Development and Evaluation of a PCR–Enzyme-Linked Immunosorbent Assay for Diagnosis of Human Brucellosis

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In order to overcome some of the limitations of conventional microbiological techniques in the diagnosis of human brucellosis, a simple PCR–enzyme-linked immunosorbent assay (PCR-ELISA) was developed. After amplification of a 223-bp sequence of a gene that codes for the synthesis of an immunogenetic membrane protein specific for the *Brucella* genus (BCSP31), the digoxigenin-labeled amplified product was hybridized with a biotinylated capture probe which was complementary to the inner part of the amplicon. The hybrid was captured on streptavidin-coated microtiter plates and detected by using an antidigoxigenin Fab-peroxidase conjugate. The detection limit of the PCR-ELISA in a background of 3.5 µg of human genomic DNA was 10 fg (two bacterial cells). The PCR-ELISA showed an analytical sensitivity higher than that of ethidium bromide staining and equal to that obtained by conventional PCR followed by dot blot hybridization. In 59 peripheral blood samples from 57 consecutive patients with active brucellosis and 113 control samples, the PCR-ELISA was found to be 94.9% sensitive and 96.5% specific, whereas the sensitivity of the blood culture was only 70.1%. Since the assay can be performed in 1 day, is very reproducible, is easily standardized, and avoids the risk of infection in laboratory workers, this PCR-ELISA seems to be a practical and reliable tool for the diagnosis of human brucellosis.

Brucellosis is a worldwide zoonosis and represents an important public health problem in many countries, especially around the Mediterranean basin and in the Middle East, India, and Central and South America (11). More than 500,000 new cases are reported each year, and according to the World Health Organization, this figure underestimates the magnitude of the problem (34). The incidence of the disease in the United States is low, although only an estimated 4 to 10% of cases are in fact recognized (8).

Due to its heterogeneous and poorly specific clinical symptomatology, the diagnosis of brucellosis always requires laboratory confirmation, either by isolation of the pathogen or by demonstration of specific antibodies. Blood cultures provide the best yield for microbiological diagnosis, with a sensitivity of 53 to 90% in cases of acute brucellosis caused by *Brucella melitensis* (2, 10, 35). However, this sensitivity is considerably reduced in patients with long-term clinical courses, in patients with focal complications, and in infections caused by *Brucella abortus* and *Brucella suis*. Furthermore, handling of these microorganisms represents a high risk for laboratory personnel, since *Brucella* spp. are class III pathogens (36, 38).

There is presently a wide battery of serological tests which can be used for diagnosis of human brucellosis, although they each have important limitations. Their sensitivity is poor in the early stage of the disease, during which the levels of antibodies can still be low, and their specificity is reduced in areas where

the disease is highly endemic, in exposed professionals, and in the frequent relapses of the disease (3, 17, 37).

Our group has recently reported that the use of a PCR technique with blood samples provides better results than conventional microbiological techniques in the diagnosis of both primary infection and relapses, as well as for focal complications of the disease (23, 24, 28). Nevertheless, interpretation of the results of conventional PCR assays is fairly subjective, normally requires an agarose gel electrophoresis as well as handling of toxic products such as ethidium bromide, and sometimes requires a dot or Southern blot analysis for interpretation of uncertain results. These approaches are often time-consuming and poorly suited for use in general diagnostic laboratories (14, 18, 41). In an attempt to avoid all of these difficulties, we have now developed a microplate PCR-hybridization assay (PCR–enzyme-linked immunosorbent assay [PCR-ELISA]) and have evaluated its diagnostic yield with peripheral blood samples from patients with brucellosis.

MATERIALS AND METHODS

Clinical specimens. From January 1999 to December 2001, a total of 59 peripheral blood samples were taken from 57 consecutive patients with active brucellosis diagnosed in the Infectious Diseases Unit of Carlos Haya University Hospital, Málaga, Spain, before starting appropriate antibiotic treatment. Two of the patients provided two samples each, one corresponding to the initial episode and the other corresponding to a relapse. The diagnosis of brucellosis was established according to one of the following criteria: (i) isolation of *Brucella* spp. in blood culture or other clinical samples or (ii) the presence of a compatible clinical picture together with the demonstration of specific antibodies at significant titers or seroconversion. Significant titers were considered to be a Wright's seroagglutination titer of $\geq 1/160$ or an immunocapture-agglutination anti-*Brucella* test titer of $\geq 1/320$.

Control blood samples were obtained from 113 subjects, composed of 30 patients with febrile syndromes of other defined etiologies which had initially

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involved a differential diagnosis with brucellosis (10 patients with bacteremia [4 with primary *Staphylococcus aureus*, 3 with *Staphylococcus epidermidis*, and 1 each with *Proteus mirabilis*, *Klebsiella pneumoniae*, and *Escherichia coli*], 5 patients with vertebral osteomyelitis [2 tuberculous, 2 *S. aureus*, and one *E. coli*], 2 patients with Q fever, 2 patients with *Plasmodium falciparum* malaria, 1 patient with acute cytomegalovirus infection, 1 patient with Mediterranean exanthematic fever, 1 patient with extrapulmonary tuberculosis, 1 patient with pyogenic liver abscess caused by *E. coli*, 4 patients with different autoimmune diseases, 1 patient with metastatic prostate adenocarcinoma, and 2 patients with lymphoma [1 with Hodgkin and one with non-Hodgkin lymphoma]), 41 asymptomatic patients with a history of brucellosis treated correctly during the previous 12 months and who were discharged after 1 year of follow-up with no evidence of relapse, 14 asymptomatic subjects professionally exposed to *Brucella* infection with persistent high titers of antibruella antibodies, and 28 healthy subjects (blood donors) with no history of brucellosis or exposure to *Brucella* spp.

Bacteriological and serological techniques. Two blood cultures, as well as a serological battery including the rose Bengal plate agglutination test, Wright's seroagglutination test, and immunocapture-agglutination test, were done for all of the patients with active brucellosis, febrile syndromes of other etiologies, or a previous history of brucellosis. Blood cultures were processed in a semiautomatic BACTEC 9240 system (Becton Dickinson Diagnostic Instrument Systems, Sparks, Md.), according to standard techniques (25), with incubation being maintained for 30 days and blind subcultures performed on chocolate agar and brucella agar (Biomedics, San Sebastian de los Reyes, Madrid, Spain) after 10, 20, and 30 days. These subcultures were incubated at 37°C in a 5 to 10% CO₂ atmosphere for 3 days. If growth appeared, the suspected colonies were identified by colonial morphology; Gram staining; oxidase, catalase, and urease tests; and positive agglutination with specific antiserum. All isolated strains were sent to the National Brucellosis Reference Laboratory in Valladolid, Spain, for definitive identification and biotyping. The rose Bengal plate agglutination test and Wright's seroagglutination test were performed according to previously described techniques (1). The determination of total anti-*Brucella* antibodies was made by an immunocapture-agglutination test (Brucellacapt; Viracell SL, Santa Fe, Spain) according to the manufacturer's instructions (26).

Isolation of DNA from clinical blood samples. A modification of the method described by Miller et al., and adapted by us, was used (21). Briefly, 1 ml of blood, collected in sodium citrate and stored at -20°C, was suspended in 0.5 ml of erythrocyte lysis solution (320 mM saccharose, 5 mM Mg₂Cl₂, 1% Triton X-100, and 10 mM Tris HCl [pH 7.5]), mixed, and centrifuged at 15,000 × g for 2 min. The supernatant was discarded, and the leukocyte pellet was washed with 1 ml of sterile Milli-Q water. The supernatant was then removed, and to ensure elimination of any remains of the heme compound, 100 µl of an H₂O₂ (30%, wt/wt) solution (Sigma Chemical Company, Madrid, Spain) was added to the pellet and incubated for 2 to 5 min at room temperature. The H₂O₂ was then removed with the tip of the pipette. Template DNA was obtained from the leukocytes as follows. Four hundred microliters of nucleic lysis buffer (60 mM NH₄Cl and 24 mM Na₂ EDTA [pH 8.0]) containing proteinase K (10 mg/ml) (Sigma) and sodium dodecyl sulfate (10%) was added to the pellet, 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 to each tube. The tubes were shaken for 30 s, 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 alcohol were added, and the tubes were inverted several times until the DNA was precipitated. DNA was recovered by centrifuging the samples at 15,000 × g for 10 min, and the pellets were rinsed with 1 ml of 70% ethanol, dried, and suspended in 40 µl of sterile Milli-Q water. The concentration and purity of the DNA were then determined spectrophotometrically by readings of A₂₆₀ and A₂₈₀.

Extraction of genomic DNA from *B. abortus* B-19. Bacterial cells were washed twice with phosphate-buffered saline (PBS) and pelleted by centrifugation. The pellet of bacteria was suspended in a solution containing 68 µl of 20-mg/ml lysozyme (Sigma), 40 µl of 10% sodium dodecyl sulfate, 80 µl of lysis buffer (375 mM NH₄Cl, 120 mM Na₂-EDTA [pH 8.0]), and 157 µl of sterile Milli-Q water, mixed, and incubated for 30 min at 37°C. After this incubation, 40 µl of 10-mg/ml proteinase K (Sigma) was added, mixed gently by inverting the tube several times, and incubated for 30 min at 55°C. Purification and precipitation of bacterial DNA were done as for the blood samples (described above). The concentration and purity of the DNA were then determined spectrophotometrically by readings of A₂₆₀ and A₂₈₀.

DNA amplification. A 223-bp fragment from the conserved region of the gene which encodes an immunogenic membrane protein of 31 kDa of *B. abortus* specific to the *Brucella* genus and present in all its biovars (20) was amplified. A pair of 21-nucleotide primers, B4 (5' TGG CTC GGT TGC CAA TAT CAA 3')

and B5 (5' CGC GCT TGC CTT TCA GGT CTG 3') (Amersham Pharmacia Biotech, Barcelona, Spain), described by Baily et al. were used in the amplification process (4). PCR was performed in a 50-µl mixture containing template DNA; PCR buffer (10 mM Tris HCl [pH 8.4], 50 mM KCl, 1.5 mM MgCl₂); a 200 nM concentration of each of the PCR primers; a 200 µM concentration (each) of dATP, dCTP, and dGTP; 190 µM dTTP; 10 µM digoxigenin-11'-dUTP (Roche Diagnostics, Barcelona, Spain); and 1.25 U of *Taq* polymerase (Roche Diagnostics). The reaction was performed in a DNA thermal cycler without mineral oil (model 2400; Perkin-Elmer, Norwalk, Conn.). PCR consisted of a preheating at 93°C for 5 min; 35 cycles of 90°C for 1 min, 60°C for 30 s, and 72°C for 1 min; and incubation at 72°C for 7 min. Positive controls based on DNA from *B. abortus* B-19 were included in all the tests, as were negative controls which contained all of the elements of the reaction mixture except DNA. Fifty microliters of each PCR-amplified sample was loaded onto each lane on a 2% agarose gel and stained with 2 µg of ethidium bromide per ml to determine the sizes of the amplified products. To guarantee the reliability of the results, all samples were processed in duplicate.

Detection of PCR products by digoxigenin-ELISA. The reaction was performed on commercial streptavidin-coated plates (2.5 µg/ml) (Viracell SL). The plates were prepared in large lots with minimal well-to-well variations and were provided stabilized and dry packed to avoid day-to-day variations. Briefly, 40-µl aliquots of the PCR product were mixed with 80 µl of 1× SSC (0.15 M NaCl plus 0.015 M sodium citrate)-0.5% Tween 20 containing a 5' biotin-labeled probe (5'-TCAGACGTTGCCTATTGGGCC-3') (Amersham Pharmacia Biotech). Denaturation (94°C for 15 min) and hybridization (55°C for 60 min) were carried out in solution in the thermal cycler. A 50-µl aliquot of the reaction volume was added in duplicate to the wells of streptavidin-coated microtiter plates and incubated at 37°C for 30 min. Plates were washed two times with 200 µl of 0.1× SSC-0.1% sodium dodecyl sulfate per well and two times with 200 µl of PBS (pH 7.2)-0.05% Tween 20 per well. A 50-µl aliquot of antidigoxigenin Fab-peroxidase conjugate diluted 1:3,500 in a peroxidase stabilizer buffer (Viracell SL) was added to each well and incubated at 37°C for 30 min. After four washings with 200 µl of PBS (pH 7.2)-0.05% Tween 20, color was developed by the addition of 50 µl of tetramethylbenzidine (Viracell SL). The color reaction was stopped after incubation for 15 min at 37°C by the addition of 50 µl of 0.5 M H₂SO₄ (16). The A₄₅₀ of each specimen was measured in an ELISA reader (MR-3100T; Dynex Technologies Inc., Chantilly, Va.) as a net value after subtracting the value for the blank. Each PCR-digoxigenin-ELISA was performed with positive (100 ng of DNA from *B. abortus* B-19) and negative (distilled water) controls. All experiments were performed in duplicate, following contamination-free guidelines to prevent false-positive results.

An assay was considered positive if the mean optical density value was more than three standard deviations above the mean value for the healthy controls. As the mean absorbance value and standard deviation for the 28 healthy subjects studied was 0.12 ± 0.04, a sample was considered positive when the absorbance of the two measurements was greater than 0.3. In the event of discordant results between duplicate PCRs, i.e., one positive and one negative, the amplification procedure was repeated with a different extraction.

Statistical analysis. Data were analyzed with the help of SPSS 9.0 for Windows. To evaluate the assay precision, the respective intra- and interassay coefficients of variation (CVs) were calculated. Sensitivity, specificity, positive and negative predictive values, and likelihood ratios and their 95% confidence intervals were calculated by a Roc Curve Analyzer program as described by Centor (6).

RESULTS

Reproducibility. As a preliminary evaluation of the diagnostic performance of the PCR-ELISA, its reproducibility was determined. Twenty-three negative controls and 22 positive controls consisting of 100 ng of *B. abortus* DNA were amplified in the same run to measure the intra-assay precision. To calculate the interassay precision, 38 negative and 22 positive control samples were amplified on consecutive days in seven different assays. The absorbances of the negative controls were 0.089 ± 0.005 for the intra-assay repetitions and 0.098 ± 0.022 for the interassay determinations, with CVs of 6 and 22.4%, respectively. The positive controls showed absorbances of 3.1 ± 0.16 for the intra-assay repetitions and 3.05 ± 0.35 for the interassay determinations, with CVs of 5 and 11.5%, respec-

FIG. 1. Analytical sensitivity of PCR followed by agarose gel electrophoresis and ethidium bromide staining (A), dot blot hybridization (B), and PCR-ELISA (C) for 10-fold serial dilutions of pure *B. abortus* B-19 cultures ranging from 100 ng to 10 fg (corresponding to 20,000,000 to 2 bacterial cells) artificially spiked with 3.5 μ g of human genomic DNA.

tively (Fig. 1). The absorbance value for the healthy controls (blood donors) was 0.12 ± 0.04 . None of these samples had a value greater than the A_{450} cutoff value.

Analytical sensitivity. The detection limit was determined by PCR amplification of a 10-fold serial dilution of a pure culture of *B. abortus* B-19. The detection limit of the method was 10 fg of DNA, which is the equivalent of approximately two bacterial cells. The sensitivity of the method remained unchanged after adding human DNA to the sample at the usual concentration for a clinical sample. PCR-ELISA showed an analytical sensitivity higher than that of ethidium bromide staining and equal to that obtained by conventional PCR followed by dot blot hybridization (Fig. 1).

Detection of *Brucella* spp. in clinical specimens. Of the 57 patients included in the study, 40 (70.2%) had positive blood cultures, and the other 17 (29.8%) were diagnosed based on clinical and serological criteria. All of the strains isolated were identified as *B. melitensis*. Forty-four patients (77.2%) had fever with no apparent focus, and 13 (22.8%) had different focal complications. The most relevant clinical and epidemiological data are shown in Table 1.

Wright's seroagglutination test and the immunocapture-agglutination test had titers within the diagnostic range in 70.2 and 91.2% of cases, respectively. Both tests were negative or showed titers below the diagnostic range in 5.3% of cases.

Of the 59 samples from the patients with brucellosis, 56 (94.9%) were positive in the PCR-ELISA and 3 (5.1%) were negative. The three false-negative results corresponded to three patients with positive blood cultures. Of the 56 positive

samples, 49 (87.5%) were positive at the first measurement, and 7 (12.5%) were positive at a second measurement after an initially doubtful result. The absorbance for positive samples was 1.69 ± 0.74 . Two patients had a relapse, at 4 and 5 months after concluding their therapeutic schedules, respectively. One had compatible symptoms and positive blood cultures, and the other developed lumbar spondylitis and had a marked rise in titer from previous serologies. The PCR-ELISA was initially positive in both patients, became negative at the end of treatment, and was again positive in the relapse.

Four control samples (3.5%) had a positive PCR-ELISA. These were from one patient with a hepatic abscess due to *E. coli* and *Streptococcus intermedius*; two patients with a history of brucellosis 6 months previously with no clinical, serological, or bacteriological evidence of relapse; and one patient with sequelae from a brucellar spondylitis correctly treated 3 years previously. Thus, the sensitivity and specificity of the PCR-ELISA were 94.9 and 96.5%, respectively. Table 2 shows the diagnostic yield of the PCR versus the conventional cultures.

DISCUSSION

Despite the advances resulting from the introduction of new semiautomatic techniques for processing blood cultures and the availability of many serological tests for the detection of specific antibodies, there still are important problems in the diagnosis of brucellosis. Blood cultures lack sufficient sensitivity, and serological tests are not very specific in areas where the disease is highly endemic. On the other hand, a high proportion of brucellosis patients report nonspecific symptoms after conclusion of their treatment, and as there are no well-defined criteria for complete recovery from brucellosis, it is often difficult to decide whether the disease in these patients is following a favorable course or, on the other hand, is relapsing (2, 37).

Previous studies have demonstrated that the amplification of

TABLE 1. Clinical features of patients with brucellosis

Characteristic	Value
% Male/female	73.2/26.8
Mean age (yr) ^a	48.9 (19–81)
Mean duration of symptoms (wk) ^a	4.3 (1–54)
No. (%) with:	
Fever	57 (100)
Chills	45 (72.3)
Sweating	44 (70.9)
Hepatomegaly	17 (34.2)
Splenomegaly	14 (28.2)
Focal complications	13 (22.8)
No. (%) with focal complications as follows:	
Osteoarticular ^b	9 (69.2)
Hepatic abscess	1 (7.7)
Splenic abscess	1 (7.7)
Orchepididymitis	1 (7.7)
Suppurative lymphadenitis	1 (7.7)

^a Value in parentheses is the range.

^b Six cases of spondylitis and one case each of coxitis, sternoclavicular arthritis, and peripheral arthritis.

TABLE 2. Diagnostic yield of PCR-ELISA with peripheral blood samples^a

Test	Sensitivity	Specificity	Positive predictive value	Negative predictive value	Positive likelihood ratio	Negative likelihood ratio
PCR-ELISA	94.9 (89.3–100)	96.5 (93.2–99.9)	93.3 (87.0–99.6)	97.4 (94.4–100)	27.3 (10.4–61.6)	0.05 (0.02–0.16)
Blood culture	69.5 (57.7–81.2)	100	100	82.9 (75.6–90.1)	ND	0.31 (0.21–0.45)

^a Values are percentages. Values in parentheses are 95% confidence intervals. ND, not done for mathematical reasons (division by zero).

specific sequences of *Brucella* DNA by PCR is a much more sensitive technique than blood cultures and that their positivity is very specific for active illness (23, 40). However, most PCR procedures followed by gel agarose electrophoresis and dot blot or Southern hybridization have a limited number of samples that can be appropriately analyzed during one electrophoresis run.

In order to improve the detection of *Brucella* spp. by PCR, we first developed and then optimized a simple hybridization-based microtiter plate enzyme immunoassay for the detection of amplified *Brucella* DNA and then evaluated its yield compared with conventional microbiological techniques with a large sample of patients with brucellosis.

The precision of our PCR-ELISA can be considered good, as the intra-assay variation was lower than 10% and the inter-assay variation was around 10 to 20%, percentages which are similar to, or even lower than, those for other PCR assays based on similar principles (12, 29).

Bearing in mind that the inoculum normally found in patients with *Brucella* bacteremia is very small, the detection capacity of any PCR technique for the diagnosis of brucellosis should be very high. We have previously shown that conventional PCR is able to amplify 10 fg of bacterial DNA. Nevertheless, dot blotting was required for the correct signal identification of the smallest amount of inoculum on agarose gel electrophoresis (28). Furthermore, in clinical samples, the high concentrations of human DNA in peripheral blood samples occasionally interfere with the desired amplification (22). Our evaluation of the analytical sensitivity of the PCR-ELISA technique showed it to amplify correctly 10 fg of bacterial DNA, and this amplification was not obstructed by the presence of high concentrations of leukocyte DNA. Others, using similar methods, have reported the detection capacity of PCR-ELISA to be similar to that found with the combined use of conventional PCR and Southern hybridization (16, 19, 39).

The sensitivity of blood cultures in the diagnosis of brucellosis ranges from 53 to 90%. In the present study it was 70.1%, which is markedly lower than the 94.9% for the PCR-ELISA. This is especially relevant if we consider that the sample of patients with brucellosis was very representative of the true clinical situation, as it included not only patients in the acute phase of the disease but also others with a long disease evolution, with and without focal complications.

The existence of three false-negative results in patients with positive blood cultures is hardly surprising, bearing in mind that the amount of blood in each blood culture bottle is 8 to 10 ml, which is a much greater volume than the 0.5 ml for the amplification sample. Sample size is a major limitation for PCR-based assays; the use of very small samples from patients with small concentrations of circulating brucellae could result

in the absence of amplifiable target DNA in the sample to be studied (15, 39).

Although this study was not designed to analyze the usefulness of the PCR-ELISA during the posttreatment follow-up of patients with brucellosis, the positivity of the test in the two patients with relapses appears to confirm our previous data concerning the usefulness of molecular techniques in the early diagnosis of relapses (23).

The specificity of the PCR-ELISA can be considered good, although the existence of four patients with false-positive results requires a search for an explanation. Previous studies, as well as our own results with the B4 and B5 primers, have demonstrated the high specificity of the technique with a wide panel of microorganisms. Only the DNA from *Ochrobactrum* spp., pathogens very closely related phylogenetically to *Brucella* spp., has been amplified with these primers (4, 5, 30). The test was positive in only one patient with a disease different from brucellosis. Nevertheless, this patient, who had a polymicrobial hepatic abscess due to *E. coli* and *S. intermedii*, habitually consumed nonpasteurized dairy products and had low titers of antibrucella antibodies (Coomb's antibrucella test titer, 1/40). Thus, taking into account that cross-amplification with *E. coli* or *S. intermedii* has not been reported before, it is possible that the patient was coinfecting with *Brucella* spp., since the existence of subclinical and self-limiting episodes of infection is common in regions of endemicity (9, 27). Likewise, given the high sensitivity of the technique, the positivity of the test in the two patients with brucellosis treated correctly 4 and 5 months previously and who recovered favorably with no evidence of relapse could be due to the amplification of bacterial remains or nonviable, intramacrophage microorganisms. Nevertheless, these findings suggest caution in the interpretation of a positive result during the early posttherapeutic period, even though in a previous study of the follow-up of a cohort of 30 patients we demonstrated that this is an uncommon event (23).

As well as the high sensitivity and specificity of this PCR-ELISA, there is also the advantage that with this procedure the detection of the PCR product is rapid, easy, and objective; it requires no electrophoresis apparatus, UV light, or darkroom, and furthermore the use of toxic chemical agents such as ethidium bromide is avoided. Moreover, the technique allows the simultaneous handling of a large number of samples and can be automated, making it very attractive for use in any clinical laboratory (7, 31, 33). *Brucella* spp. are class III pathogens requiring special protection measures. PCR-based assays almost completely obviate the need for direct handling of the pathogen, thus drastically reducing the risk of infection of laboratory personnel (13, 32, 36). Finally, any sample can be stored at -20°C until processing, thus enabling it to be col-

lected by any physician and either processed immediately or stored and safely sent to another laboratory if necessary.

In conclusion, the high sensitivity and specificity of this PCR-ELISA, together with its speed, versatility in sample handling, and risk reduction for laboratory personnel, make this technique a very useful tool for the diagnosis of brucellosis.

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