Comparison of Culture and Multiplex PCR Technique for Detection of Brucella abortus and Brucella melitensis from Human Blood Samples

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**Article information**

<table>
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<th>Article history:</th>
<th>Abstract</th>
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<td>Received: 27 Nov 2012</td>
<td><strong>Background:</strong> To compare culture methods with multiplex PCR technique for identification of <em>Brucella abortus</em> and <em>Brucella melitensis</em> from suspicious patients with clinical history of brucellosis and positive serological test (Rose Bengal test and serum agglutination test).</td>
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<td>Accepted: 9 Jan 2013</td>
<td><strong>Materials and Methods:</strong> In this study, 160 blood samples from patients suspected of Brucellosis with high serum titers of 1/80 were studied. All samples were cultured in Brucella-specific media. Brucella species were identified by using microbiological methods. DNA was extracted with Phenol-chloroform DNA extraction method. IS711 was amplified simultaneously using three specific primers and obtained patterns were analyzed.</td>
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<td>Available online: 12 Feb 2013</td>
<td><strong>Results:</strong> From 160 samples, 47.5% (76) were culture positive cases from which 43 cases were <em>B. melitensis</em> and 33 were <em>B. abortus</em>. With the PCR technique 108 were detected positive from which 45.3% were <em>B. abortus</em> and 54.6% were <em>B. melitensis</em>. It should be noted that all 76 samples with positive culture were also identified by PCR.</td>
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<td>ZJRMS 2013; 15(12): 5-8</td>
<td><strong>Conclusion:</strong> Generally, use of the molecular technique multiplex PCR in addition to increased speed and accuracy and less false results than bacterial culture method, is able to identify different species of brucella. This will facilitate the treatment process.</td>
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**Keywords:**

Brucella abortus
Brucella melitensis
Multiplex PCR
Culture method

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**Introduction**

*Brucella* is an intracellular parasite of the disease brucellosis throughout the world [1]. This Gram-negative coccobacillus consists of 10 species, of which *B. abortus*, *B. melitensis*, *B. ovis*, *B. canis* and *B. suis* are pathogenic for humans. Also, *B. microti*, *B. inopinata*, *B. ceti* and *B. pinnipedialis* are isolated from animals but can occasionally cause disease in man [2]. Among these species, the main pathogens for humans are *B. melitensis* and *B. abortus* [3, 4]. *Brucella melitensis* is a highly contagious disease in sheep and goat [5]. This species is the most important zoonosis in humans [1]. Human infections due this species are widespread. The epidemics caused by these two species in developing countries is common and causes great damages [3].

This disease has extremely debilitating side effects that can even cause death. So timely and accurate diagnosis is an important factor to identify bacteria, in addition to clinical epidemiology and positive signs, we need related to the laboratory evidence [6, 7]. Current tests used in diagnosis of brucella include: 1. Culture methods, 2. Polymerase chain reaction and 3. Serological methods based on detecting antibodies against brucella.

Definitive diagnostic of brucellosis can be made with blood culture, lymph, bone marrow and other body fluids and secretions [8]. But what has been reported from previous studies is that blood culture and body fluids have low sensitivity and also serum analysis need long periods of incubation [9, 10]. Moreover, serological tests have high false positive and false negative rates [9]. Since serological tests have cross-reactions with common infections such as *Vibrio cholerae* and even *Yersinia enterocolitica* and *Francisella tularensis*, these tests are not very practical [11]. We have chosen to test polymerase chain reaction method for detection of Brucella because of the deficiencies in traditional methods [12, 13]. Multiplex PCR is a method that can identify small nucleotide differences and from small amounts of samples, therefore is very time and cost efficient [7, 14]. In this study we compare culture and multiplex PCR technique for detection of *brucella abortus* and *B. melitensis* from human blood samples.

**Materials and Methods**

**Clinical specimens**

Clinical specimens were collected from suspicious patients with clinical history of brucellosis and positive serological test (Rose Bengal test and serum agglutination test) who had measurable antibody titers 1/80. Ten ml blood was taken from each patient. Five ml for culture and 5 ml for extracting DNA. 5 ml of blood was mixed with EDTA and transferred to laboratory. It should be...
mentioned that we considered 1/80 level of Wright test, which is equivalent to four positives, that is more than 200 international units of antibody, to determine if someone was patient.

**Bacterial cultures**: Five ml blood drawn of patients was added to deionized distilled water containing 0.5% sodium citrate. Gently mixed and centrifuged at 4000×g for 15 minutes. The supernatant was discarded and the pellet was transferred to Brucella agar plates (Germany’s Merck brand). It was incubated at 37°C with 5% carbon dioxide for 7 days [15, 16]. After 7 days, colonies were analyzed by grams staining, Ziehl Neelsen staining, microscopy and colony morphology. Also hydrogen peroxidase and catalase and oxidase tests were performed. Fuchsin dye in the presence of growth was evaluated. All colonies were confirmed to be brucella spp. by multiplex polymerase chain reaction (multiplex PCR).

**Isolation of DNA from clinical blood samples**: We used a modification of the method described by Queipo-Ortuno [17]. Briefly, 0.5 ml of blood with 1 ml of erythrocyte lysis solution (320 mM Saccharose, 5 mM MgCl₂, 1% Triton X-100, 10 mM Tris HCl [pH=7.5]) was mixed and centrifuged at 15,000×g for 2 min. The supernatant was discarded, and above steps were repeated for four times until the pellet lost all reddish coloring. Four hundred micro liters of nucleic lysis buffer (10 mM Tris-Hcl’ 1% SDS’ 10 mM EDTA’ 10 mM Sodium citrate. Gently mixed and centrifuged at 4000×g for 15 minutes. The supernatant was discarded and the pellet was transferred to Brucella agar plates (Germany’s Merck brand). It was incubated at 37°C with 5% carbon dioxide for 7 days [15, 16]. After 7 days, colonies were analyzed by grams staining, Ziehl Neelsen staining, microscopy and colony morphology. Also hydrogen peroxidase and catalase and oxidase tests were performed. Fuchsin dye in the presence of growth was evaluated. All colonies were confirmed to be brucella spp. by multiplex polymerase chain reaction (multiplex PCR).

**Primer**: The presence of the mobile genetic element IS711 (Gen Bank accession no.M94960) has been a useful target for molecular characterization of classical terrestrial Brucella species based on the number and distribution of IS711 copies within the bacterial genomes [14]. The following primers were selected for simultaneous detection of *B. abortus, B. melitensis* [18]:

IS711: 5'-TGCCGATCACCTAAGGGGCTTCA-3'  
B1-F: 5'-AAATCGCGTCCTTGCTGGTCTGA-3'  
B2-F: 5'-GACGAACGGAATTTTCCAATCCC-3'

**PCR amplification**: Each PCR reaction mixture contained 15 µl master mix 2X (AmbioGen Co, Denmark) that contained 1X PCR buffer, 1.5 mM MgCl₂, 1 µl template DNA (0.5 µg), 0.15 mM dNTP, 1.25 U Taq DNA polymerase, 20 pmol of each forward and reverse primers and sterile distilled water up to 50 µl.

PCR were performed in a Gene Amp PCR system (Eppendorf, USA) according to the following program: pre-denaturation for 5-min at 94°C followed by 30 cycles of denaturation at 94°C for 45 sec, annealing at 66°C for 45 sec and extension at 72°C for 60 sec, followed by final extension at 72°C for 5 min.

Then, the PCR products were analyzed using electrophoresis technique on 1.5% agarose gel for 1 hour at 85 volt and 25mA, stained by SYBER green and visualized under UV transilluminator. Finally, amplification products were further evaluated by sequencing and restriction digestion procedures.

Extracted genomes of vaccine strains of *B. abortus* B-19 and *B. melitensis* Rev-1 were used as positive control and a suspension containing all of the reagents except template as negative control. All PCRs were carried out in duplicate.

**Statistical analysis**: The results were analyzed as positive or negative PCR amplification reaction for each bacterium separately, as well as for two or three bacteria simultaneously. Descriptive analyses were performed and results are presented as numbers.

Statistical analysis was conducted to determine how many samples were positive for each bacterium, as well as those positive for two bacterial species. Perspective analyses were performed and data rounded numerical values (percentage) was documented.

**Results**

In this study, 160 suspicious patients had shown symptoms of the disease for on average 20 days. There was no significant difference between sex and rate of infection by brucellosis. Most of patients were in direct contact with livestock.

Time to positive culture in the samples that grew was 3±0.7 (mean±SD) days. 47.5% of tests were positive, among which 56.5% (43 cases) were *B. melitensis* and 44.5% (33 cases) were *B. abortus*. After 10 days, plates without any colonies were considered as negative. 108 (67.5%) were positive by multiplex PCR method (Fig. 1). The isolation rate for *B. melitensis* was 54.6% (59 cases) and 45.3% (49 cases) for *B. abortus* (Table 1).

**Figure 1.** Agarose gel electrophoresis of PCR amplified products generated from DNA samples. Lane 1 shows DNA size marker (100bp DNA ladder). Lanes 2 and 3 show 733 bp *B. melitensis* and 494 bp *B. abortus* amplification product. Lane 4 is negative control.
Table 1. Result of PCR and culture method in blood specimens of suspicious patients to brucellosis.

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<tr>
<th>Organism</th>
<th>PCR N(%)</th>
<th>Culture N(%)</th>
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<tr>
<td>Positives results</td>
<td>108(67.5)</td>
<td>76(47.5)</td>
</tr>
<tr>
<td>B. abortus</td>
<td>49(45.3)</td>
<td>33(44.5)</td>
</tr>
<tr>
<td>B. melitensis</td>
<td>59(54.6)</td>
<td>43(56.5)</td>
</tr>
<tr>
<td>Negative Results</td>
<td>52(42)</td>
<td>167(52.2)</td>
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<td>Total</td>
<td>160(100)</td>
<td>160(100)</td>
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Discussion

Brucellosis is a major health problem in developing and Mediterranean countries [8]. This disease can spread directly and indirectly from infected animals to humans. Since brucellosis has no specific symptoms in humans, it is important to be diagnosed early by laboratory methods [19, 20]. Bacterial isolation and culture is always required for diagnosis and biotyping of strains. For the definitive diagnosis of brucellosis, the choosing a tissue sample for diagnosis depends on the clinical signs observed.

In the case of clinical brucellosis, valid samples include aborted fetuses (spleen, lung, and stomach), vaginal secretions, fetal membranes, milk,colostrum, sperm, blood and fluid collected from arthritis. For liquid samples (blood or milk), sensitivity is increased by the use of a specific medium like the brucella medium, originally described for use with human blood cultures. Growth may appear after 3 days, but if cultures did not grow were usually considered negative after 10 days of incubation. But the new methods for identification and sometimes typing of brucella have been developed which are in use in certain diagnostic laboratories such as PCR based methods [18]. Nevertheless, as a general rule, brucellosis multiplex PCR techniques show a lower diagnostic sensitivity than culture methods.

In this study, 160 blood samples of suspected patients with brucellosis were evaluated with two methods. Accuracy and speed of multiplex PCR method was confirmed in this study.

In a study by Yu et al. [21], molecular methods based on polymerase chain reaction was evaluated and routine use of PCR for diagnosis was recommended in the clinical laboratories. A rapid and sensitive PCR method was reported that does not require laboratory biosafety L1. They showed multiplex PCR to be an efficient method our study also show the same performance for multiplex PCR method. In another study by Kang et al. [22], multiplex PCR was introduced as an accurate, sensitive and rapid detection for brucella was introduced. Our research also shows it to be more sensitive than culture methods.

In this study there was no significant difference between sex and infection rate by brucellosis. Cetinkaya et al., brucellosis serological methods for detection of brucellosis, found that there is relationship between age, sex and infection rate [23, 24]. According to the results, the multiplex PCR technique was better at all stages of diagnosis disease. Multiplex PCR method is more efficient, faster and more accurate than culture methods.

Acknowledgements

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Authors’ Contributions

All authors had equal role in design, work, statistical analysis and manuscript writing.

Conflict of Interest

The authors declare no conflict of interest.

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