MAJOR ARTICLE

Comparison between LightCycler Real-Time Polymerase Chain Reaction (PCR) Assay with Serum and PCR–Enzyme-Linked Immunosorbent Assay with Whole Blood Samples for the Diagnosis of Human Brucellosis

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Background. To overcome some of the limitations of conventional microbiological techniques, polymerase chain reaction (PCR)–based assays have been proposed as a useful tool for the diagnosis of human brucellosis.

Methods. A single-blinded comparative study was undertaken that compared 2 different PCR assays: a SYBR Green I LightCycler-based Real-Time PCR assay (LC-PCR; Roche Diagnostic) with serum samples and a PCR–enzyme-linked immunosorbent assay (ELISA) with whole blood samples. Both assays amplify a 223-bp sequence of a gene that codes for the synthesis of an immunogenetic membrane protein specific for *Brucella* genus (BCSP31). We analyzed the diagnostic yield of these assays with 60 samples obtained from patients with active brucellosis and 37 samples obtained from a control group composed of patients with febrile syndromes of other defined etiologies, asymptomatic subjects with past brucellosis or exposure to *Brucella* infection who had persistently high titers of anti-*Brucella* antibodies, and healthy subjects.

Results. The sensitivities of LC-PCR with serum samples, PCR-ELISA with whole blood samples, and blood cultures were 93.3%, 90%, and 65%, respectively. Three control samples (8.1%) had a positive PCR-ELISA result, and 2 of these samples (5.4%) also had positive LC-PCR results. The specificity and positive likelihood ratios were 94.6% and 17.3, respectively, for LC-PCR and 91.9% and 11.1, respectively, for PCR-ELISA.

Conclusions. The diagnostic yield of LC-PCR with serum samples was higher than that of PCR-ELISA with whole blood samples. The speed and technical simplicity of LC-PCR in serum samples make it a useful alternative to blood cultures for patients with suspected brucellosis and negative or doubtful serological test results.

Brucellosis is a widespread and economically important zoonosis causing a high degree of morbidity in humans [1, 2]. The disease is endemic in the Mediterranean basin, the Middle East, India, and Central and South America. Furthermore, the constant growth in migration and tourism, together with the variable incubation period of the disease, explain the appearance of cases of human brucellosis in countries where the animal disease has been eradicated [3, 4].

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The clinical picture of brucellosis is very nonspecific, and its diagnosis requires laboratory confirmation [5, 6]. However, blood cultures—the "gold standard" for diagnosis—lack sensitivity, and serological tests are not specific enough in areas of endemicity [6, 7].

PCR-based assays hold much promise as key tools for the diagnosis of brucellosis in the near future [8]. Our group has recently reported that the use of both conventional PCR techniques and the PCR-ELISA assay with whole blood samples provides better results than conventional microbiological techniques for the diagnosis of both primary infection and relapses of brucellosis [9, 10].

Real-time PCR assays quantitatively monitor PCR products as they accumulate during thermal cycling [11, 12]. Because this technology does not require post-

amplification handling, much faster assays are possible. A SYBR Green I LightCycler-based Real-Time PCR (LC-PCR; Roche Diagnostic) combines rapid amplification and sequence-specific detection of amplicons in an automated and standardized format. In the present study, we compare the diagnostic yields of an LC-PCR with serum samples and a PCR-ELISA assay with whole blood samples for the diagnosis of human brucellosis.

PATIENTS AND METHODS

From April 2002 through March 2003, a total of 60 whole blood and serum samples were obtained from 59 consecutive patients who received a diagnosis of active brucellosis in the Infectious Diseases Unit of Carlos Haya University Hospital (Malaga, Spain). One patient provided 2 samples (one sample corresponding to the initial episode, and the other corresponding to a relapse). The diagnosis of brucellosis was established according to either of the following criteria: (1) isolation of *Brucella* species from culture of blood or other clinical samples and (2) 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 test result of ⩾1:160 or immunocapture-agglutination anti-*Brucella* test result of ⩾1:320.

Control blood samples were obtained from 37 subjects. These included 11 patients with febrile syndromes of other defined etiologies that initially involved a differential diagnosis of brucellosis: 2 patients with liver abscess (1 case due to *Escherichia coli* and 1 case due to *Streptococcus intermedius*), 2 patients with bacteriemia (1 due to *E. coli* and 1 due to *Salmonella enterica* serotype Typhimurium), 2 patients with giant cell arteritis, and 1 patient each with acute cytomegalovirus infection, chronic hepatitis C, coxofemoral arthritis due to *Staphylococcus epidermidis*, seminoma, and liver metastasis due to a colonic adenocarcinoma. The 37 subjects also included 7 asymptomatic patients with a history of brucellosis treated according to usual antibiotic regimens >12 months earlier, 9 asymptomatic subjects exposed to *Brucella* infection because of their professions (i.e., farmers, shepherds, and veterinary, abbatoir, or laboratory workers with persistently high titers of anti-*Brucella* antibodies), and 10 healthy subjects with no history of brucellosis or exposure to *Brucella* species.

Bacteriological and serological techniques. Two blood cultures, a rose Bengal card agglutination test, a Wright's seroagglutination test, an immunocapture-agglutination test, an LC-PCR assay, and a PCR-ELISA assay were performed for all 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). If no growth was detected with the usual 5-day protocol, incubation was maintained for 15 days, and blind subcultures were performed on chocolate agar and *Brucella* agar (Biomedics) after 7 and 15 days. These subcultures were incubated at 37°C in a 5%–10% CO_2 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, according to usual techniques [13]. For definitive identification, all isolated strains were sent to the National Brucellosis Reference Laboratory in Valladolid, Spain. Definitive identification and biotyping were performed by study of the oxidative metabolism against different substrates, CO_2 requirement, H_2S production, urea hydrolysis, growth in the presence of thionine and basic fuchsin, and phage sensitivity.

The rose Bengal card agglutination test and Wright's seroagglutination test were performed according to previously described techniques [14]. The determination of total anti-*Brucella* antibodies was made by an immunocapture-agglutination test (Brucellacapt; Vircell SL), following the manufacturer's instructions [15].

DNA extraction. All samples for the PCR-ELISA and LC-PCR assays were extracted at the same time as the samples used for blood cultures and stored at −20°C until processing. For the PCR-ELISA, 0.5 mL of the blood collected in sodium citrate was resuspended in 1 mL of erythrocyte lysis solution (320 mmol/L saccharose, 5 mmol/L $MgCl_2$, 1% Triton X-100, and 10 mmol/L Tris HCl [pH, 7.5]) and mixed and centrifuged at 15,000 *g* for 2 min. The supernatant was discarded, and the leukocyte pellet was washed with sterile water and incubated with H_2O_2 (30% w/w) (Sigma) for 2–5 min at room temperature. The H_2O_2 was then removed with the tip of the pipette. Template DNA was obtained from the leukocytes as follows: 400 μL of nucleic lysis buffer (60 mmol/L NH_4Cl and 24 mmol/L Na_2 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. Purification and precipitation of DNA were performed as reported previously [9]. For the LC-PCR assay, DNA was extracted by boiling. Two hundred microliters of serum sample were placed in a 0.5-mL microcentrifuge tube and centrifuged for 15 min at 15,000 *g*. The supernatant was eliminated, and the pellet was resuspended in 200 μL of sterile water and centrifuged for 10 min at 15,000 *g*. The supernatant was eliminated, and the pellet was resuspended in 40 μL of sterile water and subjected to boiling in a water bath for 10 min, cooled on ice, and centrifuged briefly for 10 s at 15,000 *g* before storing at −20°C until use; 2 μL of the suspension was used for PCR.

Once the DNA was extracted, the PCR-ELISA was performed with use of our previously reported technique [16]. In brief, this consists of amplification of a 223-bp fragment from the gene coding for the synthesis of an immunogenic membrane protein of *Brucella abortus*, BCSP31. This protein is specific to

the genus *Brucella* and is present in all of its biovars. The amplification was performed with the primers B4 and B5, as described by Baily et al. [17]. After amplification, 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.

LC-PCR. The primers were the same for LC-PCR as for PCR-ELISA. LC-PCR was set up in a final volume of 20 μL with the FastStart DNA Master SYBR Green I Kit (Roche Diagnostic), including heat-activatable *Taq* polymerase, with 4 mmol/L $MgCl_2$, each primer at 0.5 μmol/L and 2 μL of extracted DNA template. Following polymerase activation (95°C for 10 min), 45 cycles were run with 10 s denaturation at 95°C, 10 s annealing at 60°C, and 9 s extension at 72°C. The temperature transition rate was 20°C per s for all steps. The double-stranded PCR product was measured once every cycle immediately after the 72°C incubation (extension step) by detection of fluorescence associated with the binding of SYBR Green I to the amplification product. Fluorescence curves were analyzed with the LightCycler software, version 3.5 (Roche Diagnostic).

Melt-curve analysis was performed immediately after the amplification protocol under the following conditions: 0 s at 95°C (hold time on reaching temperature), 15 s at 71°C, and 0 s at 95°C. Temperature change rates were 20°C per s (except for the final step, which had a temperature change rate of 0.1°C per s). The peak melting temperature obtained represented the specific amplified product. To guarantee the reliability of the results, all samples were processed in duplicate. The test was considered to have positive results if the signal from the amplified product was clearly visible in both samples. Each assay was performed using positive and negative controls (DNA from *B. abortus* B-19 and distilled water, respectively) and sterile procedures following contamination-free guidelines to prevent false-positive results. To avoid potential subjectivity, the *Brucella* infection status of each patient was unknown by the personnel doing the PCR assays.

Statistical analysis. Data were analyzed with SPSS software, version 11.0 for Windows (SPSS). Sensitivity, specificity, positive and negative predictive values, likelihood ratios, and 95% CIs were calculated using the Twobytwo 1.0 analyzer program (Robert M. Centor and Jerry Keightley).

RESULTS

Of the 59 patients included in the study, 41 (69.5%) were men, and 18 (30.5%) were women. The mean age (±SD) was 43.2 ± 15.7 years (range, 15–75 years). The mean duration (±SD) of the symptoms prior to diagnosis was 51.2 ± 73.6 days: in 15 patients (25.4%), the duration was <2 weeks; in 27 (45.7%), the duration was between 2 weeks and 1 month; in 9 (15.2%), the duration was 1–3 months; and in the remaining 8 (13.5%), the duration was >3 months. Forty patients (67.7%) had fever with no apparent focus, and 19 (32.2%) had ⩾1 focal complication (10 patients had spondylitis, 3 had orchiepididymitis, and 1 each had sternoclavicular arthritis, ankle arthritis, hepatic abscess, meningoencephalitis, prostatic abscess, and erythema nodosum).

Blood culture results were positive in 39 episodes (65%). In the remaining 21 episodes (35%), the diagnosis of brucellosis was established on the basis of clinical and serological criteria. All isolated strains were identified as *B. melitensis*. The mean time (±SD) to detection by the blood culture system was 5.6 ± 3.4 days. Growth of the microorganism was detected by the system before the seventh day of incubation in 67.1% of cases, with the remaining 32.9% of cases requiring blind subcultures onto a plate.

Wright's seroagglutination test and the immunocapture-agglutination test had titers within the diagnostic range in 66.1% and 92.5% of cases, respectively. Both tests had negative results or showed titers below the diagnostic range in 7.4% of cases.

Of the 60 samples from the patients with brucellosis, 56 (93.3%) had positive LC-PCR results, and 54 (90%) had positive PCR-ELISA results. LC-PCR results and PCR-ELISA results were both positive for 54 samples. However, 2 samples (3.3%) that had negative PCR-ELISA results had positive LC-PCR results. In all samples with positive LC-PCR results, *Brucella*-specific amplicons could be distinguished by their characteristic melting temperature of 87.9°C in melting curve analysis.

One patient experienced relapse, with blood cultures positive for *Brucella*, 3 months after concluding anti-*brucella* treatment. Both PCR-ELISA and LC-PCR results were initially positive in this patient, became negative at the end of treatment, and were again positive during the relapse.

Three control samples (8.1%) had positive PCR-ELISA results, 2 of which (5.4%) also had positive LC-PCR results. The sensitivity for LC-PCR and PCR-ELISA was 93.3% and 90%, respectively, and the specificity was 94.6% and 91.9%, respectively. Table 1 shows the diagnostic yield of both PCRs, compared with conventional cultures. The 3 persons with false-positive PCR-ELISA or LC-PCR results had been previously exposed to *Brucella* infection. The first subject was a farmer with a history of brucellosis 4 years earlier with a seronegative, HLA B27–positive spondyloarthropathy, immunocapture-agglutination test titers of 1:80, and no clinical or bacteriological evidence of relapse; the second subject was a shepherd with Caroli disease, *E. coli* bacteriemic cholangitis, and immunocapture-agglutination test titers of 1:160; and the third subject was an asymptomatic woman with negative serological anti-*Brucella* test results. The third subject's family usually con-

Table 1. Diagnostic yield of SYBR Green I LightCycler-based Real-Time PCR (LC-PCR) in serum and PCR-ELISA in peripheral blood samples.

Diagnostic method	Sensitivity	Specificity	PPV	NPV	Positive LR	Negative LR
LC-PCR	93 (83–97)	95 (81–98)	97 (92–100)	90 (80–99)	17.3 (5.2–62.5)	0.07 (0.02–0.16)
PCR-ELISA	90 (79–95)	92 (77–97)	95 (89–100)	85 (74–96)	11.1 (4.2–32.3)	0.10 (0.05–0.22)
Blood culture	65 (52–75)	100	100	64 (51.4–76.2)	ND[a]	0.35 (0.24–0.48)

NOTE. Data are percent of samples with positive results (95% CI). Data were calculated by Twobytwo 1.0 analyzer program. LR, likelihood ratio; NPV, negative predictive value; PPV, positive predictive value.

[a] Not done for mathematical reasons (division by zero).

sumed unpasteurized cheese, and 2 members of the family had recently received diagnoses of brucellosis.

DISCUSSION

Despite advances in sensitivity and in time-to-detection resulting from the introduction of new semiautomatic systems for processing blood cultures, such cultures still show no growth in 20%–30% of patients with brucellosis. This percentage is much higher in patients with a prolonged clinical course or those who have previously received antimicrobial therapy [5, 7, 18].

A large number of different tests have been used for the serological diagnosis of brucellosis, thus demonstrating the lack of an ideal technique. The sensitivity of these serological tests ranges from 65% to 95%, but their specificity in areas of endemicity is low, because of the high prevalence of antibodies in the healthy population. Moreover, most serological tests can produce cross-reactions with other bacteria, and they also have important limitations during the early phases of the disease, in persons whose professions involve exposure to *Brucella* species, in patients with a recent history of brucellosis, and in patients who experience relapse [6, 7].

Because microorganisms of the *Brucella* genus are facultative intracellular pathogens and the innoculum found in patients is normally small [19], most studies of PCR assays involving human brucellosis have been undertaken with whole blood samples. However, Zerva et al. [20] reported a greater sensitivity in serum samples than in whole blood samples, even though the amount of circulating DNA in serum is presumably lower.

The use of real-time amplification and detection of nucleic acids in sealed capillary tubes in LC-PCR has significantly increased test efficiency, compared with PCR-ELISA, in 2 ways. First, amplification and detection of *Brucella* DNA can be completed within 1 h after extraction, compared with >6 h with conventional PCR amplification followed by amplicon detection in gel electrophoresis or digoxigenin ELISA. Second, being a sealed system, real-time PCR assay decreases the risk of carryover contamination [21].

Considering the advantages of real-time PCR, the fact that there are theoretically fewer *Taq* polymerase inhibitors in serum than in whole blood, and the fact that the DNA extraction process is easier in serum than in whole blood samples, we compared the diagnostic yield of PCR-ELISA in whole blood samples with that of LC-PCR in serum samples in a cohort of patients with brucellosis. The sensitivity of both LC-PCR and PCR-ELISA was far higher than that of blood cultures. This is especially relevant if we consider that, in the present study, the sample of patients with brucellosis was very representative of the true clinical situation, because it included not only patients in the acute phase of the disease, but also others with a long evolution of disease, with and without focal complications.

The specificity of both PCR assays can be considered good, although the existence of 2 control samples with false-positive LC-PCR results merits comment. We cannot be absolutely sure that contamination was not the cause of these false-positive results, and *Brucella*-specific DNA might have existed in these samples. The fact that 2 of the subjects with false-positive results had false-positive results according to both LC-PCR and PCR-ELISA—performed with independent samples, different extraction processes, and on different days—suggests that contamination was unlikely. Previous studies have demonstrated the high specificity of the B4 and B5 primers with a wide panel of microorganisms. Only DNA from *Ochrobactrum* species—a pathogen very closely related phylogenetically to *Brucella* species—has been amplified with these primers [22]. If we further consider that asymptomatic infection with *Brucella* species is not unusual [23, 24] and that the 2 patients, although asymptomatic, were strongly exposed to infection, an asymptomatic infection is more likely than a false-positive test result. One of the main problems associated with molecular diagnosis of some infectious diseases is the interpretation of certain results. The high sensitivity of PCR-based methods enables amplification of the remains of clinically irrelevant circulating DNA in asymptomatic subjects [25].

Although the control group contained fewer persons than did the group with brucellosis, this should not represent any relevant bias or alter the results. In fact, because the efficacy of diagnostic tests should ideally be studied in those situations that pose the most diagnostic problems in daily clinical practice, we expressly included in the control group a similar number of patients with febrile syndromes of other etiologies involving a differential diagnosis with brucellosis, as well as asymptomatic

patients with a history of brucellosis and subjects professionally exposed to *Brucella* infection, without overrepresenting healthy control subjects. The control group thus included a considerable number of subjects in whom the reliability of the serological tests commonly used for the diagnosis of brucellosis is very low. In conclusion, our results suggest that LC-PCR that uses serum samples is at least as sensitive as PCR-ELISA that uses whole blood samples and that the speed and simplicity of real-time PCR could be useful for patients with a high suspicion of brucellosis but whose serological test results are negative or inconclusive.

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