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Single-Step PCR for Detection of *Brucella* spp. from Blood and Milk of Infected Animals

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A versatile method for the extraction of *Brucella* DNA and PCR are presented as reliable tools for the detection of *Brucella* spp. from body fluids of infected animals. Two oligonucleotides homologous to regions of the gene encoding for an outer membrane protein (*omp-2*) were designed to detect the pathogen from milk and/or blood of infected goats, bovines, and human patients. The sensitivity of our test and its ability to detect the pathogen in samples from the field reveal a promising advance in the diagnosis of brucellosis in animals and humans.

Brucellosis continues to be of great health significance and economic importance in many countries. It is caused by gram-negative bacteria of the genus *Brucella*. *Brucella melitensis* is the leading cause of brucellosis in goats in Mexico (3), and it is the most important causal agent of brucellosis in humans (20).

Infections in animals caused by *Brucella* spp. frequently result in abortions and diminished levels of milk production. Once the acute period of the disease is over, animals may present with little or no disease symptoms, and *Brucella* cells can chronically be located in the supramammary lymphatic nodes and mammary glands of 80% of infected animals; thus, animals continue to secrete the pathogen in their body fluids (4, 6, 25, 26, 28). In Mexico humans often consume milk in the form of raw milk and cheese; therefore the pathogen could be easily acquired by consumers. Therefore, there is no doubt that control of this disease in animals will have an immediate effect on the incidence of this disease in humans.

The diagnosis of brucellosis is currently based on serological and microbiological tests. It is well known that serological methods are not always sensitive or specific (7, 14, 27). Moreover, they have repeatedly been reported to cross-react with antigens other than those from *Brucella* spp. (7, 16, 27). Microbiological isolation and identification are the most reliable methods of diagnosing for brucellosis. However, these procedures are not always successful, are cumbersome, and represent a great risk of infection for laboratory technicians (20).

PCR (29) provides a promising option for the diagnosis of brucellosis. It is a potentially useful method that has been used alone or in combination with labeled probes for the detection of *Brucella* spp. from isolated bacteria (10, 15, 17) or highly contaminated aborted tissues (9). We have previously reported the first development of a sensitive diagnostic PCR test for the detection of *Brucella abortus* in naturally infected bovines (23). Here we report a reliable, highly sensitive, and specific single-step PCR test for the detection of *Brucella* spp. in body fluids obtained from infected livestock and human patients. The report also describes a simplified method of extracting *Brucella* DNA from milk and blood samples. These procedures should prove to be useful for the early detection of *Brucella* infections in animals and should provide a basis for the development of more effective procedures for early detection of brucellosis in humans.

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MATERIALS AND METHODS

Biological samples. (i) Positive samples. Reference *Brucella* strains were kindly donated by recognized laboratories (Table 1). Goats artificially inoculated or naturally infected with *B. melitensis* were used in the study. The animals were positive by the card and complement fixation tests at a 1:64 dilution and were later confirmed to be positive by microbiological isolation and identification of *B. melitensis* biovar 1. Blood and milk from those animals were taken and processed as described below. In addition to goats, samples from naturally infected bovines and human patients were also obtained.

(ii) Negative samples. The bacterial strains used as negative controls were selected either because they were closely related to the genus *Brucella* or because of their reported cross-reactivity in serological tests (Table 1). Also included as negative controls were DNAs extracted from human, goat, and bovine blood or milk samples from individuals who were nonvaccinated, apparently never exposed to *Brucella* spp., and, according to clinical, serological, and microbiological tests, free of *Brucella* infection.

Serological testing. Nine milliliters of blood from goats, bovines, and humans infected with *Brucella* spp. was obtained by jugular or vein puncture and was dispensed in 3-ml aliquots; 3 ml was not treated with anticoagulants such as heparin or sodium citrate and was used to obtain sera to perform the card and complement fixation tests as described elsewhere (1).

Microbiological analysis. Three milliliters of the dispensed blood containing heparin or sodium citrate as the anticoagulant were inoculated onto Ruiz-Castañeda biphasic medium as described elsewhere (28). Blood cultures were incubated in the vertical position at 37°C. Aliquots were plated onto standard *Brucella* agar weekly during a 3-week period. Samples from the fatty upper surface layer of milk samples were also plated onto antibiotic-supplemented *Brucella* agar, and the plates were incubated at 37°C under aerobiosic and vellobiotic conditions (8). Suspect *Brucella* colonies were identified by conventional biochemical and serological methods (1).

DNA extraction from blood samples. Heparin or sodium citrate was added to 3 ml of each of the blood samples that were obtained, and the DNA was extracted as follows. Four hundred microliters of the sample were taken and centrifuged at 4,000 × *g* for 3 min. The cell pellets were resuspended in 1 ml of erythrocyte lysis solution (155 mM NH_4Cl, 10 mM $NaHCO_3$, 100 mM disodium EDTA [pH 7.4]), mixed, and centrifuged as described above. Treatment with

TABLE 1. Species and biovars used in the study

Strain	Species and biovar	Origin[a]	Source	Country	PCR result
544	*B. abortus* 1	CVL	Reference	United Kingdom	+
99S	*B. abortus* 1	CVL	Reference	United Kingdom	+
S19	*B. abortus* 1	CVL	Vaccine strain	United Kingdom	+
RB51	*B. abortus* 1	VMRC	Rough mutant	United States	+
86/8/59	*B. abortus* 2	CVL	Reference	United Kingdom	+
Tulya	*B. abortus* 3	CVL	Reference	United Kingdom	+
292	*B. abortus* 4	CVL	Reference	United Kingdom	+
B3/96	*B. abortus* 5	CVL	Reference	United Kingdom	+
870	*B. abortus* 6	CVL	Reference	United Kingdom	+
63/75	*B. abortus* 7	CVL	Reference	United Kingdom	+
C68	*B. abortus* 9	CVL	Reference	United Kingdom	+
16M	*B. melitensis* 1	CVL	Reference	United Kingdom	+
Rev 1	*B. melitensis* 1	CVL	Vaccine strain	United Kingdom	+
63/9	*B. melitensis* 2	CVL	Reference	United Kingdom	+
Ether	*B. melitensis* 3	CVL	Reference	United Kingdom	+
1330	*B. suis* 1	CVL	Reference	United Kingdom	+
S2 China	*B. suis* 1	CVL	Vaccine strain	United Kingdom	+
Thompsen 1	*B. suis* 2	CVL	Reference	United Kingdom	−
686	*B. suis* 3	CVL	Reference	United Kingdom	−
40/67	*B. suis* 4	CVL	Reference	United Kingdom	−
ERt80	*B. suis* 5	CVL	Reference	United Kingdom	+
RM6/66	*B. canis*	CVL	Reference	United Kingdom	−
63/290	*B. ovis*	CVL	Reference	United Kingdom	−
NZ-P2037	*R. loti*	CFN	Reference	Mexico	−
	A. tumefaciens	CFN	Reference	Mexico	−
	Y. enterocolitica O:9	INIFAP	Reference	Mexico	−
	Y. enterocolitica O:3	INIFAP	Reference	Mexico	−
	V. cholerae O1	INDRE	Reference	Mexico	−
	O. anthropi	INIFAP	Field strain	Spain	−
	Noninfected goat				−
	Noninfected bovine				−
	Noninfected human				−

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erythrocyte lysis solution was repeated until the leukocyte pellets lost all reddish coloring.

Template DNA was obtained from either leukocyte pellets harboring *Brucella* spp. obtained from 400 μl of broth, isolated colonies, or 400-μl samples from the fatty top layer of raw milk, as follows. Four hundred microliters of lysis solution (2% Triton X-100, 1% sodium dodecyl sulfate, 100 mM NaCl, 10 mM Tris-HCl [pH 8.0]) and 10 μl of proteinase K (10 mg/ml) were added to the samples, and the contents were mixed thoroughly and incubated for 30 min at 50°C. Four hundred microliters of saturated phenol (liquid phenol containing 0.1% 8-hydroxyquinoline, saturated, and stabilized with 100 mM Tris-HCl [pH 8.0] and 0.2% 2-mercaptoethanol) (30) was added, and the contents were mixed thoroughly and centrifuged at 8,000 × *g* for 5 min. The aqueous layer was transferred to a fresh tube, and an equal volume of chloroform-isoamyl alcohol (24:1) was added; the tubes were mixed thoroughly and centrifuged at 8,000 × *g* for 5 min. The upper layer was again transferred to a fresh tube, and 200 μl of 7.5 M ammonium acetate was added and mixed thoroughly. Samples were kept on ice for 10 min and centrifuged at 8,000 × *g* for 5 min, and the aqueous content was transferred to a fresh tube. Two volumes of 95% ethanol were added, the contents were mixed, and the tubes were stored at −20°C.

DNA was recovered by centrifuging the samples at 8,000 × *g* for 5 min, the pellets were rinsed with 1 ml of 70% ethanol, dried, and resuspended in 20 μl of TE buffer (10 mM Tris-HCl [pH 8.0], 1 mM disodium EDTA). DNA concentrations were determined by measuring their A_{260}, and the samples were stored at −20°C until they were processed. RNase treatment of the samples was found to be unnecessary.

Synthetic oligonucleotide design. The nucleotide sequence of the gene coding for the outer membrane protein *omp-2* reported for *B. abortus* (12) was obtained from the GenBank database located at the National Center for Biotechnology Information of the National Library of Medicine (Bethesda, Md.). Oligonucleotides were designed in our laboratory and were synthesized by Bio-Synthesis, Inc. (Lewisville, Tex.). The forward primer (JPF) sequence is 5′-GCGCTCA GGCTGCCGACGCAA-3′, and the reverse primer (JPR) sequence is 5′-AC CAGCCATTGCGGTCGGTA-3′. Primers described elsewhere to be specific for IS*711* and *B. abortus* or *B. melitensis* were also used in the present study by following original procedures (5).

PCRs. PCR was performed in a total volume of 25 or 50 μl with 200 ng (or less) of DNA, 50 pmol of each primer, 50 mM KCl, 10 mM Tris-HCl (pH 9.0), 0.1% Triton X-100, 3 mM $MgCl_2$, 200 μM (each) the four nucleoside triphosphates (dNTPs), and 2.5 U of *Taq* polymerase (GIBCO BRL, Inc., Gaithersburg, Md., or Perkin-Elmer Cetus Co., Norwalk, Conn.). To prevent evaporation, the reaction mixture was covered with a layer of 50 μl of mineral oil. The reaction was performed in a DNA thermal cycler (Perkin-Elmer) or a PTC 150 instrument (MJ Research, Inc., Watertown, Mass.) at a denaturation temperature of 94°C for 4 min; this was followed by 35 cycles at 94°C for 60 s, 60°C for 60 s, and 72°C for 60 s and one final extension at 72°C for 3 min. PCR assays with primers designed elsewhere (5) were performed by using previously described conditions.

Eight microliters of the amplification reaction mixture was taken and fractionated in a 1.5% agarose (or 8% polyacrylamide) gel containing 1× TBE (100 mM Tris-HCl [pH 8.0], 90 mM boric acid, 1 mM disodium EDTA), stained with an ethidium bromide solution (0.5 μg/ml), and visualized under UV light (30).

Computer analysis of DNA. DNA sequence comparisons and alignments were done by using the GenBank version 84.0 database at the National Center for Biotechnology Information by using BLAST, version 1.3 (Basic Local Alignment Tool) (2). Computer analysis was performed and restriction maps were made by using the computer program DNA Strider, version 1.01 (21).

RESULTS AND DISCUSSION

From the DNA sequences obtained from the GenBank database, we chose the gene that encodes for an external membrane protein (*omp-2*) reported for *B. abortus*. This gene has a duplication (in a head-to-head array) of the open reading frame which is 85% homologous for this species (11–13). We took advantage of this peculiar genomic organization, which is uncommon among prokaryotes, by designing primers for two homologous regions present in both copies; thus, when the DNA sequences at the priming areas are conserved enough,

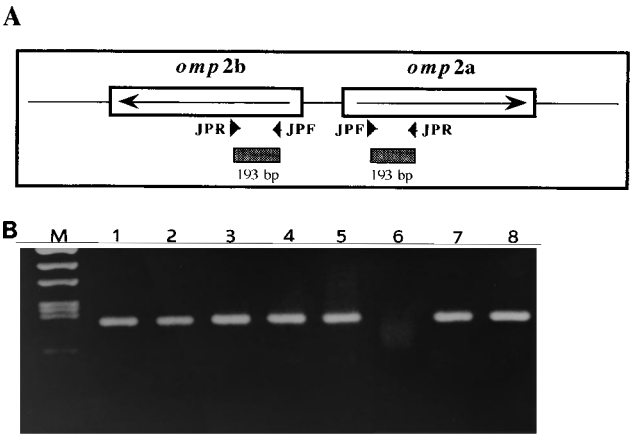

FIG. 1. Amplification map of the *omp-2* gene with primers JPF, JPR, and *Brucella* DNA. (A) Schematic representation of the two copies of the *omp-2* gene reported for *B. abortus*. The arrows indicate the orientations of both copies (*omp-2a* and *omp-2b*). Triangles represent the positions of primers JPF and JPR. Filled boxes represent the amplified fragments of 193 bp. (B) Representative PCR amplification products (for a complete list of the results, see Table 1) by using the primers described above and DNAs from *B. melitensis* rev 1, vaccine strain (lane 1), *B. melitensis* biovar 1, 16M (lane 2), *B. melitensis* biovar 2, 63/9 (lane 3), *B. melitensis* biovar 3, Ether (lane 4), *B. abortus* biovar 1, 99S (lane 5), *O. anthropi* (lane 6), *Brucella*-contaminated milk from naturally infected goats (lane 7), and blood from a goat infected with *B. melitensis* biovar 1 (lane 8). Lane M, pBR322 digested with *Alu*I.

the PCR should amplify two times the normal amount of DNA (Fig. 1A).

As predicted, primers JPF and JPR allowed (at annealing temperatures ranging from 50 to 63°C; optimum, 60°C) the amplification of an 193-bp fragment from all reference, vaccine, and field strains of *B. melitensis* tested in the present study (Fig. 1; Table 1). Our test proved to be useful for amplifying DNAs from many other *Brucella* species and biovars, excluding *Brucella suis* biovars 2, 3, and 4, *Brucella* canis, and *Brucella ovis*, which can then be detected by using a third primer that is not discussed here (19). *Brucella neotomae* was not included in the study. No amplification signal was detected when DNAs extracted from *Escherichia coli*, *Vibrio cholerae* O1, or *Yersinia enterocolitica* O:3 and O:9 or the closely related bacteria *Rhizobium loti*, *Agrobacterium tumefaciens*, and *Ochrobactrum anthropi* (Table 1) were used as templates for the PCR assay.

In order to confirm the identities of the amplified fragments, restriction analysis was carried out on the basis of the restriction map for the *omp-2* gene of *B. abortus* (12). However, definitive confirmation was obtained by sequencing amplified products, which matched perfectly the DNA sequence reported by Ficht and collaborators in 1989 (12).

Sequential dilutions of purified genomic DNA from *B. abortus* cultures showed clear amplifications with as little as 2.5×10^{-11} μg of template per reaction mixture. The sensitivity of this test was also studied by using serial dilutions of *B. melitensis* and *B. abortus* in uncontaminated milk from which DNA was later isolated by the method described here. Our primers and PCR assay were highly sensitive (detecting fewer than 10 cells in 1 ml of milk) and versatile under a variety of protocol conditions, namely, different annealing temperatures and different primer and $MgCl_2$ concentrations. We also tested a series of *Brucella*-specific primers that have been reported elsewhere (5) in PCRs with the same DNA extracted from our sequential dilutions in milk, which yielded negative results in every case. Use of these primers was successful only when PCR conditions were strictly the same as those described in a previous research report (5). The sensitivity of the test with bacteria diluted in blood was not attempted because *Brucella* cells are intracellular parasites in leukocytes; therefore, any result from these kinds of experiments could not be of practical value.

TABLE 2. Detection of *Brucella* spp. by different detection methods

Organism	No. of samples			No. of samples detected by PCR/total no. of samples	
	Total	Detected by serology	Recovered by isolation	Milk	Blood
Goat group					
I	22	14	1	11/17	19
II	15	0	0	NT[a]	2
III	3	3	3	3	3
Experimentally infected goats	3	3	3	3	3
Human patients	3	3	3	NT	3

[a] NT, not tested.

Our PCR assay with DNA (extracted by the method described here) from blood and milk from both naturally and artificially infected animals yielded the same amplified fragments of 193 bp as the ones from *Brucella* colonies isolated from the corresponding infected samples (Fig. 1B, lanes 7 and 8), whereas DNAs extracted from the blood of healthy bovines, goats, and humans, as well as from the milk of uninfected goats and bovines, failed to serve as templates in the PCRs (data not shown). These results indicate the specificity of our DNA extraction and PCR protocol for the selective detection of *Brucella* infections from body fluids in animals and humans. The lack of amplification when DNAs extracted from *E. coli*, *V. cholerae* O1, and *Y. enterocolitica* O:3 and O:9 or from the closely related bacteria *R. loti*, *A. tumefaciens*, and *O. anthropi* (Table 1) were used as templates supports these findings.

The DNA extraction method described here has been useful for determining the incidence of natural *Brucella* infection in diverse groups of animals, namely, goats, bovines, and humans. The results of *Brucella* detection from goats and humans by different methods are given in Table 2. From a group of 22 female creole goats, among which 14 tested positive by the serological Rose Bengal test, it was possible to determine by PCR that 19 of them had a *Brucella* sp. in their blood. In contrast, a *Brucella* sp. was isolated from only one of the blood samples. From the same group of goats, 11 of 17 milking goats were determined, by PCR, to be secreting the bacteria in their milk (24). In a different group, 15 goats tested negative for *Brucella* spp. by serological methods, while our PCR assay detected *Brucella* spp. in 2 of them (22). Another group of three more goats known to be naturally infected, by means of serology (Rose Bengal test, complement fixation, gel diffusion) and bacterial isolation, were confirmed to be *Brucella* carriers by the PCR described here. Moreover, three goats that were experimentally infected with *B. melitensis* 16M were all positive by serological, microbiological, and PCR assays (Table 2). In this latter case our single-step PCR allowed for the earliest detection of *Brucella* spp. of all the procedures used on the 10th day postinoculation, on average; the Rose Bengal test yielded positive results on the 18th day postinoculation, on average (19). In every PCR experiment, DNAs extracted from uninfected organisms were used as negative controls.

Ultimately, our DNA extraction and PCR procedures were useful for detecting the presence of the pathogen in three *Brucella*-infected human patients (Table 2). This finding was confirmed by a clinical diagnosis supported by serological and microbiological analyses.

Extraction of DNA from body fluids is cumbersome, mainly because of the presence of PCR inhibitors in samples taken from those liquids. Extraction procedures for *Brucella* detection are further complicated because it is an intracellular pathogen. The protocols presented here overcome these problems, making detection by PCR more applicable for brucellosis, and might also help with the detection of pathogens with similar characteristics.

It is clearly important not only to detect but also to identify the species of *Brucella* implicated in natural infections. Our laboratory has developed a method that can be used to easily distinguish *B. abortus* biovar 1 from the rest of the *B. abortus* biovars (18), and we continue working on the development of a PCR assay that would enable us to differentiate between biovars of *Brucella* species. Here, we tested a series of primers that have been reported as being useful for discriminating between *B. melitensis* and some biovars of the other clinically important *Brucella* species (5). These primers were successfully used to detect *B. melitensis* from our infected animals only when DNA was extracted by the method described here.

Since brucellosis is a zoonosis, the fight against this disease in humans and animals relies mainly on veterinary sanitation measures focused on the reduction or eradication of this disease in farm animals. A critical tool for the success of these measures is, without a doubt, an accurate and early diagnosis of the disease. The present research has compared the classical methods (serological and microbiological methods) with PCR and demonstrated the superiority of the latter technique for detecting small amounts of the pathogen in body fluids of infected organisms. Here we have demonstrated that PCR is reliable, highly sensitive, and specific for use in the accurate detection of *Brucella* spp. Our work is also the first to report a PCR that has proved to be efficient in detecting the presence of *Brucella* spp. in body fluids, namely, blood and milk from naturally infected animals. The results provide a strong basis for the tentative early diagnosis of brucellosis in humans.

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