

Detection and Differentiation of the Six *Brucella* Species by Polymerase Chain Reaction

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ABSTRACT

Background: Brucellosis is a severe acute febrile disease caused by bacteria of the genus *Brucella*. Its current diagnosis is based on clinical observations that may be complemented by serology and microbiological culture tests; however, the former is limited in sensitivity and specificity, the latter is time consuming. To improve brucellosis diagnosis we developed a test which is specific and sensitive and is capable of differentiating the six species of *Brucella*.

Materials and Methods: Four primers were designed from *B. abortus* sequences at the well-conserved *Omp2* locus that are able to amplify the DNAs of all six species of *Brucella*.

Results: Our test detected all six species of *Brucella*. Their

differentiation resulted directly from differences in the amplification patterns or was achieved indirectly using a RFLP present in one of the PCR products. The sensitivity and specificity of the new test were then determined; it was applied successfully in confirming the diagnosis of a patient whose clinical history and serology indicated infection with *Brucella*.

Conclusions: The results make possible the use of a PCR test for *Brucella* detection and differentiation without relying on the measurement of the antibodies or microorganism culture. Our first results showed that the PCR test can confirm the presence of *Brucella* in blood samples of infected patients.

INTRODUCTION

Brucellosis, a zoonosis of worldwide public health and economic importance, affects mainly domestic animals (cattle, sheep, goat, and swine), and it may be transmitted to humans by contact or consumption of contaminated dairy and meat products. The disease is caused by organisms of the genus *Brucella*, in which six closely related species have been distinguished on the basis of biochemical, physiological, and host-specificity

differences (1). However, on the basis of their high DNA sequence similarity (>90%), some authors (2) have proposed *B. melitensis* as the only species.

In humans, the signs and symptoms for brucellosis can be confused with those of other febrile diseases. The diagnosis is generally based on a history of exposure, a positive blood culture in the early disease stage, or a positive serology. Although serology remains the more commonly used test, it may result in misinterpretation when subclinical infections or persistent antibodies exist from chronic disease or previous infection in areas where brucellosis is endemic. Because of the simplicity, specificity, and sensitivity of polymerase chain reaction (PCR), studies have been recently reported using this technique for

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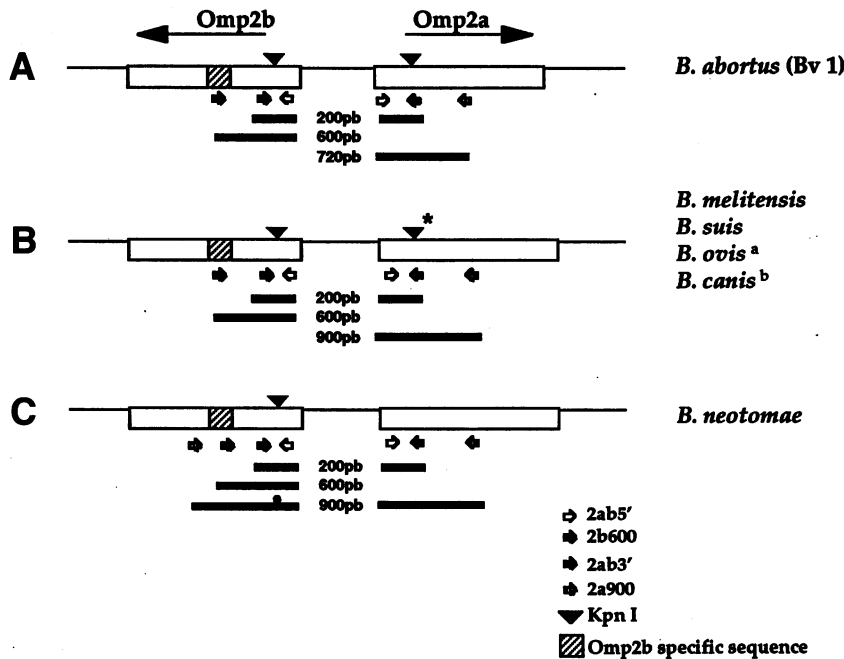


FIG. 1. Primer target sites in Omp2 locus

The Omp2 locus in all species except *B. ovis* is composed of two closely related genes, Omp2b and Omp2a. These are oriented in opposite transcriptional directions (8). A, B, and C represent the Omp2 maps reported for *B. abortus* (Bv. 1), *B. melitensis* (Bv. 1), and *B. neotomae*, respectively (9). The arrows indicate primer annealing sites and the bars represent the amplified fragment sizes. The 720-bp fragment changes in length to 900 bp for strains other than *B. abortus* (Bv. 1), whereas in *B. ovis*^(a) the 600-bp fragment is not produced as it lacks the Omp2b and in *B. canis*^(b) the 200-pb fragment is absent. In *B. neotomae*, the 2a900 primer amplifies both genes. The *KpnI* restriction site (black dot) is present in the 900-bp fragment from Omp2b.

Brucella detection, but most of them only detect the genus (3–5) or focus on the differentiation of the principal species affecting specific geographic regions (6). Nevertheless, the ability to differentiate the six *Brucella* species will be useful to carry out epidemic studies of brucellosis incidence or to investigate the disease’s aggressiveness.

In our laboratory, the successful detection of *B. abortus* genomic sequences was achieved by PCR on DNA from infected bovine blood cells (7), using a set of primers derived from the sequence of two homologous genes (Omp2a and Omp2b) coding for a major outer membrane protein (porin) of these bacteria (8). Although the Omp2 locus is well conserved within this genus, it displays enough genetic polymorphism to distinguish among species of *Brucella* (9).

We describe here a new PCR method that allows us to differentiate directly three species: *B. abortus* (Bv. 1), *B. canis*, and *B. ovis*, and indirectly the other three species: *B. suis* (Bv. 1), *B. melitensis* (Bv. 1), and *B. neotomae*, using an additional restriction fragment length polymorphism (RFLP) analysis. In addition, we report its application for *Brucella* detection in human blood.

MATERIALS AND METHODS

T4 DNA ligase and the restriction enzymes *KpnI*, *NcoI* and *NciI* were purchased from New England Biolabs (NEB; Beverly, MA) and *EcoRI*, *PstI*,

PvuII, *HindIII*, and *BamHI* were obtained from GIBCO-Bethesda Research Laboratories (BRL) (Gaithersburg, MD). RNase A was purchased from Sigma Chemical Company (St. Louis, MO), and ³⁵S-ATP from Amersham International PLC (Mexico City).

Bacterial Strains and Genomic DNA Preparation

DNAs of the six *Brucella* species were kindly provided by Dr. Thomas Fitch of Texas A & M University at College Station, TX (see ref. 9 for strain identification data). Bacterial strains others than *Brucella* used in this study were obtained from the Microbiology Department, School of Medicine, Autonomous University of Nuevo Leon, Monterrey, N.L., Mexico.

Genomic bacterial DNA was prepared as follows: bacteria from 10 ml of a culture were resuspended in 0.2 ml of lysis buffer (10 mM Tris-HCl, pH 8, 2% Triton X-100, 1% SDS, 10 mM NaCl, 1 mM Na₂ EDTA). Nucleic acids were extracted with phenol:chloroform:isoamyl alcohol (25:24:1) and treated with RNase A (final concentration, 10 µg/ml). Finally, DNA was precipitated with ammonium acetate and ethanol and resuspended in TE (10 mM Tris.Cl, pH 8, 1 mM EDTA, pH 8). The same protocol was used to obtain genomic DNA from fresh blood samples (5 ml) collected with EDTA as anticoagulant.

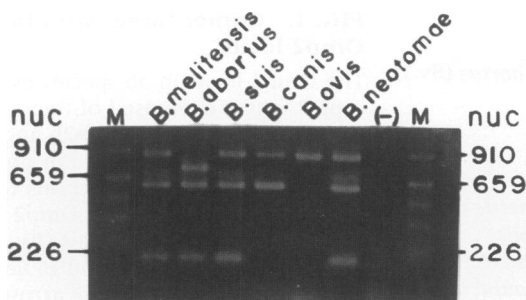


FIG. 2. Amplification of the six *Brucella* species

The amplification results of the six *Brucella* species using a set of four primers are shown. The PCR was achieved in 35 cycles, the annealing temperature was 66°C, and the extension time was 90 sec. Lane M, molecular size standard (pBR322/*AluI*).

PCR Amplification

In this study, four primers complementary to the sequence reported for *B. abortus* Omp2 locus (GenBank accession number M26034) were used in a PCR reaction that coamplifies fragments of the Omp2a and Omp2b genes. The oligonucleotides were designed in our laboratory and synthesized by BioSynthesis (Lewisville, TX). The primer annealing sites in the Omp2 locus are described in Figure 1.

The primers sequences were 2ab5': 5'actgacggatccgcgctcaggcggccgacgcaa3', 2a900: 5' actgacttcgaattgccttttcgggggcaatga 3', 2ab200: 5' actgacttcgaaaccagccattgcggtcggtagc, and 2b600: 5' actgaagcttagccgctcgatgtgtagt 3'.

Approximately 50 ng of DNA from each bacteria preparation was amplified in a total volume of 50 μ l, using 2.5 U of *Taq* DNA polymerase (Promega, Madison, WI), 25 pmol of each primer, 3 mM MgCl₂, and 200 μ M dNTPs. For each experiment, the precise PCR conditions are detailed in the footnote to the corresponding figure. The reaction lasted 35 cycles using a programmable DNA thermocycler (MJ Research, Watertown, MA). Aliquots of the PCR products were analyzed by electrophoresis on 1.5% agarose gels. For enzymatic characterization, PCR fragments were digested with some restriction enzymes, each in a final volume of 20 μ l and under the conditions suggested by the manufacturer.

Cloning and Sequencing of DNA Fragments

PCR fragments were treated to release their 5' end artificial-restriction enzyme site (introduced

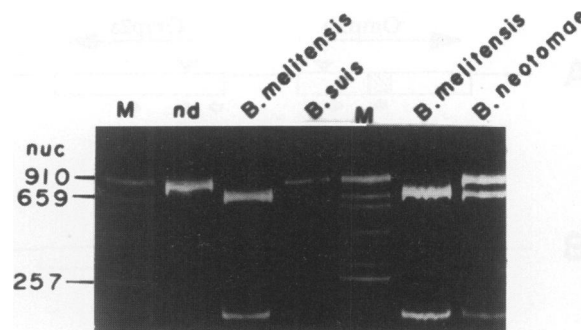


FIG. 3. Differentiation of the three undistinguishable species

The differentiation of *B. melitensis* (Bv.1), *B. suis* (Bv. 1), and *B. neotomae* using the RFLP with *KpnI* in the 900-bp fragment is shown. Lane M, molecular size standard (pBR322/*AluI*); lane nd, nondigested product.

via the primers) and inserted into the mp18 and mp19 M13 vectors between their *Bam*HI and *Hind*III sites. *E. coli* DH5 α F' competent bacteria were used for transformation (10). Individual colonies were treated to isolate their plasmid DNA, which were screened by restriction enzyme analysis (10) and nucleotide sequencing of their inserts, using the dideoxy chain termination-DNA sequencing method (11). After electrophoresis, the sequencing gel was dried and exposed on Kodak X-ray film at -70°C.

RESULTS

Detection and Differentiation of the Six *Brucella* Species

We examined whether our set of four PCR primers could be useful to amplify the DNAs from the six species that compose the genus *Brucella*: *B. abortus* (Bv. 1), *B. canis*, *B. ovis*, *B. melitensis* (Bv. 1), *B. suis*, and *B. neotomae*. As shown in Figure 2, we were able to obtain a specific amplification signal for all tested species. However, four different amplification patterns were observed: patterns I and II consisted of three electrophoretic bands (900, 600, and 200 bp); and (720, 600, and 200 bp), respectively. Pattern III was represented by two bands of 900 and 600 bp. Pattern IV corresponded to a unique PCR product of 900 bp. In these conditions, the different PCR patterns allowed us to differentially detect *B. abortus* (Bv. 1), *B. canis*, and *B. ovis*; but the three remaining species, *B. melitensis* (Bv. 1), *B. suis*, and *B. neo-*



FIG. 4. Detection limit of PCR

PCR results using decreasing amounts of DNA of *B. abortus* (Bv. 1) (as an example of all *Brucella* species) and *B. suis* (Bv. 1). Lane M, molecular size standard (pBR322/*AluI*).

tomae, were still indistinguishable using only their amplification pattern. Furthermore, sequencing of the 200-bp fragments obtained after DNA amplification of each species corroborated that the PCR products were indeed generated from the Omp2 region (data not shown) and demonstrated the specificity of our primers.

On the basis of the restriction analysis reported by Ficht (9), we were able to predict that the PCR patterns would depend on the species but not on the biovariety. This reasoning was corroborated by the results obtained with biotypes 2 and 5 of *B. melitensis* and *B. abortus*, respectively, in which the amplification pattern was type I, as expected (data not shown).

To distinguish within the three species giving pattern I, an enzymatic characterization of the 900-bp fragment was carried out; only *KpnI* restriction enzyme allowed us to find differences. The *KpnI* restriction site was present in *B. melitensis* (Bv. 1) 900-bp PCR fragment, but absent in *B. suis*. Interestingly, in *B. neotomae* we found both patterns—an intact band of 900 bp coming from the amplification of Omp2a and two additional fragments of approximately 700 and 200 bp from *KpnI* digestion of the Omp2b PCR product (Fig. 3).

Detection Limit and Specificity of the PCR Method

In the first attempts to establish our PCR method we used 50 ng of DNA. To test the detection limit, decreasing amounts of DNA were assayed. We found that 1 ng of DNA was enough to detect and differentiate all *Brucella* species; but for *B. suis*, 10 ng of DNA was needed (Fig. 4). In addition, to test the specificity of our assay, we used

TABLE 1. Specificity results of the PCR method

Strain	PCR results
<i>B. melitensis</i> bv. 1	(+, type I)
<i>B. melitensis</i> bv. 2	(+, type I)
<i>B. abortus</i> bv. 1	(+, type II)
<i>B. abortus</i> bv. 5	(+, type I)
<i>B. suis</i> bv. 1	(+, type I)
<i>B. neotomae</i>	(+, type I)
<i>B. canis</i>	(+, type III)
<i>B. ovis</i>	(+, type IV)
<i>Escherichia coli</i>	(-)
<i>Haemophilus influenzae</i>	(-)
<i>Pseudomonas spp</i>	(-)
<i>Mycobacterium tuberculosis</i>	(-)
<i>Salmonella spp</i>	(-)

our method with DNA from other bacteria; porins have been reported for some of these (12). None of the bacteria resulted positive with our PCR method (Table 1). When the same reaction mix was challenged with DNA from *B. abortus* as positive control for these assays, we obtained the expected amplification pattern, confirming that the reactions were devoid of PCR inhibitors (data not shown).

Detection of *Brucella* in Blood Samples

Genomic DNA was extracted from a blood sample of a pediatric patient who presented clinical symptomatology of *Brucella* infection and positive serology, and then it was amplified using the four-primer PCR assay. As shown in Figure 5, no amplification was observed with the DNA control extracted from a healthy control subject. In the patient, we obtained the pattern (type I) corresponding to the undifferentiated species. *KpnI* digestion of the amplified product revealed that the likely species involved was *B. melitensis*.

After medical treatment of this patient, we again isolated the DNA from a sample of his blood and performed the PCR assay. The result of this new amplification was only the band of 200 bp (data not shown). This result may be explained by the reduction of the bacteria in the blood of the patient, since, as shown in Figure 4, the decrease in DNA to 0.1 ng in the PCR reaction produced only the amplification product of 200 bp.

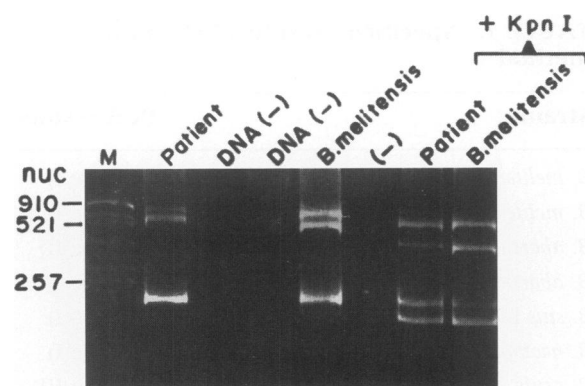


FIG. 5. Detection and differentiation of *Brucella* in blood samples

PCR reactions were carried out using 1 μ g of DNA from a brucellosis patient (Patient lanes), two uninfected individuals [DNA (-) lanes], and *B. melitensis* DNA as positive control (lane 5). A negative control (-) (lane 6) having no DNA was also included. As the patient's sample gave the undistinguishable PCR pattern (900, 600, and 200 bp), the PCR product was digested with *KpnI*. The last two lanes correspond to the results of digestion of the amplified patient specimen and *B. melitensis*, respectively, using the enzyme *KpnI*. Lane M, molecular size standard pBR322/*AluI*.

DISCUSSION

In this study we describe the development of a test for differential detection of *Brucella* species based on PCR technology. Detection is possible because of the conservation throughout the genus *Brucella* of the two Omp2 gene nucleotide sequences, which encode for outer membrane proteins of the porin family (8). Variations within the locus allowed us to design PCR primers that amplify specific-sized species products. Four different amplification patterns were obtained: I of 900, 600, and 200 bp corresponding to *B. melitensis* (Bv. 1), *B. suis*, or *B. neotomae*; II of 720, 600, and 200 bp to *B. abortus* (Bv. 1); III of 900 and 600 bp to *B. canis*, and IV of 900 bp corresponding to *B. ovis*. The three type I species could be differentiated by a *KpnI* RFLP in the 900-bp amplified product.

An important aspect of a diagnostic test is its specificity. PCR specificity depends both on the gene selected for amplification and on the primers' uniqueness. Although, the primers were designed from the Omp2 locus, no microorganisms different from *Brucella* cross-reacted. This result was consistent, regardless of whether they contained porin genes (12).

An annealing temperature of 66°C was used

to increase specificity. However, as the primers were designed to match sequences at the Omp2 locus of *B. abortus*, under this more stringent condition the sensitivity of the test was lower when trying to detect the other species of *Brucella*. This is probably because these other species' target sites in the Omp2 locus do not match perfectly to the primers. Further adjustments to the assay through species-specific primers should increase PCR sensitivity.

The development of the assay was accomplished by using purified DNA from *Brucella* strains, but as a further step we applied it to clinical samples to verify the feasibility of using it for *Brucella* diagnosis in humans. Although the application of our PCR assay in diagnosis is preliminary (only one patient was tested), it presents several advantages over the current techniques used for *Brucella* diagnosis and serotyping. Perhaps the most important advantage is the shortened time needed to perform a diagnosis (less than a single day versus the several weeks needed to isolate and culture the *Brucella* organism). It is clear that PCR will be an important tool in the diagnosis of *Brucella* because of its sensitivity and specificity. Experiments on the optimization and validation of our PCR method in a larger sampling of patients are currently underway.

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