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Specific Detection of *Brucella* DNA by PCR

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A PCR assay with primers derived from the 16S rRNA sequence of *Brucella abortus* was developed. Nine different combinations between six primers were tested. One pair of primers, which amplified a 905-bp fragment, was selected. As little as 80 fg of *Brucella* DNA was detected by this method. DNAs from all of the representative strains of the species and biovars of *Brucella* and from 23 different *Brucella* isolates were analyzed and yielded exclusively the 905-bp fragment. No amplification was detected with DNAs from 10 strains phylogenetically related to *Brucella* spp., 5 gram-negative bacteria showing serological cross-reactions with *Brucella* spp., and 36 different clinical isolates of non-*Brucella* species. Only *Ochrobactrum anthropi* biotype D yielded a PCR product of 905 bp, suggesting a closer relationship between *Brucella* spp. and *O. anthropi* biotype D. The specificity and high sensitivity of the PCR assay may provide a valuable tool for the diagnosis of brucellosis.

The members of the genus *Brucella* are gram-negative aerobic bacteria which multiply within macrophages and cause infections in animals and humans. Six nomen species (*B. abortus*, *B. melitensis*, *B. suis*, *B. ovis*, *B. canis*, and *B. neotomae*) are recognized; it has been proposed that *B. melitensis* be the genomic species (15). On the basis of 16S rRNA sequence comparisons, the genus *Brucella* has been found to be a member of the alpha-2 subdivision of the class *Proteobacteria*, closely related to *Rochalimaea*, *Rhizobium*, and *Agrobacterium* spp. (11, 12).

Brucellosis causes great economic losses and human suffering. Currently, the diagnosis of this zoonosis is based on microbiological and serological laboratory tests. Although the most specific diagnostic test is isolation of the causative organism, long incubation periods are necessary, and cultures are not always positive when other tests are positive. Thus, the use of serological methods is recommended as a means to obtain indirect proof of the diagnosis. However, the presence of antibodies does not always mean an active case of brucellosis: humans from areas where brucellosis is endemic often show weak serological responses, and vaccinated animals may yield false-positive results, and other gram-negative bacteria may cross-react with smooth *Brucella* spp. (3, 5).

Amplification of DNA by PCR is currently used for the diagnoses of several infectious diseases caused by fastidious or slowly growing bacteria. Although previous studies have demonstrated that PCR can be used to detect *Brucella* DNA (1, 7–9), a more extensive evaluation with a wide range of bacteria is necessary. This report describes a highly sensitive PCR method for the detection of *Brucella* spp., using a genus-specific primer pair derived from the 16S rRNA sequence of *B. abortus*. By testing all of the biovars and species of *Brucella* and a series of clinical and field isolates of *Brucella*, we have demonstrated the high specificity of this PCR assay. We also studied a large number of other genera, including bacteria phylogenetically or serologically related to *Brucella* spp. and including other microorganisms that, like *Brucella* spp., can produce bacteremia in humans or are intracellular pathogens.

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MATERIALS AND METHODS

Bacterial strains and growth conditions. The strains used in this study are listed in Table 1. These strains include the reference strains of all of the species and biotypes of *Brucella*, strains from four genera belonging to the alpha-2 subdivision of the class *Proteobacteria*, and five gram-negative bacteria that are known to cross-react serologically with *Brucella* spp. (3). In addition, a total of 59 clinical and field isolates were included. Clinical samples were obtained from different hospitalized patients in a university clinic, and field isolates were collected from infected animals (Table 2). Isolates were identified by standard methods. *Brucella* strains were grown on tryptic soy agar at 37°C for 48 h. *Agrobacterium*, *Phyllobacterium*, *Alcaligenes*, and *Ochrobactrum* strains were grown on the same medium at 26°C. Other bacteria were grown on nutrient agar at 37°C for 24 h, with the exception of those with specific growth requirements. The *Mycobacterium* sp. was grown on Löwenstein-Jensen medium and incubated at 37°C until growth became clearly visible. *Clostridium* and *Propionibacterium* spp. needed an anaerobic atmosphere, and *Neisseria meningitidis* and *Haemophilus influenzae* were cultured on chocolate agar with 10% CO_2.

Extraction of genomic DNA from bacterial cultures. A modification of the method described by Wilson (16) was used. Briefly, cells were resuspended in 0.5 ml of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0), heat killed at 80°C for 15 min, and incubated at 37°C for 1 h with 0.5% sodium dodecyl sulfate and proteinase K (200 μg/ml). Cell wall debris, denatured proteins, and polysaccharides were removed by precipitation with 5 M NaCl and CTAB-NaCl solution and incubated at 65°C for 10 min. DNA was extracted by a standard protocol with phenol-chloroform-isoamyl alcohol, precipitated with isopropanol, washed with 70% ethanol, and dried (13). The pellet of DNA was redissolved in 50 to 100 μl of distilled sterile water. The concentration and purity of the DNA were determined spectrophotometrically by readings of A_{260}, A_{280}, and A_{310}. Samples were diluted up to 0.08 μg/μl and stored at −20°C. Negative controls with distilled water instead of cells were treated exactly as the samples were.

Primer design. The 16S rRNA sequences of *B. abortus* (EMBL accession number X13695), *Rochalimaea quintana* (M11927 and M73228), *Rochalimaea vinsonii* (L01259 and M73230), *Rochalimaea saintelizabethsina* (L01260), *Bradyrhizobium japonicum* (S46916), *Agrobacterium tumefaciens* (M11223), *Rhodopseudomonas globiformis* (M59066), and *Rhodopseudomonas palustris* (M59068) were aligned and compared, allowing the design of six oligonucleotides potentially specific for *Brucella* spp. (Table 3). The oligonucleotides were synthesized on a model 391 DNA synthesizer (Applied Biosystems, Inc.). Nine different combinations between the forward (F1, F3, and F4) and the reverse (R1, R2, and R3) primers were tested. The expected sizes of the amplification products were as follows: F1-R1, 803 bp; F1-R2, 820 bp; F1-R3, 833 bp; F3-R1, 844 bp; F3-R2, 861 bp; F3-R3, 874 bp; F4-R1, 888 bp; F4-R2, 905 bp; and F4-R3, 918 bp.

DNA amplification by PCR. Amplification reaction mixtures for the pair F4-R2 were prepared in a volume of 25 μl containing 50 mM KCl–10 mM Tris-HCl (pH 9.0)–0.10% Triton X-100 (1× reaction buffer; Promega Biotec), 200 μM each deoxynucleoside triphosphate, 0.5 μM each primer, 0.5 U of *Taq* DNA polymerase (Promega Biotec), and 1 μl of template DNA (80 ng). The magnesium concentration was optimized at 1 mM $MgCl_2$ (see Results). The mixture was overlaid with 50 μl of mineral oil (Sigma Chemical Co.), and the amplification was performed in a Gene ATAQ Controller System (Pharmacia Biotech, S.A.). Negative controls containing all of the reagents but lacking template DNA were routinely processed exactly as described above to monitor

TABLE 1. PCR results with DNAs from *Brucella* strains and from bacteria phylogenetically or serologically related to *Brucella* spp.

Bacterium	Strain	Origin[a]	PCR
Brucella abortus biovar 1	2308	NADC	+
Brucella abortus biovar 1	RB51	G. Schuring	+
Brucella abortus biovar 2	86/8/59	INRA	+
Brucella abortus biovar 3	Tulya	INRA	+
Brucella abortus biovar 4	292	INRA	+
Brucella abortus biovar 5	B3196	INRA	+
Brucella abortus biovar 6	870	INRA	+
Brucella abortus biovar 9	87/46	INRA	+
Brucella melitensis biovar 1	16M	CVL	+
Brucella melitensis biovar 2	63/9	INRA	+
Brucella melitensis biovar 3	Ether	INRA	+
Brucella suis biovar 1	1330	INRA	+
Brucella suis biovar 2	Thomsen	INRA	+
Brucella suis biovar 3	686	INRA	+
Brucella suis biovar 4	40	INRA	+
Brucella suis biovar 5	513	INRA	+
Brucella ovis	Bow-CO_2^-	INRA	+
Brucella neotomae	56239	INRA	+
Brucella canis		INRA	+
Agrobacterium tumefaciens[b]	10854	ICPB	−
Agrobacterium rhizogenes[b]	020-AP	IVIA	−
Agrobacterium vitis[b]	565-5	IVIA	−
Phyllobacterium sp.[b]	8225	LMG	−
Phyllobacterium myrsinacearum[b]	$2t_2$	LMG	−
Phyllobacterium rubiacearum[b]	$1t_1$	LMG	−
Ochrobactrum anthropi biotype A[b,c]	3331	LMG	−
Ochrobactrum anthropi biotype D[b,d]	3301	LMG	+
Alcaligenes denitrificans[b]	15173	ATCC	−
Alcaligenes denitrificans[b]	27061	ATCC	−
Xanthomonas maltophilia[e]	113	CECT	−
Yersinia enterocolitica O:9[e]	E518/90	G. Waters	−
Vibrio cholerae O:1[e]		CUN	−
Salmonella urbana[e]		CUN	−
Escherichia coli O:157[e]		CUN	−
Escherichia coli O:111[f]		NIBSC	−

[a] NADC, National Animal Disease Center, Ames, Iowa; INRA, Institut Nationale de la Recherche Agronomique, Tour, France; CVL, Central Veterinary Laboratory, Weybridge, England; ICPB, International Collection of Phytopathogenic Bacteria, University of California, Davis; IVIA, Instituto Valenciano de Investigaciones Agrarias, Valencia, Spain; LMG, Culture Collection of the Laboratory for Microbiology, Ghent, Belgium; ATCC, American Type Culture Collection, Rockville, Md.; CECT, Colección Española de Cultivos Tipo, Valencia, Spain; CUN, Clínica Universitaria, Universidad de Navarra, Pamplona, Spain; NIBSC, National Institute for Biological Standards and Control, London, England.

[b] Phylogenetically related to *Brucella* spp.

[c] Type strain (10).

[d] Originally isolated from human blood (10).

[e] Serologically related to *Brucella* spp.

[f] Negative control.

contamination with *Brucella* DNA. After an initial denaturation at 95°C for 5 min, the PCR profile was set as follows: 30 s of template denaturation at 95°C, 90 s of primer annealing at 54°C, and 90 s of primer extension at 72°C, for a total of 30 cycles, with a final extension at 72°C for 6 min. The samples were analyzed by electrophoresis (70 V for 2 h) in a 0.8% agarose D1 (Hispanlab, S.A.) gel in TBE buffer (89 mM Tris-HCl, 89 mM boric acid, 2.0 mM EDTA, pH 8.0). PCR amplification products were detected by visualization of the bands under UV light after the gel was soaked with 2 μg of ethidium bromide per ml. Each sample was tested twice in different independent experiments.

Nucleotide sequence determination. DNA sequences were obtained by the dideoxy-chain termination method of Sanger et al. (14). DNAs to be used as sequencing templates were PCR products purified with the Wizard PCR Preps DNA Purification System (Promega Biotec). The templates were resuspended in a sequencing buffer supplied in the Sequenase 2.0 DNA sequencing kit (United States Biochemical Corp.). Sequencing reactions were performed according to the manufacturer's instructions, and products were electrophoresed on 6% polyacrylamide gels and exposed to XAR-2 X-ray film at room temperature.

TABLE 2. PCR results with DNAs from clinical and field isolates

Species (no. of isolates)	Source	PCR
Achromobacter sp. (1)	Human	−
Acinetobacter calcoaceticus (1)	Human	−
Brucella melitensis (13)	Human	+
Brucella melitensis (5)	Bovine	+
Brucella ovis (5)	Ram	+
Campylobacter sp. (1)	Human	−
Candida albicans (1)	Human	−
Citrobacter freundii (1)	Human	−
Clostridium sp. (1)	Meat	−
Enterococcus faecalis (2)	Human	−
Escherichia coli (2)	Human	−
Haemophilus influenzae (2)	Human	−
Klebsiella pneumoniae (2)	Human	−
Listeria monocytogenes (1)	Meat	−
Mycobacterium tuberculosis (2)	Human	−
Neisseria meningitidis (1)	Human	−
Ochrobactrum anthropi (1)	Human	−
Propionibacterium sp. (1)	Human	−
Proteus mirabilis (1)	Human	−
Pseudomonas aeruginosa (1)	Human	−
Pseudomonas maltophilia (1)	Human	−
Salmonella enteritidis (1)	Human	−
Salmonella typhi (1)	Human	−
Salmonella typhimurium (2)	Human	−
Serratia marcescens (1)	Human	−
Shigella sp. (1)	Human	−
Staphylococcus aureus (2)	Human	−
Staphylococcus epidermidis (1)	Human	−
Streptococcus faecalis (1)	Human	−
Streptococcus pneumoniae (1)	Human	−
Streptococcus viridans (1)	Human	−
Xanthomonas sp. (1)	Human	−

RESULTS AND DISCUSSION

To select the optimum pair of primers which would specifically amplify *Brucella* DNA, nine different combinations between the forward and reverse primers were tested. Both *B. abortus* 2308 DNA and *Escherichia coli* O:111 DNA (negative control) were used as templates in these initial optimization studies. Since amplification conditions for the different combinations of the oligonucleotides could be different, the influence of the following PCR parameters was assayed: annealing temperature from 50 to 58°C, Mg^{2+} concentration from 0.5 to 2.5 mM, *Taq* polymerase concentration from 0.25 to 0.75 U per reaction, and number of cycles of amplification. The strongest, most specific, and most reproducible amplification was achieved with the F4-R2 pair under the conditions described in Materials and Methods (Fig. 1). Therefore, all subsequent

TABLE 3. Sequences and locations of primers used for PCR amplification

Primer	Sequence	Location[a]
F1	5′-TGC TAA TAC CGT ATG TGC TT-3′	148–167
F3	5′-GGG AAC GTA CCA TTT GCT A-3′	107–125
F4	5′-TCG AGC GCC CGC AAG GGG-3′	63–79
R1	5′-TAA CCG CGA CCG GGA TGT-3′	932–949
R2	5′-AAC CAT AGT GTC TCC ACT AA-3′	947–966
R3	5′-TCC AGC CTA ACT GAA CCA TA-3′	960–979

[a] Based on the nucleotide sequence of the *B. abortus* 16S rRNA described by Dorsch et al. (6).

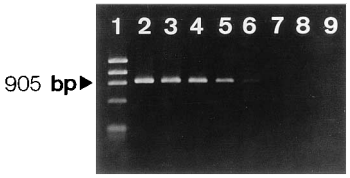

FIG. 1. Sensitivity of PCR assay for detection of *B. abortus* 2308 DNA. Lanes 2 to 9, different amounts of template DNA, as follows: 800 pg (lane 2), 80 pg (lane 3), 8 pg (lane 4), 800 fg (lane 5), 80 fg (lane 6), 8 fg (lane 7), 0.8 fg (lane 8), and no template DNA (lane 9). Lane 1, ϕX174 DNA/*Hae*III marker (Boehringer-Mannheim, S.A.). The 905-bp PCR product is indicated.

experiments were performed with these primers and under these conditions.

To confirm the identity of the PCR product, the nucleotide sequence of each end of the fragment was determined and compared with the *Brucella* 16S rRNA sequence described by Dorsch et al. (6). The results confirmed the amplification of the correct fragment (data not shown).

We also tested the ability of the PCR assay to amplify DNAs from other strains of *Brucella*. Under the optimal conditions described in Materials and Methods, all of the representative strains of the different species and biovars of *Brucella* and 23 *Brucella* isolates yielded only the predicted 905-bp fragment of amplified DNA (Tables 1 and 2). This result is in agreement with that of De Ley et al. (4), who found that the genus *Brucella* forms a very tight genetic cluster, and with the proposal of a single genomic species for *Brucella* (15).

To determine the specificity of the primers selected, DNAs from 10 different strains phylogenetically related to *Brucella* spp. and from 5 gram-negative bacteria showing serological cross-reactions with *Brucella* spp. (Table 1) were assayed. No amplification products were observed, with the sole exception of *Ochrobactrum anthropi* LMG 3301 DNA, which yielded a PCR product of 905 bp. The genus *Ochrobactrum* (formerly in CDC group Vd) is also a member of the alpha-2 subdivision of the class *Proteobacteria* and is the closest known neighbor of the genus *Brucella* (10). However, it is unlikely that *O. anthropi* would cause a false-positive result in a test for *Brucella* spp. with the PCR assay described here, since *O. anthropi* has rarely been found to be pathogenic (2, 10). In addition, it is significant that DNAs from *O. anthropi* LMG 3331 (type strain) and from a clinical isolate of *O. anthropi* (from blood of an immunocompromised patient) were not amplified (Tables 1 and 2). The clinical isolate was characterized by the National Collection of Type Cultures (Public Health Laboratory Service, London, England) as belonging to biotype A. The genus *Ochrobactrum* is a broad group, and it has been reported that the relative binding ratios of *O. anthropi* LMG 3331 (biotype A) DNA with *O. anthropi* LMG 3301 (biotype D) DNA and *Brucella* DNA are 41 and 20 to 30%, respectively (10). In this regard, our results confirm the genetic heterogeneocity of the genus *Ochrobactrum* and suggest a closer relationship between *Brucella* spp. and *O. anthropi* biotype D (4). The PCR specificity was also evaluated by using a total of 36 isolates of other genera different from *Brucella* (Table 2), including intracellular pathogens and microorganisms able to cause bacteremia. All samples tested were also negative, confirming the high specificity of the PCR assay proposed.

Finally, the threshold sensitivity of the PCR assay was determined by testing serial dilutions of *B. abortus* 2308 DNA from 80 ng to 0.08 fg. As little as 80 fg of DNA was detected after 40 cycles of amplification (Fig. 1). Similar findings with respect to the amount of *Brucella* DNA detected by PCR have been obtained by using primers based on the genes encoding a 43-kDa outer membrane protein (8) and a 31-kDa *B. abortus* antigen (1). In those reports, the authors suggested that 60 to 100 fg of bacterial DNA is equivalent to 20 cells.

In conclusion, on the basis of its sensitivity and specificity, this PCR method based on the sequence of the 16S rRNA of *B. abortus* could provide a useful diagnostic tool for brucellosis.

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