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Serum Is the Preferred Clinical Specimen for Diagnosis of Human Brucellosis by PCR

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Human brucellosis poses a significant public health problem in many developing countries and requires fast and accurate diagnosis. A PCR assay amplifying part of the 31-kDa *Brucella abortus* antigenic protein gene sequence was developed and applied to whole-blood and serum samples from 31 brucellosis patients and 45 healthy individuals. All patients except one had detectable *Brucella* DNA in either whole blood or serum (combined sensitivity, 97%), but the assay sensitivity was higher with serum samples (94%) than with whole-blood samples (61%). The assay specificity was excellent (100%). A confirmatory PCR assay targeting another *Brucella* gene region (*omp-2*) was also developed but lacked sensitivity. Serum is the optimal specimen for the diagnosis of brucellosis by PCR, a choice that leads to assay simplification and shortens turnaround time.

Brucellosis is still an important zoonosis of both public health and economic significance in many developing countries. Half a million new cases are reported worldwide each year, but according to the World Health Organization, these numbers greatly underestimate the true incidence of human disease (22). As the clinical picture of human brucellosis is extremely variable, diagnosis can be established only by laboratory methods. Since the disease constitutes a serious infection necessitating treatment with a prolonged course of antibiotics, accuracy and short turnaround time are required for these tests (21).

Blood cultures represent the "gold standard" of laboratory diagnosis. Automated systems have been reported to detect more than 95% of *Brucella melitensis*-positive cultures within 7 days of incubation (23). Unless this technology is not available, prolonged incubation, blind subcultures, and special growth media are no longer required (23). Ironically, however, the technology indeed is lacking in developing countries or rural areas where the disease is prevalent. In addition, due to their comparatively long doubling time, *Brucella* species grow slowly on primary cultures and subcultures, while their inert biochemical profiles hamper fast identification of isolates (9). Distinct disease conditions like focal, relapsing, or chronic disease and disease caused by species other than *B. melitensis* are characterized by low blood culture yields and pose special diagnostic problems (1, 2). Consequently, detection and identification of *Brucella* spp. in clinical specimens by cultures may still be a difficult task with significant delays.

Several agglutination tests (Rose Bengal, Wright's tube, Wright's card, and Wright-Coombs) and indirect immunofluorescence, complement fixation, and enzyme-linked immunosorbent assays are also available for diagnosis of brucellosis (3, 14, 24). The standard, with which all other methods should be compared, is Wright's tube agglutination test (1, 14). A broad range of test sensitivity, low specificity in areas of endemicity, lack of usefulness in diagnosing chronic disease and relapse, presence of cross-reacting antibodies, and lack of timeliness constitute problems associated with brucellosis serology (14, 24). Most significantly, though, there is no standardization of antigen preparations and methodology, even for the "standard" Wright's tube agglutination test.

As for other fastidious pathogens, molecular methodology offers an alternative way of diagnosing brucellosis. Nucleic acid amplification techniques, like PCR, characterized by high sensitivity and specificity and short turnaround time can overcome the limitations of conventional methodology. Only a few studies in the literature (12, 17, 20), however, address direct detection of *Brucella* spp. in clinical specimens of human origin. This study was initiated by a recent reemergence of brucellosis due to *B. melitensis* in Greece (16). Our aim was to develop a diagnostic PCR assay and define the optimal clinical specimen for this test. For this purpose, peripheral blood samples, i.e., whole blood and serum, from confirmed brucellosis cases were examined retrospectively.

Clinical specimens. Peripheral blood specimens were collected from 31 consecutive brucellosis patients diagnosed over periods of 6 and 4 months, respectively, in the University Hospital, Ioannina, Greece, and the Hospital of Infectious Diseases, Thessaloniki, Greece. All patients presented with clinical signs compatible with brucellosis. Diagnosis was established by positive blood cultures and/or serology. All patients were adults occupationally exposed to *Brucella* (age range, 21 to 74 years [mean, 52 years]; disease duration range, 1 week to 90 days [mean, 35 days]). Blood samples were obtained at the time of diagnosis before initiation of treatment. Forty-five healthy adults undergoing a routine evaluation for peripheral blood lipids constituted the control group.

Bacteriological and serological techniques. Blood cultures were processed with either the BACTEC 9050 or BacT/Alert system and were incubated for 7 days without blind subcultures. Blood culture specimens were obtained from 24 patients.

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Brucella spp. were isolated from 13 patients (54%). All isolates were identified as *B. melitensis* biotype 2 according to standard methodology (9). The serological diagnosis was established by Wright's tube agglutination test (*Brucella* Antigen; Sanofi Diagnostics Pasteur, Marnes la Coquette, France). A titer equal to or greater than 1/160 was considered significant. All patients tested positive by serology, while controls were negative.

Isolation of DNA. Peripheral blood samples from patients and controls were collected in EDTA and without anticoagulant. All samples were aliquoted and stored at −20°C until tested. A 0.5-ml portion of anticoagulated whole blood was mixed with 1 ml of erythrocyte lysis solution (320 mM saccharose, 5 mM $MgCl_2$, 1% Triton X-100, 10 mM Tris-HCl [pH 7.5]) and centrifuged at 15,000 × *g* for 2 min. The cell pellet was washed with 1 ml of water four times. DNA was isolated from serum (200 μl) and whole-blood pellets with an IsoQuick Nucleic Acid Extraction Kit (ORCA Research, Inc., Bothell, Wash.).

DNA amplification by two different PCR protocols. Two PCR assays targeting different gene regions of *Brucella* spp. were developed. The first assay, designated BCSP31-PCR, represented the diagnostic assay, while the second, designated OMP-PCR, was intended to be used for confirmation of results obtained with the first assay. Their respective primers have been reported before (4, 11). The BCSP31-PCR assay (4) amplifies a 223-bp sequence of the gene encoding the 31-kDa *Brucella abortus* antigen, which is conserved in all *Brucella* species. The OMP-PCR assay (11) amplifies a 193-bp sequence of the gene (*omp-2*) encoding an outer membrane protein in all *Brucella* species except *B. suis* biovars 2 to 4, *B. ovis,* and *B. canis*. For the BCSP31-PCR assay, isolated DNA (7.5 μl) was examined in a total volume of 37.5 μl containing 0.025 U of *Taq* DNA polymerase (Promega, Madison, Wis.) per μl, 50 mM KCl, 10 mM Tris-HCl (pH 8.4), 1 mM $MgCl_2$, a 200 μM concentration of each deoxynucleoside triphosphate (Promega), and a 500 nM concentration of each of the primers, B4 and B5 (4), in a programmable thermocycler (Progene; Techne, Princeton, N.J.). Amplifications were performed for 40 cycles with denaturation at 90°C (1 min), annealing at 60°C (30 s), and extension at 72°C (1 min). They were preceded by a 5-min incubation at 93°C and followed by a final 7-min extension step at 72°C. For the OMP-PCR assay, isolated DNA was amplified as described above except for the concentrations of $MgCl_2$ (3 mM) and primers (JPF and JPR, 300 nM each) (11). This PCR consisted of an initial 4-min incubation step at 94°C, followed by 35 cycles with denaturation at 94°C, annealing at 60°C, and extension at 72°C (each for 1 min) and a final 5-min extension step at 72°C. Extraction of DNA from clinical samples and amplification of isolated DNA were performed at least twice. For the detection of inhibitors, all samples were tested undiluted as well as diluted 1:10 in water. The positive control was genomic DNA isolated from a *B. melitensis* reference strain (strain WD-1, *B. melitensis* biotype 2; Laboratory of Clinical Microbiology, Hospital of Infectious Diseases, Thessaloniki, Greece). Water was used as the negative control. Amplicons were detected by fluorescence after electrophoresis in a 2% agarose gel in the presence of ethidium bromide (2 μg/ml). All standard precautions recommended for prevention of contamination with DNA and amplicons were undertaken (10).

BCSP31-PCR is sensitive and specific. Serial dilutions of isolated genomic *B. melitensis* WD-1 reference strain DNA were used for the optimization of the BCSP31-PCR assay. After minor modifications, the analytical sensitivity originally reported for this primer pair by Baily et al. (4) (15 to 150 fg of DNA) was reproduced. All patient and control samples were examined by this assay. Eighteen out of 31 brucellosis patients (58%) were PCR positive with both whole-blood and serum samples, 11 (36%) were positive only with serum samples, and 1 (3%) was positive only with the whole-blood sample. One patient (3%) tested PCR negative with both whole-blood and serum samples. The diagnostic sensitivities thus were 97% for the combined serum and whole-blood PCR assays, 94% for the serum assay, and 61% for the whole-blood assay.

Inhibitors were often detected in whole-blood specimens. Four out of 19 whole-blood specimens (21%) were PCR positive only when examined diluted. No inhibition was observed with serum samples. All whole-blood and serum samples obtained from the control group tested negative with the BCSP31-PCR, conferring an assay specificity of 100%.

The analytical sensitivity of the OMP-PCR assay using isolated genomic *B. melitensis* reference strain WD-1 DNA was 1 log lower (150 to 1.500 fg of DNA) than the sensitivity of the BCSP31-PCR assay. All attempts to improve the analytical sensitivity by changing assay parameters were unsuccessful.

Serum and whole-blood samples from 10 brucellosis patients were examined by the OMP-PCR assay. They were selected for being positive by the BCSP31-PCR assay with both whole blood and serum. Only in 4 out of 10 whole-blood specimens and in 6 out of 10 serum specimens was the 193-bp band amplified. The presence of inhibitors in PCR-negative specimens was ruled out by examining samples diluted in water. In order to exclude the possibility of inefficient DNA extraction, aliquots of the original samples were thawed and DNA was reextracted and used as a template for both PCR assays (BCSP31-PCR and OMP-PCR). The same results were obtained. The diagnostic sensitivities of the OMP-PCR assay for whole-blood and serum specimens thus corresponded to 40 and 60%, respectively. No examination of further patient samples was undertaken due to apparent insufficient test performance. Specificity was tested by examining whole-blood and serum samples from 16 controls. All were OMP-PCR negative (specificity, 100%).

The results of this retrospective study show that a sensitive and specific one-step diagnostic PCR assay, BCSP31-PCR, was developed. The optimal clinical specimen for this test was not whole blood but serum, which leads to assay simplification and also indicates that human brucellosis is characterized by a high degree of bacterial DNAemia. The second PCR developed, the OMP-PCR assay, did not demonstrate satisfactory sensitivity to be used as a confirmatory test; therefore, further examination of specimens by this test was discontinued.

Only three reports in the literature (12, 17, 20) have evaluated the application of PCR for the diagnosis of human brucellosis, and they all used the primers described by Baily et al. (4). The first study (12) examined samples from 20 brucellosis patients diagnosed by serology. Mononuclear cells were isolated from EDTA-whole blood; DNA was extracted with a lysis buffer containing proteinase K and used directly for PCR without purification. All patients tested positive; however, two suc-

cessive rounds of PCR were required in order to enhance band intensity, an approach prone to lead to contamination with amplicons. All controls were negative, and specificity was further confirmed by Southern hybridization and restriction endonuclease analysis.

Another study (20) examined peripheral blood samples from 47 brucellosis patients retrospectively. Specimens were collected in sodium citrate, depleted of red blood cells, and digested with a proteinase K-containing lysis buffer, and DNA was extracted by a salting-out procedure. Excellent sensitivity (100%) was reported in comparison to blood culture and serology (70 and 84%, respectively). Extensive washing of cell pellets, determination and adjustment of the isolated DNA concentration (13), and incubation of DNA with H_2O_2 (19) were recommended for avoiding false negatives; however, this method of optimization resulted in a lengthy, complicated procedure. The specificity was 98%, but the only "false-positive" specimen originated from a control subject who soon developed brucellosis. All positive results were confirmed by hybridization.

Finally, a short report (17) described a study involving a small number of brucellosis patients that tried to reproduce results obtained with the methodology described above (20). The use of identical procedures, however, did not reproduce the previous results; the sensitivity and specificity were 50 and 60%, respectively. Different inoculum sizes and degradation of target DNA in clinical samples due to different storage conditions were assumed to account for discrepant results, as did the well-known fact (18) that in-house PCR results are difficult to reproduce in different laboratories.

None of these previous studies examined the possibility of amplifying *Brucella* DNA in serum samples. However, the use of serum instead of whole-blood samples offers several advantages for nucleic acid amplification methods. Inhibition by anticoagulants, hemoglobin, human DNA, or any other substance present in whole blood but not in serum is circumvented. Red blood cell lysis, washings by centrifugation, and measurement and adjustment of isolated DNA concentrations are not required. Overall, the procedure is simplified and turnaround time is shorter, while sensitivity may be increased. Regarding the origin of pathogen nucleic acids in serum samples, most probably they are released in the circulation as breakdown products during bacteremia. Several studies have documented the presence of circulating pathogen DNA in serum samples. (5, 6, 8, 15).

The excellent sensitivity (100%) previously reported for whole-blood specimens (20) or isolated leukocytes (12) was not reproduced in our study when whole-blood specimens were tested by BCSP31-PCR. However, considering the complexity of PCR methods and differences between procedures, these results are not surprising. Despite use of the same primer pair, parameters like sample selection, anticoagulants, storage conditions, sample pretreatment methods, extraction methods, and finally the actual PCR assay all were variable.

In accordance with previous results (4, 12, 20), the BCSP31-PCR assay specificity was excellent. Further specificity testing (e.g., involving other significant bacteria, patients with fever of unknown origin, or hybridization after PCR) was not performed, since these studies have already been conducted (4, 12, 20). Instead, a different approach was chosen for confirmation of results, namely, the application of a second PCR. OMP-PCR was selected for its reported excellent performance (analytical sensitivity of 25×10^{-11} μg of DNA and fewer than 10 cells/1 ml of milk) (11). Additionally, this method appears to be the only PCR amplifying *Brucella* spp. but not *Ochrobactrum anthropi*, a rare cause of bacteremia in severely immunosuppressed or debilitated patients (7). Neither the analytical nor the diagnostic sensitivity was reproduced in our study. The possibility that our patients were infected by *O. anthropi* is not reasonable. The *Brucella* species and biovars not amplified by this assay have not been associated with animal or human disease in Greece. Different specimens, sample pretreatment, and DNA extraction methods could account for discrepant results in comparison to the original report (11) but not for the differences obtained with analytical sensitivity.

In Greece, according to the National Epidemiological Surveillance Center (Ministry of Health), more than 85% of all human brucellosis cases are diagnosed by serology only (16). Automated blood culture technology is still not available in many rural areas; therefore, clinicians rely on serological diagnosis. Laboratories use various, often not standardized, serological tests, which inevitably leads to false-positive and false-negative results. Additionally, the interpretation of serological testing for brucellosis is far from straightforward, especially in areas of endemicity. Physicians well acquainted with brucellosis recommend not relying on results obtained with a single test or a single serum specimen (24). As a consequence, disease diagnosis is often delayed.

Under these circumstances, a reference laboratory performing a standardized and quality-controlled PCR test on shipped serum specimens can greatly improve timely diagnosis and prompt the initiation of appropriate treatment. The short turnaround time of this serum one-step PCR (less than 4 h) compares favorably with that of blood cultures and Wright's tube and Wright-Coombs tests (3 to 7 days, 24 h, and 48 h, respectively). Finally, costs of in-house PCR methods are low for laboratories already equipped with the necessary infrastructure.

In conclusion, these results show that serum samples should be used preferentially over whole blood for the molecular diagnosis of human brucellosis. This choice of specimen simplifies the procedure and decreases turnaround time, while sensitivity and specificity are excellent. Further studies to evaluate assay performance prospectively are in progress. The application of this method for the presently problematic diagnosis of chronic, focal, and relapsing brucellosis will be of significant clinical utility.

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