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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 100%, 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.

Brucellosis is a zoonosis transmittable to humans. The disease exists worldwide, especially in the Mediterranean basin, the Middle East, India, and Central and South America (16), and is an important public health problem in these areas. World Health Organization figures put the number of new cases of brucellosis at more than 500,000 per year (11).

The clinical picture of brucellosis is very nonspecific and may, moreover, show great variability (7). Its diagnosis, therefore, requires microbiological confirmation by means of the isolation of the germ or demonstration of the presence of specific antibodies by serological tests.

Peripheral blood is the clinical sample most commonly used for isolation of *Brucella* spp. With acute forms produced by *Brucella melitensis*, the number of positive results from blood cultures is usually high, i.e., 70 to 80% of samples (2). This figure is notably reduced, however, with samples from patients with long illness and focal complications (meningitis, endocarditis, spondylitis, orchiepididymitis, etc.) and with samples from patients whose infections were caused by *Brucella abortus* and *Brucella suis*, where the percentage of positive samples rarely exceeds 30 to 50% (6, 12). Furthermore, culture of blood is a time-consuming procedure requiring a prolonged incubation (23). In addition, *Brucella* spp. are class III pathogens, since their handling poses considerable risk to laboratory personnel (19).

A large number of different tests have been used for the serological diagnosis of brucellosis, thus demonstrating the lack of an ideal technique (24). The sensitivities of the serological tests range from 65 to 95%, but their specificities in areas where brucellosis is endemic are low because of the high prevalence of antibodies in the healthy population (3). Moreover, most serological tests can produce cross-reactions with other bacteria (24) and also exhibit important limitations with samples taken in the early phases of the disease, from persons exposed professionally, from patients with a recent history of brucellosis, and from patients who relapse (3, 12).

Amplification of DNA by PCR is currently used to diagnose several infectious diseases caused by fastidious or slowly growing bacteria. Although prior studies have demonstrated the possibility of detecting small amounts of *Brucella* DNA in pure cultures and animal samples by means of PCR (9, 21), information concerning the use of this technique in the diagnosis of human brucellosis is scarce.

In this study we investigated the potential role of a single-step peripheral-blood-based PCR assay to diagnose human brucellosis and compared it with conventional diagnostic methods.

MATERIALS AND METHODS

Clinical specimens. A total of 50 peripheral blood samples were obtained from 47 consecutive patients with brucellosis diagnosed in the Infectious Diseases Unit of "Carlos Haya" Regional Hospital, Malaga, Spain, over a period of 18 months. Three of the patients provided two samples each: one corresponding to the initial episode and the other corresponding to a relapse. The samples were taken before adequate antibiotic treatment was begun. In 35 cases (70%) the diagnosis of brucellosis was established by isolation of *Brucella* in blood culture, and in the other 15 cases (30%) diagnosis was based on a compatible clinical picture together with the presence of high titers of antibrucella antibodies or a fourfold or greater increase in the initial titers in two paired serum samples drawn 2 to 3 weeks apart. High titers were considered to be $\geq$1/160 for Wright's seroagglutination test and $\geq$1/320 for Coomb's antibrucella test.

Control blood samples were obtained from 60 subjects, composed of 15 patients with febrile syndromes of other defined etiologies initially involving a differential diagnosis with brucellosis (six cases of bacteremia, which were three cases of *Escherichia coli* infection, one case of *Klebsiella pneumoniae* infection, one case of *Proteus mirabilis* infection, and one case of *Staphylococcus aureus* infection; two cases of acute cytomegalovirus infection; and one case each of secondary lues, psittacosis, inflammatory bowel disease, pyogenous spondylitis, Still's disease, infected aortic aneurysm, and *Coxiella burnettii* infection), 20 asymptomatic subjects either professionally exposed or with a history of brucellosis in the previous 12 months and with persistent high titers of antibrucella

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antibodies, and 25 healthy subjects with no history of brucellosis or exposure to *Brucella*.

Bacteriological and serological techniques. Two blood cultures, Wright's seroagglutination test and Coomb's antibrucella 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 (1), 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 subcultured 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 Brucelosis, Valladolid, 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 erythrocyte lysis solution (320 mM saccharose, 5 mM Mg_2Cl, 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 NH_4Cl, 24 mM Na_2-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 at room temperature, and 100 μl of ammonium acetate (7.5 M) was added, followed 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 A_{260} and A_{280}.

DNA amplification. The PCR target sequence of 223 bp present on a gene encoding a 31-kDa *B. abortus* antigen was selected for amplification. This sequence 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, 50 μl of reaction mixture contained 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 nM each; Pharmacia LKB, Barcelona Spain), 1.25 U of *Taq* polymerase (Boehringer), 2 to 4 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 denaturation 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 expected 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 processed exactly as described above to monitor contamination with *Brucella* DNA and were negative in all experiments. Positive controls with genomic DNA isolated from a suspension of *B. abortus* B-19 and *B. melitensis* Rev-1 kindly supplied by the Department of Agriculture of the Regional Government of Andalucia were also included in each experiment. All PCRs were carried out in duplicate.

Purification and sequencing of PCR product. In order to confirm the identities of the amplified fragments, the PCR products were purified and sequenced. Template DNA was obtained by PCR amplification as described above.

(i) Purification. The 223-bp PCR product was purified with a Centricon-100 device (Amicon, Beverly, Mass.). The reaction mixture was diluted with 2 ml of TE buffer (10 mM Tris [pH 8.0], 1 mM EDTA) and placed into a Centricon-100 device, and the unit was spun at 1,000 × *g* for 20 min. After the initial spin, the retentate was diluted with another 2 ml of TE buffer and centrifuged as described above. The retentate containing the PCR product was recovered by inverting the Centricon device and centrifuging the reaction mixture at 500 × *g* for 2 min (13).

(ii) Sequencing. The DNA sequence of the PCR product was determined with a Thermo Sequenase kit (Thermo Sequenase cycle sequencing with 7-deaza-dGTP and fluorescent 1 dye primer labeling). The assay was performed according to the protocol provided with the Thermo Sequenase sequencing kit (Amersham International, Amersham, United Kingdom).

Dot blot analysis. The specificity of the amplification PCR assay was determined by dot blot hybridization of PCR products. Samples of 10 μl were spotted onto a Hybond-N^+ nylon membrane (Amersham International) with a Biot-Dot apparatus (Bio-Rad Laboratories, Madrid, Spain). Hybridization was made at 55°C for 3 h with 5-pmol/ml probe BR-1 (5′-TCA GAC GTT GCC TAT TGG GCC-3′), derived from the amplified sequence of *Brucella*, which had been labeled with fluorescein at its 5′ end. Washing was performed at the same temperature as hybridization (twice for 15 min each time at 55°C) according to the specifications of the manufacturer (Tropix Inc., Bedford, Mass.). Finally, membranes were exposed to a radiographic film for 15 min.

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

Characteristic	Values
No. of patients/no. of samples studied	47/50
Demographics	
Male/female	37/10
Mean age in years (range)	37.9 (14–91)
Clinical characteristics	
Mean duration of symptoms in days (range)	25.5 (2–120)
No. (%) of patients with fever	50 (100)
No. (%) of patients with focal forms[a]	12 (25.5)
Diagnostic tests	
No. (%) of patients with titers ≥1/160 by Wright's seroagglutination test	37 (74)
No. (%) of patients with titers ≥1/320 by Coombs' antibrucella test	32 (64)
No. of patients with positive blood cultures[b]	35 (70)

[a] Two cases of prostatitis, one case of pneumonitis, and nine osteoarticular cases (two cases of sacroiliitis, two cases of oligoarthritis, two cases of olecranon bursitis, two cases of monoarthritis, and one case of spondylitis).

[b] *B. melitensis* was isolated from all cultures.

RESULTS

Of the 47 patients with brucellosis, 13 (27.6%) acquired their infections through direct contact with livestock, 14 (29.8%) acquired their infections by consuming nonpasteurized dairy products, 15 (31.9%) acquired their infections possibly from either of these two sources of infection, and the remaining 5 (10.6%) acquired their infections from an unknown source. Three of these patients (6.4%) had relapses 5, 9, or 10 months after concluding treatment, for a total of 50 cases.

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 other 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 initial 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 specificity 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 history of fever accompanied by a new increase in the titer of antibrucella 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

Result of routine procedure	No. of samples (%)	
	Positive by PCR	Negative by PCR
Positive by serological test[a] and blood culture	27 (54)	0
Negative by serological tests[b] and positive by blood culture	8 (16)	0
Positive by serological tests and negative by blood culture	15 (30)	0
Total	50 (100)	0 (100)

[a] Either Wright's seroagglutination test (titer, ≥1/160) or Coombs' antibrucella test (titer, ≥1/320).

[b] 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).

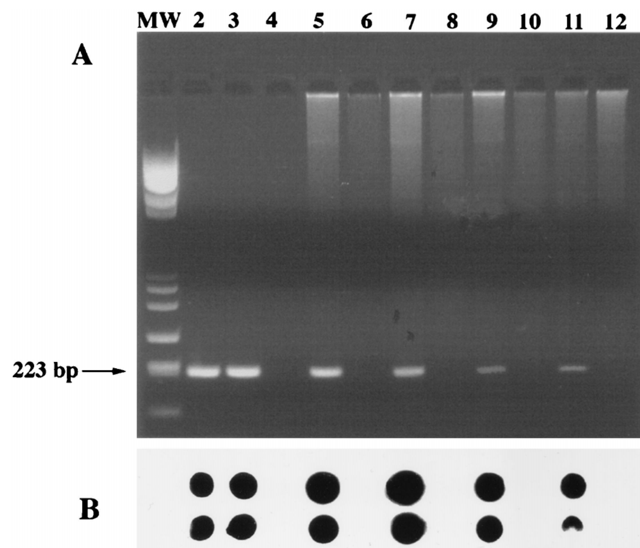

FIG. 1. (A) Agarose gel electrophoresis and ethidium bromide staining. Lane MW, DNA ladder (223 bp); lanes 2 and 3, positive controls (*B. abortus* B-19 and *B. melitensis* Rev-1, respectively); lane 4, no DNA added; lanes 5 and 7, DNAs from two patients with brucellosis and positive blood cultures; 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 psittacosis; lane 12, DNA from a patient with past brucellosis 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 serological tests are performed. The cost in personnel and material of this diagnostic approach is similar to that required to make a PCR-based probe.

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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