of primers: forward primer, 5′-ATTCTCAATCTCTCGGTGCT3′, and reverse primer, 5′-CTGACGGATTCTGACAA3′. The purified PCR product was cloned into the pQE-30 UA vector (Qiagen, Germany) according to the manufacturer’s instructions. Competent *E. coli* M15 cells harboring the lacZ repressor-encoding plasmid pREP4 were transformed with ligation mixture according to the standard protocol (19). Transformed *E. coli* M15 cells were selected on Luria-Bertani (LB) agar plates containing 100 and 25 μg/ml of ampicillin and kanamycin, respectively. M15 cells harboring the pQE-30 UA vector with a 282-bp insert (called pQ10) were grown overnight at 37°C in LB broth containing ampicillin (100 μg/ml) and kanamycin (25 μg/ml). The overnight culture was diluted 20 times with LB broth containing the aforesaid antibiotics and grown at 37°C with shaking (200 rpm). Gene expression was induced by 1 mM IPTG after the absorbance (A600) reached 0.5. After 4 h of induction, the cells were harvested by centrifugation at 4,000 × g for 20 min. Expression of the recombinant protein was checked by SDS-PAGE.

**Protein purification and Western blotting.** The solubility of the r10-kDa protein was determined according to the QiAexpressionist protocol (Qiagen, Germany), and the insoluble recombinant protein was purified under denaturing conditions. Various fractions were analyzed on 15% SDS-PAGE, and the fractions containing purified protein were pooled. The purified protein was quantified by the method of Lowry et al. (11) with bovine serum albumin (BSA) as a standard.

The purified recombinant 10-kDa protein was resolved on 15% SDS-PAGE and transferred electrophoretically onto a nitrocellulose membrane (25). Blocking of the membrane was done with 5% defatted milk powder in phosphate-buffered saline (PBS) (pH 7.2; 136 mM NaCl, 2.5 mM KH2PO4, 6.9 mM K2HPO4) overnight at 4°C. After washing the membrane with PBS containing 0.05% Tween 20 (PBS-T), the blot was probed with a 1:2,000 dilution of anti-His antibody-HRP conjugate for 1 h at 37°C. The membrane was washed with PBS-T, and the reaction was developed using 3,3'-diaminobenzidine tetrahydrochloride (DAB)-H2O2 as a substrate.

**Diagnostic assays for antibody detection.** All 408 serum samples were tested for brucellosis by RBPT and STAT as briefly described below.

**RBPT.** Equal volumes (20 μl) of RBPT colored antigen and the test serum were mixed on a clean glass slide with the help of a clean sterile toothpick (2). The slide was observed after 1 min for the formation of clumps. The formation of clear clumps indicated a positive test, while the absence of clumps was considered a negative reaction.

**STAT.** Plain antigen of *B. abortus* S99 was used according to the standard protocol (2). Two-fold serial dilutions (1:10 to 1:640) of the sera were prepared in phenol saline (0.8 ml of phenol saline was added to the first tube and 0.5 ml to the remaining tubes; then, 0.2 ml of serum was added to the first tube and mixed, and 0.5 ml was transferred to the next tube; further volumes of 0.5 ml were transferred to subsequent tubes to give a series of double dilutions). 0.5 ml of plain antigen of *B. abortus* S99 was added to each tube. After mixing, all the tubes were incubated at 37°C for 24 h. A titer of 1:40 or above was considered positive for brucellosis.

**Development of p-ELISA.** To determine the optimum antigen concentration and antibody dilution for p-ELISA, a checkerboard titration analysis was done using pooled brucellosis-negative and -positive bovine sera. Standard 96-well polystyrene plates (Polypropnunc, Denmark) were coated with purified r10-kDa protein (50 ng/well) in carbonate-bicarbonate buffer (pH 9.6) overnight at 4°C. Unbound antigen was removed by decanting the plate after incubation. After 2 h of blocking with 1% BSA at 37°C, the plates were washed 3 times with 0.05% Tween 20 in PBS. The plates were washed 5 times, and 5 μg of OPD and 20 μl of H2O2 in 10 ml of phosphate-citrate buffer (pH 5) was used as a substrate for colorimetric reaction. After development of sufficient color, the reaction was stopped by adding 50 μl of 2.5 M H2SO4, and the optical density at 492 nm (OD492) was read. The pooled positive and negative serum samples were used simultaneously in each plate at the time p-ELISA was performed as a positive control and for determination of the ELISA cutoff value, respectively.

**FIG. 1.** SDS-PAGE analysis of 10-kDa protein expression in *E. coli* M15 cells. Lane 1, protein molecular mass marker (kDa); lane 2, uninduced cells; lane 3, IPTG-induced cells; lane 4, purified r10-kDa protein. The arrow on the right indicates the position of the r10-kDa protein.
Cloning and expression of the 10-kDa gene fragment. The gene fragment (282 bp) encoding the immunodominant region of BP26 was amplified by PCR, and the amplicon was cloned into the pQE-30 UA vector. The expression cassettes were transformed into competent E. coli M15 cells for protein expression. The inserted positive clones were subjected to expression studies, and the clone exhibiting the highest level of expression of recombinant protein was selected for production of the recombinant protein. Study of the time course of expression of the recombinant protein revealed maximum expression after 4 h of induction with 1 mM IPTG. SDS-PAGE analysis performed to determine the solubility of the r10-kDa protein revealed that the recombinant protein was insoluble, indicating the formation of inclusion bodies.

Protein purification and Western blotting. Since the r10-kDa protein (with N-terminal 6×His residues) was localized in inclusion bodies, it was solubilized by urea (8 M) and purified using Ni-NTA matrix immobilized metal affinity chromatography (IMAC). Fractions collected during different steps of purification were analyzed by SDS-PAGE. The size of the purified protein on SDS-PAGE was consistent with the predicted molecular mass of the r10-kDa protein (Fig. 1). The authenticity of the r10-kDa protein was verified by Western blot analysis using monoclonal anti-polyhistidine antibodies directed toward the polyhistidine tags in the r10-kDa protein (Fig. 2). Downstream processing of 2 liters of culture yielded ~10 mg of purified r10-kDa protein.

Serological results. Group I serum samples (n = 70) were collected from small herds in which brucellosis was never reported and the animals were healthy. Out of 70 presumptively negative samples, 58 (82.85%) samples were negative by all three tests, while 9 (12.85%), 7 (10%), and 3 (4.28%) samples were positive by RBPT, STAT, and p-ELISA, respectively (Table 1). Of the three samples that tested positive by p-ELISA in the negative group, two were positive by STAT, while all three were positive by RBPT. The RBPT and STAT systems resulted in more false positives than p-ELISA (Table 1). In group I, the specificity of p-ELISA versus RBPT (100%) and STAT (98.41%) was more than the specificity of RBPT versus STAT (95.08%) (Table 2). Out of 308 random serum samples (group II), 105 samples were found to be brucellosis positive by p-ELISA versus 111 and 123 by STAT and RBPT, respectively. However, 15 random samples that were brucellosis negative by both STAT and RBPT tested positive by p-ELISA (Table 1). In group III, 9 and 29 serum samples were positive by RBPT and STAT, while none of them was positive by p-ELISA (Table 1).

**RESULTS**

**TABLE 1. Comparison of RBPT, STAT, and p-ELISA in different combinations**

| Sample group (no.) | RBPT-negative, STAT-negative, p-ELISA-negative | RBPT-negative, STAT-positive, p-ELISA-positive | RBPT-positive, STAT-negative, p-ELISA-negative | RBPT-positive, STAT-positive, p-ELISA-negative | RBPT-negative, STAT-negative, p-ELISA-positive | RBPT-negative, STAT-positive, p-ELISA-positive | RBPT-positive, STAT-negative, p-ELISA-negative | RBPT-positive, STAT-positive, p-ELISA-negative |
|--------------------|-----------------------------------------------|-----------------------------------------------|-----------------------------------------------|-----------------------------------------------|-----------------------------------------------|-----------------------------------------------|-----------------------------------------------|-----------------------------------------------|
| Negative (70)      | 58                                            | 2                                             | 2                                             | 4                                             | 0                                             | 0                                             | 1                                             | 3                                             |
| Random (308)       | 152                                           | 68                                            | 25                                            | 18                                            | 10                                            | 15                                            | 12                                            | 8                                             |
| Vaccinated (30)    | 0                                             | 0                                             | 8                                             | 1                                             | 0                                             | 0                                             | 0                                             | 21                                            |
| Total (408)        | 210                                           | 70                                            | 35                                            | 23                                            | 10                                            | 15                                            | 13                                            | 32                                            |

DISCUSSION

We describe here a truncated 10-kDa protein of *B. abortus* that may be useful for diagnosis of brucellosis. The truncated 10-kDa protein, an immunodominant region of the BP26 protein of *B. abortus*, was cloned in the pQE-30 UA vector and expressed by IPTG induction. The expressed protein was found to be insoluble and was purified by Ni-NTA column chromatography. The purified recombinant protein was used for antibody detection in bovine sera by p-ELISA.

In group I, containing the presumptive brucellosis-negative samples, 9 (12.85%), 7 (10%), and 3 (4.28%) samples tested positive by RBPT, STAT, and p-ELISA, respectively (Table 1). The maximum number of samples that tested negative by p-ELISA in this group indicates that p-ELISA is more specific than RBPT and STAT. Out of 308 random serum samples (group II), 105 samples were found to be brucellosis positive by p-ELISA versus 111 and 123 by STAT and RBPT, respectively. Fifteen samples that tested negative by both STAT and RBPT tested positive by p-ELISA (Table 1), which is indicative of the higher sensitivity of p-ELISA. However, due to the lack of culture-positive serum samples in the present investigation, we have not described the sensitivity of p-ELISA. Further study...
with culture-proven cases and clinical correlation would be relevant for justification of higher sensitivity of p-ELISA.

All 30 vaccinated samples (group III) were reported to be brucellosis negative by p-ELISA, while RBPT and STAT resulted in 9 (30%) and 29 (96.6%) false-positive cases, respectively (Table 1). STAT was clearly unable to distinguish between B. abortus strain 19-vaccinated animals and naturally infected animals. The retention of seroagglutination titers in a significant number of cattle vaccinated with strain S19 creates a diagnostic problem of differentiation between vaccinal and infection titers (12, 15). RBPT utilizes B. abortus whole cells as the antigen and detects mainly IgG antibodies (14). Cernyseva et al. reported that IgG1 antibody plays an active role in RBPT, while IgM and IgG2 can be detected by STAT (4). The S19 vaccine induces antibody response to the O antigens and can be best used as a screening tool (22). The negativity of STAT is associated with the ability to produce and maintain a STAT antigen reagent with relatively stable sensitivity, to interpret the agglutination reaction correctly, and to reproduce the results. Further, STAT and RBPT are not suitable for differentiating Brucella-infected and -vaccinated animals. The results of this study indicate that 10-kDa recombinant protein in p-ELISA format may prove to be a good reagent for diagnosis of bovine brucellosis; however, further investigation is required, with bacteriological and clinical correlation. The recombinant 10-kDa protein is also a specific antigen and can be used to distinguish naturally infected bovines from vaccinated ones. The r10-kDa protein, as a genus-specific protein, also shares ~99% identity with its counterpart in B. melitensis; therefore, the test reagent may be useful for diagnosis of human brucellosis, but this needs to be further investigated.

**REFERENCES**

1. Afzal, M., A. Mirza, and M. Jahanmir. 2000. Immune response of buffaloes to vaccination with Brucella abortus strain 19. Rev. Sci. Tech. Off. Int. Epiz. 19:367–370.
2. Alton, G. G., L. M. Jones, and D. E. Pietz. 1975. Laboratory techniques in brucellosis. Monogr. Ser. World Health Organ. 58:1–163.
3. Bagues, M. P. J., C. M. Marin, J. M. Blasco, I. Morion, and C. Gamazo. 1992. An ELISA with Brucella lipopolysaccharides 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. 20:233–241.
4. Cernyseva, M. I., E. N. Knjazeva, and S. Egorova. 1977. Study of the plate agglutination test with rose bengal antigen for the diagnosis of human brucellosis. Bull. World Health Organ. 55:669–674.
5. Ciuchi, F., R. Adone, and P. Pasquale. 2002. Coombs antiglobulin test using Brucella abortus 99 as antigen to detect incomplete antibodies induced by B. abortus RB51 vaccine in cattle. Clin. Diagn. Lab. Immunol. 9:1398–1399.
6. Cloeckaert, A., et al. 1996. Cloning, nucleotide sequence, and expression of the Brucella melitensis bg26 gene coding for a protein immunogenic in infected sheep. FEMS Microbiol. Lett. 140:139–144.
7. Corbel, M. J. 1997. Brucellosis: an overview. Emerg. Infect. Dis. 3:213–221.
8. Corbel, M. J., F. A. Stuart, and R. A. Brewer. 1984. Observation of serological cross-reaction between smooth Brucella species and organisms of other genera. Dev. Biol. Stand. 56:341–363.
9. Ihan, Z., et al. 2008. Comparison of culture and PCR for the detection of Brucella melitensis in blood and lymphoid tissues of serologically positive and negative slaughtered sheep. Lett. Appl. Microbiol. 46:301–306.
10. Lindler, L. E., et al. 1996. Cloning of a Brucella melitensis group 3 antigen gene encoding Omp28, a protein recognized by the humoral immune response during human brucellosis. Infect. Immun. 64:2490–2494.
11. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with folin phenol reagent. J. Biol. Chem. 193:265–275.
12. MacMillan, A., W. I. Greiser, V. Moening, and L. Mathias. 1990. A competitive enzyme immunoassay for brucellosis diagnosis. Dtsch. Tierarztl. Wochenschr. 97:85–89.
13. Memish, Z. A., M. Almuneef, and M. W. Mah. 2000. Comparison of the Brucella standard agglutination test with the ELISA IgG and IgM in patients with Brucella bacteremia. Diagn. Microbiol. Infect. Dis. 40:129–132.
14. Nielsen, K. 2002. Diagnosis of brucellosis by serology. Vet. Microbiol. 90:447–459.
15. Nielsen, K., J. W. Chorwngonrodzky, J. R. Duncan, and D. R. Bundle. 1989. Enzyme-linked immunosorbent assay for differentiation of antibody re-

**TABLE 2. Statistical comparison of RBPT, STAT, and p-ELISA**

| Parameter* | Negative samples (n = 70) | Random samples (n = 308) |
|------------|--------------------------|-------------------------|
|            | RBPT vs STAT             | RBPT vs p-ELISA         |
|            | RBPT vs STAT             | RBPT vs p-ELISA         |
|            | RBPT vs STAT             | RBPT vs p-ELISA         |
|            | RBPT vs STAT             | RBPT vs p-ELISA         |
|            | RBPT vs STAT             | RBPT vs p-ELISA         |
| Agreement (%) | 88.57 91.43            | 91.43 84.42 77.92      |
| Kappa value   | 0.4366 0.4656          | 0.3617 0.6697 0.5282    |
| PPV (%)      | 57.14 100               | 66.66 83.78 76.19      |
| NPV (%)      | 92.06 91.04             | 92.53 84.77 78.81      |
| Sensitivity (%) | 44.44 33               | 28.57 75.6 65.04      |
| Specificity (%) | 95.08 100              | 98.41 90.27 86.48     |

* PPV, positive predictive value; NPV, negative predictive value.

*pp-ELISA, to interpret the agglutination reaction correctly, and to maintain a STAT antigen reagent with relatively stable sensitivity, to interpret the agglutination reaction correctly, and to reproduce the results. Further, STAT and RBPT are not suitable for differentiating Brucella-infected and -vaccinated animals. The results of this study indicate that 10-kDa recombinant protein in p-ELISA format may prove to be a good reagent for diagnosis of bovine brucellosis; however, further investigation is required, with bacteriological and clinical correlation. The recombinant 10-kDa protein is also a specific antigen and can be used to distinguish naturally infected bovines from vaccinated ones. The r10-kDa protein, as a genus-specific protein, also shares ~99% identity with its counterpart in B. melitensis; therefore, the test reagent may be useful for diagnosis of human brucellosis, but this needs to be further investigated.
sponse of cattle naturally infected with *Brucella abortus* or vaccinated with strain 19. Am. J. Vet. Res. 59:5–9.

16. Perry, M. B., and R. D. Bundle. 1990. Antigenic relationship of the lipopolysaccharides of *Escherichia hermannii* strains with those of *Escherichia coli* O157:H7, *Brucella melitensis* and *Brucella abortus*. J. Infect. Immun. 58:1391–1395.

17. Renukaradhya, G. J., S. Isloor, and M. Rajasekhar. 2002. Epidemiology, zoonotic aspects, vaccination and control/eradication of brucellosis in India. Vet. Microbiol. 90:183–195.

18. Rossetti, O. L., A. I. Arrese, M. L. Boschiroli, and S. L. Cravero. 1996. Cloning of *Brucella abortus* gene and characterization of expressed 26-kilodalton periplasmic protein: potential use for diagnosis. J. Clin. Microbiol. 34:165–169.

19. Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

20. Schoerner, K., K. Waterberg, and M. Rollinhoff. 1990. Differentiation of serological response of *Yersinia enterocolitica* serotype O9 and *Brucella* species by immunoblot or enzyme-linked immunosorbent assay using whole bacteria and *Yersinia* outer membrane proteins. J. Clin. Microbiol. 28:1570–1574.

21. Seco-Mediavilla, P., et al. 2003. Epitope mapping of *Brucella melitensis* BP26 immunogenic protein: usefulness for diagnosis of sheep brucellosis. Clin. Diagn. Lab. Immunol. 10:647–651.

22. Serra, J., and M. Vinas. 2004. Laboratory diagnosis of brucellosis in a rural endemic area in northeastern Spain. Int. Microbiol. 7:53–58.

23. Stevens, M. G., S. G. Hennager, S. C. Olsen, and N. F. Cheville. 1994. Serologic response in diagnostic tests for brucellosis in cattle vaccinated with *Brucella abortus* 19 or RB51. J. Clin. Microbiol. 32:1065–1066.

24. Sutherland, S. S., and J. Searson. 1990. The immune response to *Brucella abortus*: the humoral immune response, p. 65–81. In K. Nielsen and J. R. Duncan (ed.). Animal brucellosis. CRC Press, Inc., Boca Raton, Fla.

25. Towbin, H., T. Stanelin, and J. Gordon. 1979. Electrophoretic transfer of protein from polyacrylamide to nitrocellulose sheets—procedure and some applications. Proc. Natl. Acad. Sci. U. S. A. 76:4350–4354.

26. Wellingshausen, N., et al. 2006. Rapid detection of *Brucella* spp. in blood cultures by fluorescence in situ hybridization. J. Clin. Microbiol. 44:1828–1830.

27. Yagupsky, P. 1999. Detection of brucellae in blood cultures. J. Clin. Microbiol. 37:3437–3442.

28. Yagupsky, P., N. Peled, K. Riesenbey, and M. Sanai. 2000. Exposure of hospital personnel to *Brucella melitensis* and occurrence of lab acquired disease in an endemic area. Scand. J. Infect. Dis. 32:31–35.

29. Young, E. J. 1983. Human brucellosis. Rev. Infect. Dis. 5:821–842.