Rapid Laboratory Confirmation of Human Brucellosis by PCR Analysis of a Target Sequence on the 31-Kilodalton *Brucella* Antigen DNA

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We developed a PCR-based assay for the rapid and specific laboratory diagnosis of human brucellosis directly from whole blood. Specimens were collected in EDTA tubes from 17 patients with acute serologic brucellosis and 3 patients with chronic relapsing brucellosis as determined by serologic tests and the patient's clinical picture. DNA was extracted from peripheral mononuclear cells obtained from the blood of patients with brucellosis and control individuals. Specific primers for the PCR amplification of a 223-bp region on the sequence encoding the 31-kDa immunogenic *Brucella abortus* protein (BCSP 31) were used. All amplicons had the expected size of 223 bp. The specificity of amplification was determined by Southern hybridization and restriction endonuclease analysis. DNA extracted from blood taken from 30 healthy individuals as well as from 9 patients with typhoid fever did not show any amplification with the primers used. The test proved to be rapid and specific for the laboratory confirmation of acute human brucellosis. Further studies must be conducted to assess the utility of this test on additional patients with chronic relapsing brucellosis as well as patients under treatment.

Human brucellosis is a widespread zoonosis manifested by a septicemic febrile illness or localized infection of bone, tissue, or organ systems. It is transmitted by the ingestion of raw or unpasteurized milk and other dairy products, by direct contact with infected animal tissues, or by accidental ingestion, inhalation, or injection of the *Brucella* culture. Brucellosis is endemic in Lebanon and other Mediterranean countries.

The variable symptoms, the paucity of distinctive physical signs, and the occurrence of subclinical and atypical infections in both the acute and the chronic stages make the clinical diagnosis of human brucellosis difficult. Clinicians therefore rely substantially on laboratory confirmation, even though they are confident in most of the cases that the clinical picture is highly suggestive of brucellosis. The culture techniques are time-consuming and lack sensitivity for patients with chronic infections (5), and handling of the organism in the laboratory is hazardous. Conventional serologic techniques are insensitive for patients with chronic infections, although newer techniques may be an improvement. Cross-reactions with other gram-negative bacteria occur, giving false-positive results (5). In areas endemic, where brucellosis is a significant proportion of the well population may be seropositive (3); moreover, the interpretation of stable titers in the low or intermediate range (20 to 160) is difficult (2).

We have proposed a PCR-based method for the direct detection of *Brucella* organisms in clinical specimens. The PCR target sequence of the order of 223 bp present on a gene encoding a 31-kDa *Brucella abortus* antigen was selected for amplification because it is conserved in all *Brucella* species. The primers B4 and B5 used to amplify the target sequence were described previously by Baily et al. (1). These primers were tested on DNAs extracted from *Branhamella catarrhalis*,

Yersinia enterocolitica, *Campylobacter jejuni*, *Enterobacter aerogenes*, *Haemophilus influenzae*, *Legionella pneumophila*, and *Escherichia coli*, and no DNA amplification was detected (1). Moreover, a computer search of the GenBank and EMBL sequence databases did not identify any significant homology with the primers used (1). An estimated amount of 60 fg of *Brucella* DNA, equivalent to 20 bacterial cells, was detected by the method of Baily et al. (1).

In the present study, blood was collected from 20 patients with brucellosis. Seventeen patients had acute serologic brucellosis as determined by the clinical picture of brucellosis and an agglutination titer equal to or greater than 1:160, which was determined according to the manufacturer's specifications (Gamma Biologicals Inc., Houston, Tex.). The other three patients were considered to have chronic relapsing brucellosis, as determined by an agglutination titer of 1:160 lasting for more than a year, a history of brucellosis, undulating fever, and localized symptoms. Cultures were performed on two specimens from patients contracting acute brucellosis; one was culture positive and one was culture negative. Peripheral mononuclear cells were separated with Ficoll-Hypaque (Pharmacia LKB, Uppsala, Sweden), and total DNA was extracted by using a lysis buffer containing Triton X-100 detergent (0.5%) and proteinase K enzyme (2.5 mg/ml) (4). Positive controls were DNA extracts obtained from *Brucella melitensis* ATCC 23456 biotype 1 and *Brucella canis* ATCC 23365. Negative controls consisted of DNA extracts obtained from nine blood specimens taken from patients with typhoid fever and from 30 healthy individuals. Agglutination titers were determined at random for blood taken from 10 negative control patients and all typhoid patients. All were negative for *Brucella* species by serology. Total DNA extraction was done as described above.

PCR was done on all DNA extracts as described by Baily et al. (1), with slight modifications. A second PCR was done on all amplified products by using 10 μl of amplicons from the first PCR and the same PCR conditions in order to enhance the intensities of the bands. A Minicycler (MJ Research, Water-

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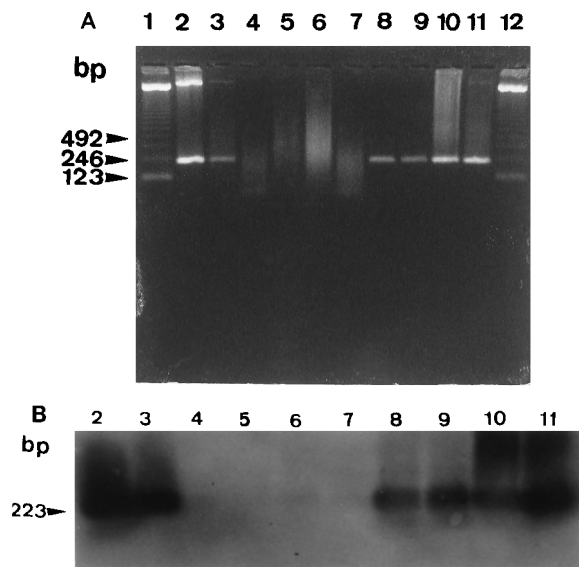
FIG. 1. (A) PCR products derived from *Brucella* DNA in patients with brucellosis. Lane 1, marker (123-bp ladder); lane 2, positive control (*B. melitensis* ATCC 23456); lane 3, positive control (*B. canis* ATCC 23365); lane 4, no DNA added; lanes 5 and 6, DNAs from two healthy individuals; lane 7, DNA from a typhoid fever patient; lanes 8 to 11, DNAs from three patients with acute brucellosis (lanes 8 to 10) and one patient with chronic brucellosis (lane 11); lane 12, marker (123-bp ladder). (B) Southern hybridization of *Brucella* amplicons with a digoxigenin-labeled probe. Lane 2, positive control (*B. melitensis* ATCC 23456); lane 3, positive control (*B. canis* ATCC 23365); lane 4, no DNA added; lanes 5 and 6, DNA from two healthy individuals; lane 7, DNA from a typhoid fever patient; lanes 8 to 11, DNA from three patients with acute brucellosis (lanes 8 to 10) and one patient with chronic brucellosis (lane 11).
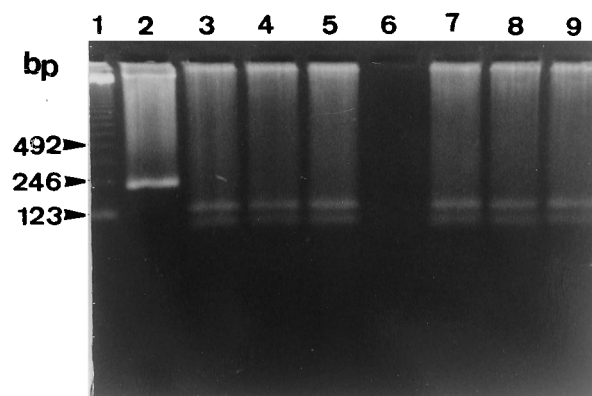

FIG. 2. Digested *Brucella* PCR products with *Hae*III restriction endonuclease. Lane 1, marker (123-bp ladder); lane 2, undigested positive control (*B. melitensis* ATCC 23456); lane 3, digested positive control (*B. melitensis* ATCC 23456); lane 4, digested positive control (*B. canis* ATCC 23365); lane 5, digested DNA from one patient with chronic brucellosis; lane 6, blank; lanes 7 to 9, digested DNA from three patients with acute brucellosis.

town, Mass.) was used for PCR amplification. Amplicons were electrophoresed on a 1% agarose (Sigma, St. Louis, Mo.) gel, stained with ethidium bromide (1 mg/ml), observed under UV light, and photographed with type 667 Polaroid film. The specificity of the amplification was confirmed by Southern hybridization by the method of Popovic et al. (6). A digoxigenin-labeled 25-mer *Brucella* probe (5'-CAAGGTGGAAGATTT GCGCCTTCTG-3') derived from the amplified sequence was used. The specificities of the amplicons were further confirmed by restriction analysis of the amplicons with *Hae*III restriction endonuclease. The assay was done according to the manufacturer's specifications (Pharmacia Biotech, Piscataway, N.J.). Digested DNA was electrophoresed on a 2.5% Nusieve agarose (FMC Bioproducts, Rockland, Maine) gel, the gel was stained with ethidium bromide, and the bands were visualized under UV light and photographed.

Our data have shown that PCR is useful for the rapid detection of *Brucella* DNA directly in blood specimens obtained from persons with brucellosis. The amplicons obtained from the blood of our positive controls as well as from the blood of 17 patients with acute serologic brucellosis and 3 patients with chronic relapsing brucellosis had the expected size of 223 bp by first and second PCRs. Figure 1A shows the amplicons obtained after a second PCR. The second PCR was shown, however, to enhance the intensities of the bands. This may be due to the reduced effect of inhibitors found in the first PCR. Southern hybridization and restriction analysis further confirmed the specificity of the amplification. Our data have shown that all amplicons hybridized with a digoxigenin-labeled probe derived from within the amplified sequence (Fig. 1B). Moreover, all amplified products were digested with *Hae*III

endonuclease, and the restriction patterns of digested *Brucella* DNAs from brucellosis patients matched those of the *Brucella* strains from the American Type Culture Collection (Fig. 2). DNA obtained from the blood of healthy individuals and patients with typhoid fever was not amplified with the *Brucella* primers.

The assay proved to be sensitive because it detected *Brucella* DNA sequences directly in blood specimens from patients with agglutination titers ranging between 1:160 and 1:5,120. The assay is also specific for *Brucella* species because DNA obtained from patients with acute and chronic brucellosis was amplified, and a 223-bp fragment similar to that from the positive controls was generated. Furthermore, all amplicons hybridized with the *Brucella* digoxigenin-labeled probe and had the same restriction pattern as that of *Brucella* strains from the American Type Culture Collection. The assay is also rapid because it can provide results to the clinician in less than 24 h. Moreover, it eliminates the hazards of handling the organism in the laboratory. Further work is required to assess the utility of this test for more chronic cases of infection and to follow up patients under treatment.

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