

Comparative evaluation of nested PCR for diagnosis of human brucellosis

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Research note

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Abstract

Objectives: Brucellosis is a worldwide zoonotic disease with high morbidity in the absence of treatment. The early diagnosis of brucellosis is efficient to prevent chronic infections. The aim of this study is evaluation of nested PCR efficiency in comparison with conventional methods for diagnosis of human brucellosis. A total of 120 patients with brucellosis symptoms were included in this study. Serological and microbiological tests and nested PCR were used for detection of *Brucella* bacteria.

Results: Based on serological tests, 60.83% (73/120) of individuals were positive for brucellosis which only 8.33% of cases were confirmed by blood culture. Among them, 55% of cases were positive in serum agglutination test (SAT \geq 1:160) and Coombs (C-SAT \geq 1:160) tests. Furthermore, 7 negative SAT cases were positive in C-SAT as evidence for chronic brucellosis. Also, 68.18% and 56.06% of SAT positive samples were positive in blood nested PCR and serum nested PCR respectively. The sensitivity of blood nested PCR was more than serum nested PCR, SAT \geq 1:160 and blood culture ($P<0.001$). The specificity of the blood and serum nested PCR was 100% compared with blood culture and SAT \geq 1:160. Our findings highlight high performance of nested PCR for diagnosis of both acute and chronic brucellosis.

Introduction

Brucellosis is one of the most common zoonotic diseases with the annual occurrence of half a million cases worldwide[1].

Brucella as a Gram-negative, facultative, intracellular pathogen can infect the wide variety of animals and human. The most common species of pathogenic in human are including *Brucella(B) melitensis*, *B. abortus*, *B. suis*, *B. ovis*, *B. canis* and *B. neotomae* [2]. The prevalence of brucellosis especially *B. melitensis* is still high in several regions of Iran[1]. The main symptoms of human brucellosis are including high fever, headache, chills, malaise, myalgia and even arthralgia of the large joints [3].

Based on symptoms and clinical presentation time, brucellosis can be classified as acute (0–2 months), sub-acute (2–12 months), and chronic (>12 months) [4, 5]. The early diagnosis of brucellosis is critical for prevention of chronic infection with high mortality.

There are several approaches for diagnosis of human brucellosis such as conventional microbiological tests to isolate *Brucella* spp, serological tests for identification of anti-*Brucella* spp antibodies and molecular methods to detect *Brucella* spp. DNA [6]. Although, blood culture is considered as gold standard for diagnosis of brucellosis, it is time-consuming, unsuccessful in the case of chronic brucellosis and increases the hazards of handling the organism in the laboratory [7].

The serological tests are cost effective, rapid with high sensitivity but the presence of antibodies does not always imply to active brucellosis and people from endemic areas generally show weak serological responses[6]. Among the serological tests, Rose-Bengal test and the serum agglutination test (SAT) are most common methods to detect brucellosis [8]. 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 [9].

Recently, PCR based assays due to high sensitivity and low cost are used to detect *Brucella* DNA in clinical samples such as tissue, blood, milk [10, 11]. This study was aimed to compare the sensitivity and specificity of nested PCR with conventional diagnostic approaches of brucellosis.

Methods

Clinical specimens

Blood samples were collected from patients with clinical symptoms of brucellosis admitted to Central Laboratory of Tabriz, Iran. A total of 120 patients (61 males, 59 females) with the mean age of 39 years were included in this study.

Microbiological Methods

A 10 ml-blood specimen was obtained from hospitalized patients during fever period. Each sample was processed by conventional blood culture method, as described[12]. Briefly, the blood samples were inoculated aseptically into Castaneda's biphasic medium and incubated at 37°C with 10% CO₂ for 28 days. The bottles were observed daily for growth. Sub-culturing was done by allowing the blood broth mixture to flow over the solid phase.

The isolates were identified based on colony morphology, gram stain, modified "ZN" staining, CO₂ requirement, biochemical tests such as oxidase and urease, H₂S production for 4 days and growth in the presence of basic fuchsin (1:50,000 and 1:100,000) and thionin (1:25,000, 1:50,000 and 1:100,000) [12].

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 [13].

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. Equal amount of *B. abortus* plain antigen (0.5 ml) was added to each sample and incubated at 37° C for 20 hours in a water bath. A known positive and negative control sera were also included along with the samples. The test tubes were compared with antigen control tubes based on degree of opacity of the supernatant fluid.

For determination of titer of antibodies as the end point of serum activity, the highest serum dilution indicating 50% agglutination was recorded. To eliminate false negative results in sera, C-STA test was also performed as described. 2ME test was also carried out to eliminate the cross-reacting IgM antibodies and evaluation of *Brucella* specific IgG antibodies [13].

The serological diagnosis was established by a positive STA titer of $\geq 1:160$, Coombs anti-Brucella titer of $\geq 1:160$ and 2ME titer of $\geq 1:80$.

Isolation of DNA from blood samples

For this, lymphocytes were separated from blood by lysing the red blood cells using lysis buffer (150 mMNH₄Cl, 10 mMNaHCO₃, 1mMEDTA, pH 7.4)[14]. After that, TE buffer (Tris 1M and EDTA 0.5M) containing 10%SDS and 10 μ L proteinase K was added to samples and incubated at 42°C overnight. DNA extraction from blood samples was carried out by phenol-chloroform method as described [14]. The quantification of extracted DNA was carried out via spectroscopy at 260nm. The DNA purity was determined by the absorbance ratio of 260/280.

Detection of *Brucella* DNA 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 detection of *B. melitensis*, *B. abortus*, *B. ovis*, *B. canis* and *B. neotomae*, *B. suis* strains were designed based on genome sequences of *Brucella* bacteria in NCBI. The first round PCR reaction was performed using F1/R1 primers (F1: 5'-AAGATGGTGCCTGGACGCC-3'; R1: 5'-AAAAGCGTTCTGCGCCGGGA-3'). PCR products obtained from this reaction were used for the second PCR reaction using F2/R2 primers (F2: 5'-CTTTGTGGGCGGCTATCC-3'; R2: 5'-CGCACTATCGAGCTTGATGAG-3'). Products were analyzed on %1.5 (w/v) agarose gels. For further corroboration, the PCR product of each gene was analyzed by sequencing.

Statistical analysis

Data were analyzed using SPSS program (software version 17.0). Independent t-test was used to compare quantitative variables. Chi-square test was used to detect statistical significant difference (*P* value < 0.05). To evaluate the efficiency of nested PCR, sensitivity, specificity, and accuracy were compared to conventional diagnostic tests.

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 which also were positive in both PCR and STA tests.

Detection of Brucella antibodies by STA, 2ME and C-STA 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. Results of 2ME test were negative in 6 SAT-positive cases. Out of 66 STA positive samples, 28.78% (19 cases) had an SAT titer at least 4-fold higher than the 2ME titer. Details of results are summarized in Table 1.

Diagnosis of Brucellosis using Nested PCR

Totally, 60 (50%) of the 120 samples were positive by nested PCR performed on blood and serum samples. Out of 66 SAT positive samples, 68.18% (45 cases) were positive by blood nested PCR and 56.06% (37 cases) were positive in serum nested PCR (Table 2). 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 \geq 1:160.

Evaluation of sensitivity and specificity of Nested PCR

Efficiency of nested PCR for detecting *Brucella* DNA in blood and serum samples was compared with results of blood culture and STA \geq 1:160 through calculation of their sensitivity, specificity. The sensitivity, specificity of tests was evaluated as following:

$$\% \text{ sensitivity} = [\text{true positives} / (\text{true positives} + \text{false negatives})] \times 100 [15]$$

$$\% \text{ Specificity} = [\text{true negatives} / (\text{true negatives} + \text{false positives})] \times 100 [16]$$

Overall, 90.90% (60/66) nested PCR samples were positive in SAT test. 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 \geq 1:160 methods since all the control subjects were negative. The sensitivity of blood nested PCR was significantly more sensitive than serum nested PCR and SAT and blood culture ($P < 0.001$).

Discussion

Although, the STA is the most common procedure used for diagnosis of human brucellosis, it is not sufficiently sensitive and specific because of serological cross reactivity or weak immune responses[17]. Also, the sensitivity of blood culture is low, depending on *Brucella* species, the stage of disease and type of culture medium [18]. In the present study we compared the sensitivity and specificity of Nested PCR with conventional diagnostic methods.

In total, the *Brucella* antibody was detected in 60.83% (73/120) of patients with clinical symptoms of brucellosis through SAT, C-SAT and 2ME methods. The *Brucella* DNA was detected in 90.99% of SAT positive patients by nested PCR, while only 10 (8.33%) patients were positive in blood culture. In other study done by Roushan et al., only 10% patients had positive blood culture [4]. Hence, in this study, if blood culture was considered as the gold standard, almost 84% of positive brucellosis samples were ignored. In consistent with previous studies, our findings indicated that, culturing method is not always positive when other tests are positive [4, 18, 19].

The sensitivity and specificity of nested PCR was 100% compared to blood culture which is in agreement with other studies [20]. The high sensitivity of nested PCR suggests that PCR is reliable than blood culture for the diagnosis of acute brucellosis.

In total, the sensitivity and specificity of nested PCR compared with SAT were 79% and 100%, respectively which is consistent with several previous researches [21–23]. In this study, the sensitivity of the blood nested PCR compared with SAT was higher than serum nested PCR in consistent with other studies [24]. As a noted previously, the serum-PCR is rapid but the blood PCR showed higher sensitivity [25]. 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 diagnosis of chronic brucellosis. This findings were consistent with previous reports and emphasizes on the advantages of nested PCR for diagnosis of brucellosis in both of the early stages and chronic disease [4, 26].

Conclusion: According to our findings, nested PCR has better analytical performances than blood culture for diagnosis of brucellosis.

Limitations

However, none of methods show 100% sensitivity and specificity for diagnosis of both acute and chronic brucellosis, hence combination of serological, microbiological and molecular methods is suggested for diagnosis of human brucellosis.

Declarations

Abbreviations

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

Ethics approval and consent to participate

The study was conducted after ethical approval of the ethics committee of Tabriz University of medical science, Tabriz Iran. The blood samples were collected after written consent with a brief description about the importance of the study to the participants. Regarding to children (under 16 years of age), consent to participate was collected from the parents/guardians.

Consent for publication

Not applicable.

Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Competing interests

Authors have no conflict of interest.

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Authors' contributions

FS designed the study and drafted the work. LR wrote the manuscript and was involved in study analysis. BN interpreted data. NS performed microbial and molecular tests. All authors read and approved the final manuscript.

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Tables

Table 1: Epidemiological, serological, results of 120 patients with brucellosis symptoms

Study group(n=120)	values
Gender	
Female	59
Male	61
Age	
5-15	10
15-30	28
31-45	36
46-60	19
>60	18
SAT \geq 1/160, no. (%)	66
C-SAT	73
2-ME \geq 1/80	43
Serum nested PCR	45
Blood nested PCR	55

Table2: Comparison of the nested PCR results with SAT, C-SAT and 2-ME tests in brucellosis patients

Titer	SAT (%)	C-SAT (%)	2-ME (%)	Serum Nested PCR (%)	Blood Nested PCR (%)
<1:80	46(38.3)	46 (38.3)	3(2.5)	0	0
1:80	9(7.5)	4(3.33)	18(15)	7(5.83)	7(5.83)
1:160	31 (25.8)	20 (16.66)	2(1.66)	17(14.16)	18(15)
1:320	20 (16.66)	16 (13.33)	3(2.5)	10(8.33)	11(9.16)
1:640	9 (7.5)	7 (5.83)	3(2.5)	1(0.83)	4(3.33)
1:1,280	9 (7.5)	10 (8.33)	1(0.83)	3(2.5)	5(4.16)

Figures

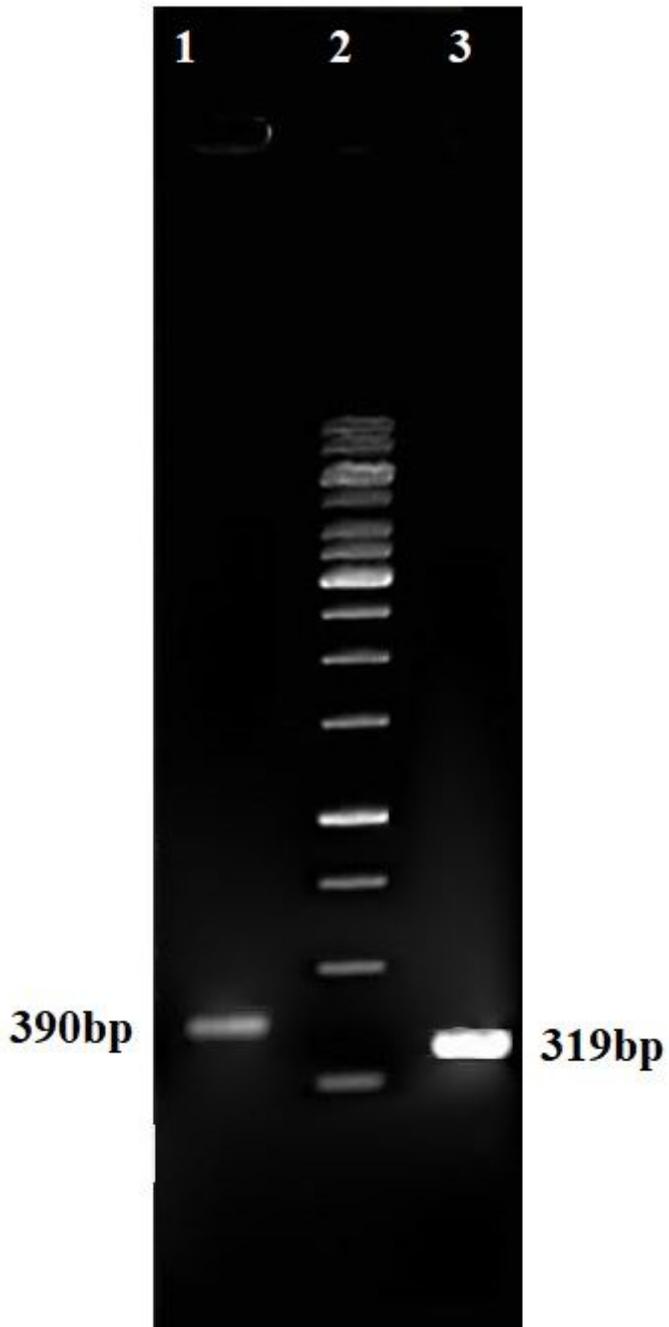


Figure 1

Results of nested PCR for identification of *Brucella* clinical isolates, Lane1: 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.