Rapid Diagnosis of Human Brucellosis by Peripheral-Blood PCR Assay

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A single-step PCR assay with genus-specific primers for the amplification of a 223-bp region of the sequence encoding a 31-kDa immunogenetic Brucella abortus protein (BCSP31) was used for the rapid diagnosis of human brucellosis. We examined peripheral blood from 47 patients, with a total of 50 cases of brucellosis, and a group of 60 control subjects, composed of patients with febrile syndromes of several etiologies other than brucellosis, asymptomatic subjects seropositive for Brucella antibodies, and healthy subjects. Diagnosis of brucellosis was established in 35 cases (70%) by isolation of Brucella in blood culture and in the other 15 cases (30%) by clinical and serological means. The sensitivity of our PCR assay was 98.3%, since it correctly identified all 50 cases of brucellosis, regardless of the duration of the disease, the positivity of the blood culture, or the presence of focal forms. The specificity of the test was 98.3%, and the only false-positive result was for a patient who had had brucellosis 2 months before and possibly had a self-limited relapse. In those patients who relapsed, the results of our PCR assay were positive for both the initial infection and the relapse, becoming negative once the relapse treatment was completed and remaining negative in the follow-up tests at 2, 4, and 6 months. In conclusion, these results suggest that the PCR assay is rapid and easy to perform and highly sensitive and specific, and it may therefore be considered a useful tool for diagnosis of human brucellosis.
antibodies, and 25 healthy subjects with no history of brucellosis or exposure to 

**Brucella.**

**Bacteriological and serological techniques.** Two blood cultures, Wright’s se-

roagglutination test and Coomb’s antibruceella test, were done for all the patients with 

active brucellosis, febrile syndromes of other etiologies, or a previous history of 

brucellosis. The serological tests were carried out according to previously 

described techniques and the blood cultures were processed by following 

usual bacteriological techniques with a BACTEC 9240 system (Becton Dickinson 

Diagnostic Instrument Systems, Towson, Md.), incubated for 30 days, and sub-

cultured in a blind manner at 10, 20, and 30 days. Brucella species were identified 

as recommended by Hausler et al. (10). All isolated strains were sent to a 

brucellosis reference laboratory (Laboratorio Regional de Brucellosis, Valladol-

lid, Spain) for definitive confirmation and biotyping.

**Isolation of DNA from clinical blood samples.** A modification of the method 
described by Miller et al. (18) was used. Briefly, 0.5 ml of blood collected in 

sodium citrate and stored at −20°C was resuspended in 1 ml of ethyrycye lysis 

solution (320 mM sarcosuch, 5 mM MgCl, 1% Triton X-100, 10 mM Tris HCl 

[ph 7.5]), mixed, and centrifuged at 15,000 × g for 2 min. The supernatant 

was discarded, and the pellet was washed with 1 ml of Milli-Q water to 

lyse the cells and centrifuged as described above. Treatment with 

water was repeated until the 

leukocyte pellet lost all reddish coloring.

Template DNA was obtained from the leukocytes as follows. Four hundred 

microliters of nucleic lysis buffer (60 mM NH₄Cl, 24 mM Na₂-EDTA [pH 8.0]) 

containing proteinase K (1 mg/ml) and sodium dodecyl sulfate (1%) was mixed 

and incubated for 30 min at 55°C. After digestion, the samples were cooled 

to room temperature, and 100 μl of ammonium acetate (7.5 M) was added, fol-

lowed by centrifugation at 15,000 × g for 10 min. The supernatant containing 

total DNA was transferred to a fresh tube. Two volumes of absolute ethanol at 

room temperature were added, and the tubes were inverted several times until 

the DNA precipitated. DNA was recovered by centrifuging the samples at 

15,000 × g for 10 min. The pellets were rinsed with 1 ml of 70% ethanol, 

dried and resuspended in 30 μl of water. The concentration and purity of the 

DNA were determined spectrophotometrically by reading A₂₆₀ and A₂₈₀.

**DNA amplification.** The PCR target sequence of 223 bp present on a gene 

encoding a 31-kDa B. abortus antigen was selected for amplification. The se-

quence has been shown to be common to all Brucella biovars (15). The primers 

B4 and B5 described previously by Baily et al. (4) were used to amplify the target 

sequence. A PCR was performed with each of the DNA extracts as described 

previously with slight modifications (4). Briefly, 10 μl of reaction mixture con-

tained 10 mM Tris-HCl (pH 8.4), 50 mM KCl, 1 mM magnesium chloride, 200 

μM each deoxyribonucleoside triphosphate (dATP, dGTP, dCTP, and dTTP), 

Boehringer, Mannheim, Germany), oligonucleotides B4 and B5 (100 ng each; 

Pharmacia LKB, Barcelona Spain), 1.25 U of Taq polymerase (Boehringer), 2 μg 

of total DNA extracted from blood samples, and 100 ng from the positive 

controls. The reaction was performed in a DNA thermal cycler (model 2400; 

Perkin-Elmer, Norwalk, Conn.) without mineral oil. After an initial denaturation 

at 93°C for 5 min, the PCR profile was set as follows: 60 s of template denatur-

ation at 90°C, 30 s of primer annealing at 60°C, and 60 s of primer extension at 

72°C for a total of 35 cycles, with a final extension at 72°C for 7 min.

A sample was considered positive when DNA with a molecular weight ex-

pected for the amplified product was seen after electrophoresis in 2% agarose to 

be fluorescent in the presence of ethidium bromide (2 μg/ml). Negative controls 

containing all of the reagents but lacking template DNA were routinely pro-

duced, and all were negative. The positive controls with preamplification of 

DNA isolated from a suspension of 

B. abortus 

were included in each experiment. All PCRs were carried out in 

duplicate.

**Purification and sequencing of PCR product.** In order to confirm the identi-

fication of the amplified fragments, the PCR products were purified and sequenced.

Template DNA was obtained by PCR amplification as described above. 

**TABLE 1. Epidemiological, clinical, and microbiological characteristics of patients with brucellosis**

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Values</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of patients/no. of samples studied</td>
<td>47/50</td>
</tr>
<tr>
<td>Demographics</td>
<td></td>
</tr>
<tr>
<td>Male/female</td>
<td>37/10</td>
</tr>
<tr>
<td>Mean age in years (range)</td>
<td>37.9 (14–91)</td>
</tr>
<tr>
<td>Clinical characteristics</td>
<td></td>
</tr>
<tr>
<td>Mean duration of symptoms in days (range)</td>
<td>25.5 (2–120)</td>
</tr>
<tr>
<td>No. (%) of patients with fever</td>
<td>50 (100)</td>
</tr>
<tr>
<td>No. (%) of patients with focal forms*</td>
<td>12 (25.5)</td>
</tr>
<tr>
<td>Diagnostic tests</td>
<td></td>
</tr>
<tr>
<td>No. (%) of patients with titers ≥1/160 by Wright’s seroagglutination test</td>
<td>37 (74)</td>
</tr>
<tr>
<td>No. (%) of patients with titers ≥1/320 by Coomb’s antibruceella test</td>
<td>32 (64)</td>
</tr>
<tr>
<td>No. of patients with positive blood cultures*</td>
<td>35 (70)</td>
</tr>
</tbody>
</table>

* Two cases of prostatitis, one case of pneumonitis, and nine osteoarticular cases (two cases of sacroiliitis, two cases of olioarthritis, two cases of oleanorarthritis, and one case of spondylitis).

B. melitensis was isolated from all cultures.

The mean duration of the symptoms before diagnosis of brucellosis was 25.5 days (range, 2 to 49 days). In 19 cases (38%) symptoms lasted less than 2 weeks, in 20 cases (40%) symptoms lasted between 2 weeks and 1 month, in 9 cases (18%) symptoms lasted between 1 and 3 months, and in the remaining 2 cases (4%) symptoms lasted more than 3 months. The only demographic, clinical, and microbiological characteristics of the patients with brucellosis are shown in Table 1. All the samples from patients with brucellosis had a positive PCR. PCR results for the three patients who had a relapse after completing the treatment were positive for both the ini-

tial episode and the relapse, although only two patients had positive blood cultures again. The sensitivity of the PCR was, therefore, 100%. Table 2 shows the diagnostic results of PCR compared with those of conventional methods for the 50 cases of brucellosis studied.

Fifty-nine of the 60 controls had a negative PCR, the speci-

ficiy therefore being 98.3%. The only subject in the control 

group whose PCR was positive had had brucellosis 2 months 

previously and was referred to our hospital with a 10-day his-
tory of fever accompanied by a new increase in the titer of 

antibruceella antibodies, strongly indicative of a relapse. The 

fever ceased spontaneously without treatment, and the blood 

cultures were repeatedly negative. This patient was monitored 

for 6 months but did not show any further symptoms, and the 

levels of antibodies fell progressively.

Clear visualization of PCR-amplified fragments was possible in all cases after electrophoresis with an agarose gel. The
TABLE 2. Comparison of the results of the PCR amplification procedure with those of routine microbiological techniques for the diagnosis of 50 cases of brucellosis

<table>
<thead>
<tr>
<th>Result of routine procedure</th>
<th>No. of samples (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Positive by PCR</td>
</tr>
<tr>
<td>Positive by serological test and blood culture</td>
<td>27 (54)</td>
</tr>
<tr>
<td>Negative by serological tests and positive by blood culture</td>
<td>8 (16)</td>
</tr>
<tr>
<td>Positive by serological tests and negative by blood culture</td>
<td>15 (30)</td>
</tr>
<tr>
<td>Total</td>
<td>50 (100)</td>
</tr>
</tbody>
</table>

* Either Wright's seroagglutination test (titer, ≥1/160) or Coombs' antibrucella test (titer, ≥1/320).
* Both Wright's seroagglutination test (titer, ≥1/160) and Coombs' antibrucella test (titer, ≥1/320).

specificities of the amplified products were confirmed by dot blot hybridization (Fig. 1). However, to confirm the identity of a PCR product, its nucleotide sequence was determined. The amplified fragment matched perfectly the DNA B. abortus (BCSP31) sequence described by Mayfield et al. (17).

![Agarose gel electrophoresis and ethidium bromide staining](image)

**FIG. 1.** (A) Agarose gel electrophoresis and ethidium bromide staining. Lane MW, DNA ladder (223 bp); lanes 2 and 3, positive controls (B. abortus B-19 and B. melitensis Rev-1, respectively); lane 4, no DNA added; lanes 5 and 7, DNAs from two patients with brucellosis and positive blood cultures; lane 6, DNA from a healthy subject; lane 8, DNA from a patient with bacteremia due to E. coli; lanes 9 and 11, DNAs from two patients with active brucellosis but negative blood cultures; lane 10, DNA from a patient with pneumococcal disease; lane 12, DNA from a patient with bacteremia but without evidence of active disease and with high serological titers of Brucella antibodies. (B) Dot blot hybridization. The PCR products of samples from positive controls and patients with brucellosis hybridized to a fluorescein-labeled probe (BR-1), demonstrating that these samples contained DNA from Brucella. No hybridization was observed in the sample from any patient from the control group. Duplicate samples were used in all cases. The photocomposition of the figure was obtained from the original Polaroid film plus the autoradiograph from dot blot hybridization with a ScanJet IICX scanner (Hewlett-Packard, Corvallis, Oreg.). After the initial image was scanned and saved as a TIFF file, the file was opened in Adobe Photoshop, version 3.0 (Adobe System, Inc., Seattle, Wash.).

DISCUSSION

As the clinical picture of human brucellosis is fairly nonspecific, it is necessary to resort to isolation of the germ, by demonstrating high levels of specific antibodies or seroconversion, in order to make a definite diagnosis. However, all these methods have serious limitations (12).

Mayfield et al. cloned the gene which codes for the production of a 31-kDa membrane protein specific to the Brucella genus (17). Recently Baily et al. developed a PCR technique capable of amplifying a region of the gene which codes for this protein. This technique showed high sensitivity, since it was able to amplify 60 fg of DNA in pure cultures of *Brucella* (4).

Although there are a few reports concerning the use of PCR techniques to diagnose animal brucellosis (14, 22), information concerning the use of this diagnostic method for human brucellosis is very limited. In the only clinical study to date, the number of patients included was small, the clinical information about them was very scarce, and the control group did not include any patient from the groups which usually pose problems in the interpretation of the results of the diagnostic tests, such as persons exposed professionally, those with a recent history of brucellosis, or carriers of antibrucella antibodies that do not exhibit evidence of active disease. Moreover, brucellosis was confirmed bacteriologically for only one of the patients (15).

In the present study we investigated the potential use of a single-step PCR assay as a rapid test for the diagnosis of human brucellosis. The sensitivity of the test was 100% for both the patients with a positive blood culture and those for whom no bacteremia could be detected in two or more peripheral blood cultures. This finding is especially important if we consider that 25.5% of patients presented with focal forms and 22% had clinical pictures of more than 1 month’s evolution, both of which manifestations are associated with a lower number of circulating microorganisms. The high sensitivity of the technique is probably related to its ability to detect 10 fg of bacterial DNA (data not shown), which equates to approximately two bacteria, a number of microorganisms possibly present in any 1-ml sample of peripheral blood from patients with clinical brucellosis.

The specificity was likewise very high, 98.3%, a figure which could even have been 100% if the criteria followed in the only false-positive case had not been so strict. This case was considered a false positive since the disease was self-limiting without the patient receiving antimicrobial treatment, although we could also have considered it a true positive, since oligosymptomatic and self-limited forms of this disease are well-documented (20).

There are at present no definite criteria to establish that brucellosis has been cured, since the presence of negative blood cultures does not exclude the presence of the disease and the antibodies may remain elevated for a long time after the conclusion of the treatment (3). With the exception of the above-mentioned patient, all the seropositive controls had a negative PCR, a fact which, in the future, may be very interesting in order to fix objective criteria for a cure.

The methodology of the previously reported PCR-based method for detecting the *Brucella* organism in human blood samples is too complex for routine use in clinical practice, since it requires a second PCR for all amplified products in order to enhance the intensities of the bands (15).

The method proposed herein can be used with a simple sample of 0.5 to 1 ml of peripheral blood without the need to separate the cells. It enables an easy extraction of the DNA with a high degree of purity. It is not necessary to employ...
hazardous organic solvents like phenol, which in previous works has been described as able to inhibit Taq polymerase (5). Finally, this method achieves an optimum visualization of the PCR product without requiring a second-stage amplification, which reduces the risks of carryover contamination (8).

One of the characteristics of brucellosis is its marked tendency to produce relapses once the correct treatment is concluded (2). The diagnosis of these relapses is difficult by conventional methods. In this study the three patients who suffered a relapse had a positive PCR, which became negative after they completed the treatment and remained negative at 2-, 4-, and 6-month follow-ups. Although these data appear very promising, further studies with a sufficiently large group of patients are necessary to determine that the test really becomes negative after the conclusion of treatment and remains so for those who have a favorable outcome but becomes positive again for those who suffer a relapse. If this can be confirmed, then PCR assay could become the method of choice for the diagnosis and follow-up of patients with brucellosis.

Finally, it is important to consider the technical difficulties and costs associated with carrying out a PCR-based probe. In our experience, this technique is not too complex; the infrastructure necessary is within the financial reach of any clinical microbiology laboratory habitually processing samples from patients with brucellosis, and a peripheral blood sample can be stored and sent to a laboratory at −20°C with complete assurance. In our center, for any patient suspected of having brucellosis, two blood cultures are obtained and the corresponding material of this diagnostic approach is similar to that required for those who suffer a relapse had a positive PCR, which became negative after they completed the treatment and remained negative at 2-, 4-, and 6-month follow-ups. Although these data appear very promising, further studies with a sufficiently large group of patients are necessary to determine that the test really becomes negative after the conclusion of treatment and remains so for those who have a favorable outcome but becomes positive again for those who suffer a relapse. If this can be confirmed, then PCR assay could become the method of choice for the diagnosis and follow-up of patients with brucellosis.

In conclusion, the peripheral-blood-based PCR assay described here is highly sensitive and specific, easy to perform, and rapid (providing a result to a clinician in less than 6 h), and it also avoids the risks to laboratory personnel associated with handling the microorganism. It may, therefore, soon become a technically feasible approach for the diagnosis of human brucellosis.

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