Evaluation of three polymerase chain reaction techniques for detection of Brucella DNA in peripheral human blood

Manal M. Baddour and Dalal H. Alkhalifa

Abstract: Brucellosis is a widespread zoonosis. Currently the diagnosis of this zoonosis is based on microbiological and serological laboratory tests. Polymerase chain reaction (PCR) has been used to detect DNA from Brucella. Different target genes, primer pairs, PCR techniques, and extraction procedures have previously been published for Brucella detection. But only a few of these primers have been used in human samples, and only one study has been carried out to compare sensitivity between them. In the present study, 3 sets of primers and 3 different PCR protocols amplifying 3 different regions of the Brucella genome were compared for detection of Brucella DNA in a peripheral-blood PCR assay to conclude which is most suitable for the clinical diagnostic laboratory. These 3 pairs of primers amplify 3 different fragments included in (i) a gene encoding a 31 kDa Brucella abortus antigen (B4/B5), (ii) a sequence 16S rRNA of B. abortus (F4/R2), and (iii) a gene encoding an outer membrane protein (omp-2) (JPF/JPR). Some modifications on the reported techniques were applied during the present work to improve the outcome. The results showed that the B4/B5 primer pair had the highest sensitivity for detection of positive samples (98%), the JPF/JPR primer pair detected 88.4% of positive samples, whereas F4/R2 primer pair was the least sensitive, being able to detect only 53.1% of positive samples. The specificity of the 3 techniques was 100%. The B4/B5 primer pair was also able to detect the smallest number of bacteria (700 cfu/mL), whereas JPF/JPR was able to detect 7 × 10^5 cfu/mL and F4/R2 was able to detect 7 × 10^7 cfu/mL. It is thus concluded that using the B4/B5 primer PCR with the suggested modifications is a robust assay, which meets the sensitivity requirements to be used for testing of human blood samples for brucellosis in the diagnostic laboratory.

Key words: Brucella, PCR, diagnosis, human blood, comparison.


M.M. Baddour.1,2 Microbiologie et Immunologie Department, Faculty of Medicine, Alexandria University.

1 Corresponding author (e-mail: mbaddour@ksu.edu.sa).
2 Present address: King Saud University, P.O. Box 22452, Riyadh 11495, Saudi Arabia.
Introduction

Brucellosis is a widespread zoonosis that is still responsible for economic losses of livestock in many areas of the world. It is transmissible to humans via contact with animals or their products. 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 (World Health Organization 1997). Since the disease constitutes a serious infection necessitating treatment with a prolonged course of antibiotics, accuracy and short turnaround time are required for the diagnostic tests (Solera et al. 1997). Brucellosis is the most frequent laboratory-acquired bacterial infection, causing severe disease in humans with unspecific clinical signs simulating other febrile illnesses, e.g., malaria, tuberculosis, and typhoid fever. Therefore, clinical diagnosis is difficult to establish. A definite diagnosis requires the isolation of *Brucella* from blood, bone marrow, or other tissues (Al Dahouk et al. 2003).

Blood cultures represent the “gold standard” of laboratory diagnosis, this requires prolonged incubation, blind subcultures, and special growth media due to their comparatively long doubling time. However, the sensitivity of this technique is low, ranging from 15% to 70% (Young 1997).

Detection and identification of *Brucella* spp. in clinical specimens by culturing is a difficult task with significant delays and hazards to laboratory personnel (Yagupsky 1999), as *Brucella* spp. are class III pathogens (Pike et al. 1965).

Thus, diagnosis is usually based on indirect serological tests. 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 (Moyer et al. 1987; Young 1995).

As for other fastidious pathogens, molecular methodology offers an alternative way of diagnosing brucellosis. Nucleic acid amplification techniques, like polymerase chain reaction (PCR), which is characterized by high sensitivity and specificity and short turnaround time, can overcome the limitations of conventional methodology. However, only a few studies in the literature (Leal-Klevezas et al. 1995; Matar et al. 1996; Queipo-Ortuno et al. 1997; Navarro et al. 1999; Zerva et al. 2001) address the direct detection of *Brucella* spp. in clinical specimens of human origin, and in these studies, different extraction procedures, different primer pairs, different target genes, and different amounts of DNA were applied.

The present study compares 3 reported PCR techniques for diagnosing brucellosis from human blood samples and determines the technique most suitable for use in a diagnostic microbiology laboratory in terms of sensitivity, specificity, robustness, and ease of implementation.

Materials and methods

Peripheral blood specimens were collected from 147 consecutive brucellosis patients diagnosed over a period of 17 months in a semi-urban hospital setting outside Riyadh, Saudi Arabia. All patients presented with clinical signs compatible with brucellosis. 3 of the patients were relapses of a previous *Brucella* infection. All patients were adults (age range, 16–75 years; mean, 34.08 years). Males constituted 66% of the cases. Diagnosis was established by positive blood cultures and (or) serology. For comparison, 50 blood samples from apparently healthy volunteers were collected and used as negative controls. The negative control samples were obtained from individuals within the same age, sex, and demographic categories as that of the *Brucella*-positive patients. The criteria used for enrollment in the negative control group included normal vital functions, lack of prior exposure to sepsis or any chronic debilitating disease, and lack of antibiotic intake during the study period.

Blood samples were obtained at the time of diagnosis but before initiation of treatment. The serological diagnosis was established by Wright’s tube agglutination test (Brucella Agglutinating Sera; Remel, Dartford, UK). A titer equal to or greater than 1/160 was considered significant. All patients tested positive by serology, whereas controls were negative.

Blood cultures, however, yielded positive results for 98 (66.7%) of the samples.

*Brucella melitensis* was kindly supplied by the Department of Microbiology (King Faisal Specialist Hospital). It was identified by biochemical tests such as positive oxidase and urease tests, negative H₂S production, no requirement for CO₂, and growth in the presence of basic thionin and fuchsin (20 μg/mL), as well as agglutination by monospecific antisera.

DNA extraction from blood samples

Peripheral blood samples from patients and controls were collected in citrated vacutainers. 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 mmol/L saccharose, 5 mmol/L MgCl₂, 1% Triton X-100, 10 mmol/L Tris–HCl (pH 7.5)) and centrifuged at 15,000 g for 2 min. The cell pellet was washed 4 times with 1 mL of water (Miller et al. 1988). DNA was isolated from whole-blood pellets with a PURE GENE Nucleic Acid Extraction Kit (Gentra Systems), as per the manufacturer’s instructions. DNA was extracted from 150 μL of pelleted cells, eluted in 30 μL of DNA hydration solution (Gentra Systems), quantified spectrophotometrically, and stored at −80 °C until it was used.

Isolation of genomic DNA from bacteria

The positive control was genomic DNA isolated from a *B. melitensis* strain, as previously described (Ausubel et al. 2002).

DNA amplification

The 3 pairs of primers that were chosen amplification regions of 3 different *Brucella* genes: (i) primers B4 ('5'-TGG CTC GTG TGC CAA TAT CAA-3') and B5 ('5'-CGC GCT TGC CTT TCA GGT CTG-3') amplified a 223 bp fragment present on a gene encoding a 31 kDa *Brucella abortus* antigen (Baily et al. 1992), (ii) oligonucleotides JPF ('5'-GCC TCT AGG CTG CCT CCG ACG CAA-3') and JPR ('5'-ACC AGC CAT TGC GGT CGG TA-3') amplified a 193 bp fragment from a gene encoding an outer membrane protein (omp-2) (Leal-Klevezas et al. 1995), and (iii) a 905 bp fragment was amplified with primers F4 and R2, derived from the 16S rRNA sequence on *B. abortus* (Romero et al. 1995).
PCR reactions for each pair of primers were carried out as follows with slight modifications from the original published protocols.

1. The B4/B5 pair — a 50 μL reaction mixture contained 10 mmol/L Tris–HCl, 2.25 mmol/L MgCl₂, 50 mmol/L KCl, 200 μmol/L (each) deoxynucleotide triphosphate (Promega), 1 μL each of primers B4 and B5 (5 pmol/μL) (TIB MOLBIOL, Berlin, Germany), 1.25 U of Taq polymerase (Promega), and 5 μL of extracted DNA. The PCR profile was set at initial denaturation at 93 °C for 5 min; then 40 cycles of template denaturation at 90 °C for 60 s, 60 s of primer annealing at 60 °C, and 60 s of primer extension at 72 °C; with a final extension at 72 °C for 7 min (Bailly et al. 1992).

2. The JPF/JPR pair — a 50 μL reaction mixture contained 10 mmol/L Tris–HCl, 2 mmol/L MgCl₂, 50 mmol/L KCl, 200 μmol/L (each) deoxynucleotide triphosphate (Promega), 0.2 μL each of primers JPF and JPR (50 pmol/μL) (TIB MOLBIOL), 1.25 U of Taq polymerase (Promega), and 5 μL of extracted DNA. The PCR profile was set at initial denaturation 94 °C for 4 min; then 40 cycles of template denaturation at 94 °C for 60 s, 60 s of primer annealing at 60 °C, and 60 s of primer extension at 72 °C; with a final extension at 72 °C for 3 min (Leal-Klevezas et al. 1995).

3. The F4/R2 pair — a 50 μL reaction mixture contained 10 mmol/L Tris–HCl, 1.5 mmol/L MgCl₂, 50 mmol/L KCl, 0.1% Triton X-100, 200 μmol/L (each) deoxynucleotide triphosphate (Promega), 0.2 μL each of primers F4 and R2 (50 pmol/μL) (TIB MOLBIOL), 0.5 U of Taq polymerase (Promega), and 5 μL of extracted DNA. The PCR profile was set at initial denaturation 95 °C for 5 min; then 35 cycles of template denaturation at 95 °C for 30 s, 90 s of primer annealing at 54 °C, and 90 s of primer extension at 72 °C; with a final extension at 72 °C for 6 min (Romero et al. 1995).

The MgCl₂ concentration was optimized for each technique separately. All PCR reactions were performed in a Mycycler (Bio-Rad Laboratories, Hercules, California, USA). In each PCR run, a positive control (obtained by DNA extraction from B. melitensis culture) and a negative control (PCR-grade water instead of the DNA) were included to monitor adequate performance of the run and absence of cross contamination. 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 (Leal-Klevezas et al. 1995; Romero et al. 1995; Matar et al. 1996; Queipo-Ortuno et al. 1997; Casanas et al. 2001). Amplicons were detected and photographed by fluorescence after electrophoresis in a 1% agarose gel (Sigma) in the presence of ethidium bromide (1 μg/mL) in a gel documentation system (Gel Doc XR; Bio-Rad Laboratories). All standard precautions recommended for the prevention of contamination with DNA and amplicons were undertaken (Kwok and Higushi 1989). To ensure the reliability of the results, all samples were processed in duplicates, and the test was considered positive if the signal from the amplified product was clearly visible in both samples. A range of positive control (B. melitensis culture) concentrations was tested with each technique to eliminate the possibility of bacterial DNA concentration (amount of target DNA in a sample) causing a false-negative effect, and positive results were obtained with all concentrations tested.

**PCR limit of detection in inoculated blood**

The detection limit of the PCR assay was evaluated for blood by the 3 studied protocols. To determine the colony-forming units (cfu), a concentrated suspension of the 48 h culture of B. melitensis was prepared in sterile saline, and 10-fold serial dilutions (10⁻¹ to 10⁻¹⁰) were made. From the last 5 dilutions, 0.1 mL suspensions were inoculated onto 2 tryptic soy agar plates and incubated at 37 °C for 5 days. Colonies on plates were counted (Leyla et al. 2003). The concentration of the undiluted Brucella culture was estimated to be 1.4 × 10¹⁰ cfu. The number of cells in the bacterial suspension was also estimated spectrophotometrically at an optical density (OD) of 600 nm. The OD of 1.4 × 10⁸ cfu/mL bacterial cells at 600 nm was determined as 0.15. To determine the sensitivity of PCR for blood, known numbers of bacterial cells were added to PCR-negative blood samples. Aliquots of 0.5 mL were extracted and processed by PCR by the different protocols as described above. These experiments were run in triplicate.

**Results**

The PCR limit of detection in inoculated blood was found to vary for the 3 tested techniques. Remarkably, the B4/B5 primer pair was able to detect 7 × 10² cfu/mL, whereas the JPF/JPR primer pair was able to detect 7 × 10³ cfu/mL and the F4/R2 primer pair was the least sensitive being able to detect 7 × 10⁸ cfu/mL (Fig. 1). Since 20 bacterial cells are equivalent to 60 fg of bacterial DNA (Bailly et al. 1992), it is thus estimated that B4 and B5 primers are able to detect 2.1 ng of DNA, primers JPF and JPR can detect 2.1 pg of DNA, and primers F4 and R2 can detect 210 pg of DNA.

This finding was depicted in our sample testing, since the B4 and B5 primers gave the best results, with only 3 false-negative results for positive samples. Among the 147 patient samples tested, 144 were positive by B4 and B5 primers (98% sensitivity), 130 were positive by JPF and JPR primers (88.4% sensitivity), and only 78 were positive by F4 and R2 primers (53.1% sensitivity). Only 55 (37.4%) samples gave positive reactions in the 3 techniques (Table 1). All these samples were among the culture-positive ones (data not shown). Out of 11 samples that gave weak bands by the B4 and B5 primers, 4 gave strong bands by the JPF and JPR primers, 6 were negative, and one gave a weak band. Interestingly though, using the F4 and R2 primers for those samples yielded 5 positive and 6 negative reactions. Of the 9 samples giving weak bands by the JPF and JPR primers, 8 gave strong bands by B4/B5 and one gave a weak band. Eight samples gave weak bands by the F4 and R2 primers, whereas all of them gave strong bands by the JPF and JPR primers and all but one gave strong bands by the B4 and B5 primers.

The 3 relapse patients were detected by the B4 and B5 primers whereas only 2 were detected by the JPF and JPR and none were detected by the F4 and R2. All samples obtained from the control group (healthy sub-
Brucellosis is not an emerging disease but rather one that is overlooked by the majority of the scientific community. Currently, the diagnosis of this zoonosis is based on microbiological and serological laboratory tests. In the present study, 66.7% of the samples were positive by blood culture, but 98% of the samples were positive by PCR using primers B4 and B5.

When performing the techniques exactly according to the published protocols, no bands were obtained, and after several trials at modifications of the techniques, positive results were finally acquired. These modifications were then used throughout the study and were as follows: increasing the number of cycles in each technique by 5 extra cycles, increasing the annealing time for the B4 and B5 primers to 60 s rather than 30 s, reducing the Taq polymerase concentration used with the JPF and JPR primers to 1.25 U instead of 2.5 U, and changing the MgCl2 concentration to the optimum value for each technique, as obtained by calibration. The amount of primers used in the JPF/JPR reaction was also reduced to decrease the intensity of formation of primer dimers, which were consistently observed when using the original concentrations. The F4/R2 primers yielded the least sensitivity, and several unsuccessful attempts were made to increase the sensitivity of the technique, including increasing the Taq polymerase concentration from 0.5 U per reaction to 1.25 U, using a range of primer concentrations from 0.5 up to 1 μmol/L, and eliminating Triton X-100 from the reaction mixture. It is speculated that increasing the number of cycles improved the diagnostic sensitivities of all primers used, as some weak bands were obtained after increasing the number of cycles that were not detectable using the original cycle numbers published. Since these bands were of the correct size and since the positive controls were always positive and the negative controls were always negative, they were considered specific bands.

Retesting the 22 samples negative by JPF/JPR by using 2.5 U of Taq polymerase lead to the development of 5 very weak bands, which were then considered positive; these bands would not have been detected had the number of cycles not been increased. It is thus concluded that using 1.25 U of Taq polymerase (together with decreasing the amount of primer from 50 to 10 pmol) would be more cost effective in this technique provided that the cycle numbers are increased.

In the present study, to eliminate the possibility of PCR inhibitors, such as heparin (which binds Taq polymerase) and EDTA (which chelates Mg²⁺ ions from the PCR mixture), sodium citrate was used as the anticoagulant. Additionally, the extraction technique was unified so that the results obtained would depend solely on the primer sensitivity. Moreover, during the primary phase of the study, different concentrations of target DNA were tested (ranging from 1 to 5 μL of control DNA) to eliminate the possibility of reaction inhibition due to excess target DNA.

Table 1. Comparative values of test results among different primer pairs.

<table>
<thead>
<tr>
<th>Primer pair</th>
<th>Positive samples</th>
<th>Control samples</th>
<th>Limit of detection (cfu/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. positive out of 147 (%)</td>
<td>No. negative out of 147 (%)</td>
<td>No. positive out of 50 (%)</td>
</tr>
<tr>
<td>B4/B5</td>
<td>144 (98.0*)</td>
<td>3 (2.0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>JPF/JPR</td>
<td>130 (88.4*)</td>
<td>17 (11.6)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>F4/R2</td>
<td>78 (53.1*)</td>
<td>69 (46.9)</td>
<td>0 (0)</td>
</tr>
</tbody>
</table>

*Sensitivity.

Specificity.
The possible cause for false negatives in the JPF/JPR protocol could, therefore, be the presence of porphyrins or the heme compounds, which remain in the DNA sample owing to the lack of sufficient washings. The F4/R2 technique was the least sensitive and needed the highest number of cells to give a positive band; all attempts at improving its results were unsuccessful. Different specimens, sample pretreatment, and DNA extraction methods could account for discrepant results from those of original reports (Leal-Klevezas et al. 1995; Romero et al. 1995) but not for the differences obtained with analytical sensitivity.

In the present study, the sensitivity of these primer pairs was different from that described in the original reports. 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. Similar to the findings of the present study, in a previous report, the analytical sensitivity of the JPF/JPR assay using isolated genomic B. melitensis control strain was lower than the sensitivity of the B4/B5 assay. All attempts to improve the analytical sensitivity by changing assay parameters were unsuccessful (Zerva et al. 2001). The presence of inhibitors in PCR-negative specimens was ruled out by examining samples diluted in water. The diagnostic sensitivities of the JPF/JPR assay for whole-blood specimens corresponded to 40% (Zerva et al. 2001).

In the present study, the use of diluted samples (1–10 in water) did not improve the overall sensitivity of the B4/B5 or JPF/JPR primer pairs, so it was not adopted as a routine (data not shown). Although the detection limit of the assay using the JPF and JPR primers was 7 × 10^5 cfu/mL, 88.4% of blood samples were found positive by PCR. It seems that this detection limit is not a problem in testing blood samples for brucellosis because enough bacterial DNA is present in blood.

Only a few reports in the literature have evaluated the application of PCR for the diagnosis of human brucellosis, and most of them used the primers B4 and B5. The first study (Matar et al. 1996) examined samples from 20 brucellosis patients diagnosed by serology. All patients tested positive; however, 2 successive rounds of PCR were required to enhance band intensity, an approach prone to lead to contamination with amplicons. Another study (Queipo-Ortuno et al. 1997), examined peripheral blood samples from 47 brucellosis patients retrospectively. Excellent sensitivity (100%) was reported in comparison with blood culture and serology (70% and 84%, respectively). Extensive sample pretreatment was recommended for avoiding false negatives; however, this resulted in a lengthy, complicated procedure. The specificity was 98%. However, since the aim of the present study was to recommend a procedure that would be practical, cost effective, have a short turn around time, have limited hazards to laboratory personnel, and be simple enough to be routinely applied in a diagnostic laboratory, these modifications do not seem suitable.

Finally, a short report by Navarro et al. (1999) described a study involving a small number of brucellosis patients that tried to reproduce results obtained with the methodology described above (Queipo-Ortuno et al. 1997). 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 the clinical samples because of different storage conditions were assumed to account for discrepant results, as did the well-known fact that in-house PCR results are difficult to reproduce in different laboratories.

The excellent sensitivity reported by Matar and Queipo-Ortuno, using the primers B4 and B5, in the diagnosis of human brucellosis has not been reproduced by other groups (Navarro et al. 1999; Zerva et al. 2001). In the present study, the diagnostic sensitivity of the B4 and B5 primers was 98% and not 100%, but it was able to detect the 3 relapse cases (which was also the case with Queipo-Ortuno et al. 1997).

In the present study, increasing the number of PCR cycles to 40 rather than 35 and increasing the annealing time to 60 s from 30 s had its toll on increasing the detection limit, since some weak bands were observed that were not seen when the 35 cycle protocol was followed.

Navarro et al. (2002) showed F4 and R2 to be the most sensitive primers, which was not the case in the present study. They found that this pair of primers was affected by the presence of human DNA. One study used the F4 and R2 primers for diagnosis of brucellosis, with a sensitivity of 72.1%, which was higher than that found in the present work (Nimri 2003). In accordance with previous results (Baily et al. 1992; Matar et al. 1996; Queipo-Ortuno et al. 1997), the B4/B5 PCR assay specificity as well as the other primer pair assays was excellent.

The short turnaround time of PCR (less than 4 h) compares favorably with that of blood cultures, Wright’s tube agglutination test, and Wright–Coombs agglutination test (3–7 days, 24 h, and 48 h, respectively). Finally, the costs of in-house PCR methods are low for laboratories already equipped with the necessary infrastructure. The requirements for performing B4/B5 primer PCR should be available for any diagnostic laboratory with the facilities to run an in-house PCR. The apparent high sensitivity of the assay (detection of 700 cfu/mL) without being affected by different concentrations of DNA; the small concentration of primer required (1 μL of a 5 pmol/μL stock); the ability to use ready-made 10× PCR buffers; and the ability to detect, with a high diagnostic sensitivity (98%), DNA extracted by a commercially available, easy-to-use DNA extraction kit (Pure Gene) from both acute samples and samples from relapsed cases — all of these combined qualify the B4/B5 primer pair to be used for the routine diagnosis of brucellosis from human blood by PCR.

References


