Comparative evaluation of nested PCR for the rapid diagnosis of human brucellosis

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Abstract

Background: Brucellosis is known as a zoonotic disease with high morbidity in the absence of treatment. The primary diagnosis of brucellosis can be effective to achieve satisfying treatment results and prevent chronic infections. This study was aimed to compare the efficiency of conventional microbiological and serological approaches with nested PCR for the rapid diagnosis of human brucellosis.

Methods: A total of 120 subjects with symptoms of brucellosis were included in the study. The sensitivity and specificity of nested PCR for detection of Brucella bacteria was compared with serological and blood culture methods.

Results: Out of 120 patients enrolled, brucellosis was detected in 60.83% (73/120) of cases based on serological tests with a blood culture confirmation in 8.33% of participants. Based on results, 55% of cases were positive in serum agglutination test (SAT ≥ 1:160), and Coombs (C-SAT ≥ 1:160) tests. Furthermore, 7 negative SAT cases were positive in C-SAT as evidence for chronic brucellosis. Results of 2-mercaptoethanol (2-ME) ≥ 1:80 test were negative in 6 SAT-
positive cases. Based on nested PCR results, 68.18% SAT positive samples were also detected by blood nested PCR and 56.06% through serum nested PCR method. The sensitivity of blood nested PCR was significantly more than serum nested PCR, SAT≥1:160 and blood culture (P<0.001). The specificity of the blood and serum nested PCR was also 100% compared with blood culture and SAT≥ 1:160. In this study, nested PCR was able to identify chronic brucellosis in SAT negative patients.

**Conclusion:** According to the findings of this study, nested PCR showed higher efficiency than blood culture method for the rapid diagnosis of human brucellosis. Also, results are indicating the high performance of nested PCR for the rapid diagnosis of both chronic and acute brucellosis.

**Keywords:** Brucellosis, SAT, C-SAT, 2ME, Nested PCR

**Background**

Brucellosis with the annual incidence of half a million cases worldwide is one of the most common zoonotic diseases (Deng et al., 2019; Golshani and Buozari, 2017).

*Brucella* as a Gram-negative intracellular pathogen can infect the wide range of animals and human (DelVecchio et al., 2002). The most common species of human brucellosis are including *Brucella(B) melitensis*, *B. abortus*, *B. canis*, *B. suis*, *B. ovis*, and *B. neotomae* (DelVecchio et al., 2002). The prevalence of brucellosis especially *B. melitensis* is still high in several regions of Iran (Djalalinia et al., 2019; Esmaeili, 2015; Golshani and Buozari 2017). Commonly, human brucellosis is recognized based on symptoms such as headache, myalgia, high fever, malaise, chills, and even arthralgia of the large joints (Fanni et al., 2013).
Based on clinical manifestations time, brucellosis is classified as acute (0 to 2 months), sub-acute (2 to 12 months), and chronic (>12 months) (Jia et al., 2017; Roushan et al., 2016). The early diagnosis of brucellosis is critical for prevention of chronic infection with high mortality.

Human brucellosis is generally detected by conventional microbiological tests to isolate *Brucella* spp, serological tests for determination of anti-*Brucella* antibodies and molecular approaches to detect *Brucella* DNA (Lucero et al., 1999; Wang et al., 2014). Although, blood culture is known as gold standard for identification of *Brucella*, it is time-consuming, unsuccessful regarding chronic brucellosis and beside this method can increase the risks of handling the pathogen in the laboratory (Al Dahouk and Karsten Nöckler, 2011; Vafaei et al., 2019).

The serological tests are cost effective, rapid with high sensitivity but the detection of antibodies is not always indicating active brucellosis and people from endemic areas generally show weak serological responses (de Glanville et al., 2017; Wang et al., 2014). Accordingly, in the endemic areas for *Brucella* spp. in animals, the diagnostic titer of a single SAT is depending on levels of endemicity (ranging from 1:80 to 1: 320) (de Glanville et al., 2017). Among the serological tests, Rose Bengal test (RBT) and the serum agglutination test (SAT) are the most common methods to detect brucellosis (Koroglu et al., 2016; Rajaii et al., 2006). However, there are limitations to detect incomplete/blocking antibodies in chronic patients by the mentioned serological tests. In such cases, human globulin Coombs test (Coombs Wright test) is performed by adding anti-human globulin (Coombs antibody) to the SAT to eliminate false negative results. In this respect, 2- mercaptoethanol (2-ME) test is suitable for prediction of the course of the disease (Dias and Dias, 2015; Mitka et al., 2007).

Recently, PCR based assays have been considered for diagnosis of *Brucella* even in blood samples with negative culture, due to low cost, high sensitivity and specificity. According to
previous reports, PCR is reliable for the early diagnosis and detection of relapse or chronic brucellosis (Kanani, 2007; Roushan et al., 2016; Tabibnejad et al., 2016).

The present study was aimed to evaluate the sensitivity and specificity of nested PCR for the rapid diagnostic of brucellosis.

**Methods**

**Clinical specimens**
A total of 120 blood specimens were obtained from patients aged 5-60 years with clinical symptoms of brucellosis from Tabriz in North West of Iran admitted to Central Laboratory of Tabriz, Iran. Demographic data of patients have been summarized in Table 1.

**Microbiological Methods**
A 10 ml blood specimen was obtained from hospitalized patients during fever period and was processed by conventional blood culture method, as described (Mangalgi and Sajjan, 2014). Briefly, the blood samples were inoculated aseptically into Castaneda’s medium, incubated at 37°C under 10% CO₂ and monitored for 28 days. The isolated bacteria were identified based on microbiological methods including gram staining, biochemical tests such as urease and oxidase and catalase. (Table 2) (Mangalgi and Sajjan 2014).

**The serological tests**
The serological tests including SAT, C-SAT and 2ME were performed on the sera samples for detection of Brucella antibody based on conventional protocol (Mangalgi et al., 2012).

In the SAT test, the sera samples were diluted up to 1/1280 dilution with 0.5% phenol saline starting from 1:10 to 1:1280. After that, each sample was incubated at 37°C for 20 hours in the
presence of 0.5 ml *B. abortus* plain antigen. The known serum samples were employed as negative and positive controls during SAT test.

The test tubes were compared with antigen control tubes for determination of the antibodies titer. To eliminate false negative results in sera, C-SAT test was also performed as described (Roushan et al., 2016). Also, 2ME test was performed to eliminate the cross-reacting IgM antibodies and detection of *Brucella* specific IgG antibodies, (Mangalgi et al., 2012; Purwar et al., 2016).

The serological diagnosis was established by a positive SAT titer of ≥1:160, Coombs anti-*Brucella* titer of ≥ 1:160 and 2ME titer of ≥ 1:80 (Roushan et al., 2016).

**DNA extraction from the blood samples**

For this, lymphocytes were separated from blood using lysis buffer (10 mM NaHCO₃, 150 mM NH₄Cl, 1mM EDTA, pH 7.4) (Ghatak et al., 2013). After that, the cells were resuspended in TE buffer (Tris 1M and EDTA 0.5M) containing 10% SDS and 10µL proteinase K; and incubated overnight at 42°C. The extraction of DNA from blood and serum samples was performed by phenol-chloroform method as described (Ghatak et al., 2013). The quality and quantity of extracted DNA was determined via agarose gel electrophoresis and spectroscopy.

**Detection of *Brucella* by Nested PCR**

The existence of *Brucella* DNA in serum and blood samples of patients was examined by nested PCR reaction. The specific primers for nested PCR were designed based on a sequence on the *brucella* genome nominated as 31-KDa cell surface protein that is conserve among *brucella* spp (Kumar et al., 2007). The first round PCR reaction was performed using F1/R1 primers for detecting a 390bp conserved fragment in the chromosome of four prevalent species in human
including *B.melitensis*, *B.abortus*, *B.suis* and *B. canis* (Roushan et al., 2014). Then PCR products obtained from this reaction were used for the second PCR reaction using F2/R2 primers. The first PCR reaction was performed using F1/R1 primers; Forward: 5'-AAGATGGTGCGCTGGACGCC-3’ and Reverse: 5’-AAAAGCGTTCTGCACGCGGA-3’ in a 25 µl reaction for 35 cycles (94°C for 1min, 60°C for 1min, 72°C for 1min) after an initial denaturation at 94°C for 4 min. The final extension was 72°C for 5 min. The nested PCR protocol was performed on PCR products of the primary reaction by F2/R2 primers; Forward: 5’-CTTTGTGGGGCGGTATCC-3’ and Reverse: 5’-CGCAGCATGCAGCTTGATGAG-3’ with the annealing temperature 59 ºC in order to detect a 319bp gene fragment in the four prevalent species. Products were examined by agarose gel electrophoresis. The positive PCR products were confirmed by sequencing.

**Statistical analysis**

Statistical analysis was carried out through independent t-test and chi-square (*P* value< 0.05). The efficiency of nested PCR was evaluated by calculation of sensitivity, specificity of nested PCR compared to conventional diagnostic methods. The sensitivity and specificity of the tests were evaluated as following:

% sensitivity = [true positives/ true positives + false negatives] × 100 (Doucette, 2015).

% Specificity = [true negatives/(true negatives + false positives)]×100 (Mahy and Van Regenmortel, 2010).

**Results**

**Results of microbiological test**
Out of 120 blood samples obtained from patients with brucellosis symptoms, only 10 cases (8.33%) were positive in blood cultures as non-pigmented, non-hemolytic and small grey colonies (Table 2) which also were positive in both PCR and SAT tests.

**Detection of Brucella antibodies by SAT, 2ME and C-SAT tests**

Of 120 patients enrolled, 60.83% (73/120) were positive brucellosis based on serological tests which 66(55%) cases were positive in both of SAT and C-SAT. In addition to, 7 cases with SAT 1:80 were positive in C-STA test indicating chronic brucellosis and false negative results in SAT test. Overall 48.48% (32/66 cases) of samples were positive in all three tests, 7 cases were positive in both STA and 2ME tests. Also, 6 SAT positive cases were negative in 2ME test. Out of 66 SAT positive samples, the SAT titer was four fold higher than the 2ME titer in 28.78% (19 cases) of samples. Details of results are summarized in Table 1.

**Diagnosis of Brucellosis using Nested PCR**

Totally, 60 (50%) of the 120 samples were positive in nested PCR performed on blood and serum samples (Figure 1). Out of 66 SAT positive samples, 68.18% (45 cases) were positive in blood nested PCR and 56.06% (37 cases) were positive in serum nested PCR (Table 3). In total, 81.66% (49/66) SAT positive cases were positive in blood and serum nested PCR. Furthermore, we had 7 nested-PCR positive cases with SAT titer 1:80 but positive in C-SAT ≥ 1:160.

**Assessment of specificity and sensitivity of Nested PCR**

Efficiency of nested PCR for detecting *Brucella* in serum and blood samples was compared with results obtained from blood culture and SAT ≥ 1:160 methods through calculation of sensitivity, specificity of the methods.
Overall, 90.90% (60/66) nested PCR samples were positive in SAT test. In compared to SAT test, the sensitivity of blood and serum nested PCR was calculated as 60.31% and 48.43%, respectively. The specificity of the nested PCR was 100% compared to blood culture and SAT ≥1:160 methods. Notably, the sensitivity of blood nested PCR was more than serum nested PCR and SAT and blood culture (P<0.001).

Discussion
Although, the serologic methods are the most common procedures used for detecting human brucellosis, however there are several limitations such as low specificity and sensitivity because of serological cross reactivity or weak immune responses (Park et al., 2012). On the other hand, the serological tests are incapable to distinguish chronic and acute patients. Thought, the blood culture is suitable for isolation and identification of brucellosis but the sensitivity of this method is low, reliant on Brucella species, the stage of disease and type of culture medium (Dadar et al., 2019; Pabuccuoglu et al., 2011). In the present study we evaluated the sensitivity and specificity of nested PCR compared with conventional diagnostic methods.

In our work, the Brucella antibody was detected in 60.83% (73/120) of patients with clinical symptoms of brucellosis through STA, C-STA and 2ME methods. The Brucella DNA was detected in 90.99% of STA positive patients by nested PCR, while only 10 (8.33%) patients were positive in blood culture similar to study done by Roushan et al., with 10% the positive blood culture (Roushan et al., 2016; Zerva et al., 2001).

Hence, in our study, if blood culture method was considered as the gold standard, almost 84% of positive brucellosis samples were ignored. In consistent with previous studies, our findings
indicated that, culture method may be negative while other tests are positive (Pabuccuoglu et al., 2011; Romero et al., 1995; Roushan et al., 2016).

The sensitivity and specificity of nested PCR was 100% in compared to blood culture which is in agreement with previous reports (Hekmatimoghaddam et al., 2013; Refaat et al., 2016). The high sensitivity of nested PCR suggests that PCR is reliable than blood culture for the rapid diagnosis of acute and chronic brucellosis. In this study, 7 cases with negative SAT and positive C-SAT test which diagnosed as chronic brucellosis were positive in nested PCR.

In total, the sensitivity and specificity of nested PCR in comparison with SAT were 79% and 100%, respectively consistent with several previous researches (Hassanain and Ahmed, 2012; Khosravi et al., 2006; Masallat et al., 2013). In this study, the sensitivity of the blood nested PCR was higher than serum nested PCR. This observation was consistent with previous report (Keid et al., 2010). As noted previously, the serum-nested PCR is more rapid than blood nested PCR due to easier DNA extraction process but the blood nested PCR has higher sensitivity (Alikhani et al., 2013; Takele et al., 2009). Totally, in the present study, nested PCR was able to identify *Brucella* DNA even in SAT negative cases but positive –CSAT indicating the ability of our nested PCR for identification of chronic brucellosis(Asaad and Alqahtani, 2012; Gemechu et al., 2011). This findings were consistent with previous reports which emphasize on the advantages of nested PCR for diagnosis of brucellosis in both of the early stages and chronic disease (Roushan et al., 2016; Zamanian et al., 2015). However, nested PCR is not applicable when *Brucella* is negative in blood.

**Conclusion:** According to the findings of this study, nested PCR showed higher efficiency than blood culture method for the rapid diagnosis of human brucellosis.
Abbreviations

SAT: serum agglutination test, 2-ME: 2- mercaptoethanol,

Competing interests

Authors have no conflict of interest.

Acknowledgments

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Table 1: The epidemiological data and serological tests results of 120 patients with brucellosis symptoms.

<table>
<thead>
<tr>
<th>Study group (n=120)</th>
<th>Values</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender</td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>59(49.16%)</td>
</tr>
<tr>
<td>Male</td>
<td>61(50.83%)</td>
</tr>
<tr>
<td>Age</td>
<td></td>
</tr>
<tr>
<td>5-15</td>
<td>10(8.33%)</td>
</tr>
<tr>
<td>15-30</td>
<td>28(23.33%)</td>
</tr>
<tr>
<td>31-45</td>
<td>36(30%)</td>
</tr>
<tr>
<td>46-60</td>
<td>19(15.83%)</td>
</tr>
<tr>
<td>&gt;60</td>
<td>18(15%)</td>
</tr>
<tr>
<td>SAT ≥ 1/160</td>
<td></td>
</tr>
<tr>
<td>C-SAT</td>
<td>66(55%)</td>
</tr>
<tr>
<td>2-ME ≥ 1/80</td>
<td></td>
</tr>
<tr>
<td>Serum nested PCR</td>
<td>73(60.83%)</td>
</tr>
<tr>
<td>Blood nested PCR</td>
<td>43(35.83%)</td>
</tr>
<tr>
<td>Clinical symptoms</td>
<td></td>
</tr>
<tr>
<td>fever</td>
<td>98(81.66%)</td>
</tr>
<tr>
<td>fatigue</td>
<td>90(75%)</td>
</tr>
<tr>
<td>headache</td>
<td>68(56.66%)</td>
</tr>
<tr>
<td>Symptom</td>
<td>Frequency</td>
</tr>
<tr>
<td>------------------------</td>
<td>-----------</td>
</tr>
<tr>
<td>chills</td>
<td>54 (45%)</td>
</tr>
<tr>
<td>nocturnal diaphoresis</td>
<td>46 (38.33%)</td>
</tr>
<tr>
<td>Weight loss</td>
<td>24 (20%)</td>
</tr>
</tbody>
</table>

Table2: The phenotypic properties of *Brucella* spp isolates recovered from blood samples.

<table>
<thead>
<tr>
<th></th>
<th>Gram staining</th>
<th>Colony morphology</th>
<th>Incubation period</th>
<th>Catalase</th>
<th>Urease</th>
<th>Oxidase</th>
<th>motility</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Brucella</em> spp.</td>
<td>Gram negative</td>
<td>non-pigmented, non-hemolytic and small grey</td>
<td>Up to six weeks, Slow growing</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>Non-motile</td>
</tr>
</tbody>
</table>

Table3: comparison of results of different diagnostic methods for human brucellosis. Serum agglutination test (SAT), Coombs Wright test (C-SAT), 2-mercaptoethanol (2-ME), nested PCR and blood culture.
<table>
<thead>
<tr>
<th>Titer</th>
<th>SAT (%)</th>
<th>C-SAT (%)</th>
<th>2-ME (%)</th>
<th>Serum Nested PCR (%)</th>
<th>Blood Nested PCR (%)</th>
<th>Blood culture (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt;1:80</td>
<td>46 (38.3)</td>
<td>46 (38.3)</td>
<td>3 (2.5)</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>1:80</td>
<td>9 (7.5)</td>
<td>4 (3.33)</td>
<td>18 (15)</td>
<td>7 (5.83)</td>
<td>7 (5.83)</td>
<td>0</td>
</tr>
<tr>
<td>1:160</td>
<td>31 (25.8)</td>
<td>20 (16.66)</td>
<td>2 (1.66)</td>
<td>17 (14.16)</td>
<td>18 (15)</td>
<td>2 (1.6)</td>
</tr>
<tr>
<td>1:320</td>
<td>20 (16.66)</td>
<td>16 (13.33)</td>
<td>3 (2.5)</td>
<td>10 (8.33)</td>
<td>11 (9.16)</td>
<td>2 (1.6)</td>
</tr>
<tr>
<td>1:640</td>
<td>9 (7.5)</td>
<td>7 (5.83)</td>
<td>3 (2.5)</td>
<td>1 (0.83)</td>
<td>4 (3.33)</td>
<td>3 (2.5)</td>
</tr>
<tr>
<td>1:1,280</td>
<td>9 (7.5)</td>
<td>10 (8.33)</td>
<td>1 (0.83)</td>
<td>3 (2.5)</td>
<td>5 (4.16)</td>
<td>3 (2.5)</td>
</tr>
</tbody>
</table>

Figure legend:
Figure 1: Results of nested PCR for identification of *Brucella* clinical isolates. **Lane 1:** the primary PCR reaction with F1R1 primers as a band of 390bp displayed in electrophoresis gel. **Lane 2:** 1kb DNA marker. **Lane 3:** nested PCR reaction using pair F2R2 primers as a sharp band of 319bp displayed in electrophoresis gel.
References


