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Original Article

Comparative Evaluation of Nested Polymerase Chain Reaction for Rapid Diagnosis of Human Brucellosis

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Abstract

Brucellosis is recognized as a zoonotic disease with high morbidity in the absence of treatment. The primary diagnosis of brucellosis can be effective in the achievement of satisfying treatment results and prevention of chronic infections. The present study aimed to compare the efficiency of conventional microbiological and serological approaches with nested Polymerase chain reaction (nested PCR) for rapid diagnosis of human brucellosis. A total of 120 subjects with symptoms of brucellosis were included in the study. The sensitivity and specificity of nested PCR for the detection of *Brucella* bacteria were compared with serological and blood culture methods. Out of 120 patients enrolled, brucellosis was detected in 73 (60.83%) cases based on serological tests with a blood culture confirmation in 8.33% of participants. Based on the obtained results, 55% of cases were positive in serum agglutination test (SAT≥1:160), and Coombs (C-SAT≥1:160) tests. Furthermore, seven negative SAT cases were positive in C-SAT as evidence of chronic brucellosis. The results of the 2-mercaptoethanol (2-ME) ≥ 1:80 test were negative in six SAT-positive cases. Based on nested PCR results, 68.18% and 56.06% SAT positive samples were also detected by blood nested PCR and serum nested PCR, respectively. The sensitivity of blood nested PCR was significantly more than serum nested PCR, SAT≥1:160, and blood culture (P<0.001). Moreover, the specificity of blood and serum nested PCR was obtained at 100%, compared to blood culture and SAT≥ 1:160. In the present study, the nested PCR was able to identify chronic brucellosis in SAT negative patients. As evidenced by the obtained results, the nested PCR showed higher efficiency for rapid diagnosis of human brucellosis, as compared to the blood culture method. Furthermore, the findings pointed to the high performance of nested PCR for rapid diagnosis of both chronic and acute brucellosis.

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

Évaluation Comparative de la Réaction en Chaîne Par Polymérase Emboîtée Pour le Diagnostic Rapide de la Brucellose Humaine

Résumé: La brucellose est reconnue comme une maladie zoonotique avec une morbidité élevée en l'absence de traitement. Le diagnostic primaire de la brucellose peut être efficace pour obtenir des résultats de traitement satisfaisants et prévenir les infections chroniques. La présente étude visait à comparer l'efficacité des approches microbiologiques et sérologiques conventionnelles avec la réaction en chaîne par polymérase emboîtée (PCR emboîtée) pour un diagnostic rapide de la brucellose humaine. Un total de 120 sujets présentant des symptômes de brucellose ont été inclus dans l'étude. La sensibilité et la spécificité de la PCR emboitée pour la détection des bactéries *Brucella* ont été comparées aux méthodes sérologiques et d'hémoculture. Sur120 patients recrutés, la brucellose a été détectée dans 73 (60,83%) cas sur la base de tests sérologiques avec une confirmation

d'hémoculture chez 8.33% des participants. Sur la base des résultats obtenus, 55% des cas étaient positifs aux tests d'agglutination sérique (SAT ≥1: 160) et Coomb (Test à l'antiglobuline) (C-SAT≥1: 160). En outre, sept cas de SAT négatifs étaient positifs dans C-SAT comme preuve de brucellose chronique. Les résultats du test 2-mercaptoéthanol (2-ME) ≥ 1:80 ont été négatifs dans six cas positifs au SAT. Sur la base des résultats de PCR emboitée, 68.18% et 56.06% d'échantillons positifs au SAT ont également été détectés par PCR emboitée dans le sang et PCR emboitée dans le sérum, respectivement. La sensibilité de la PCR emboitée dans le sang était significativement plus élevée que la PCR emboitée dans le sérum, SAT≥1: 160 et l'hémoculture (P<0,001). De plus, la spécificité de la PCR emboitée dans le sang et le sérum a été obtenue à 100%, par rapport à l'hémoculture et SAT≥1: 160. Dans la présente étude, la PCR emboitée a pu identifier la brucellose chronique chez les patients à SAT négatif. Comme en témoignent les résultats obtenus, la PCR emboitée a montré une efficacité plus élevée pour le diagnostic rapide de la brucellose humaine, par rapport à la méthode d'hémoculture. En outre, les résultats ont souligné la haute performance de la PCR emboitée pour le diagnostic rapide de la brucellose chronique et aiguë.
Mots-clés: Brucellose, C-SAT, 2ME, PCR emboitée, SAT

1. Introduction

Brucellosis with an annual incidence of half a million cases worldwide is one of the most common zoonotic diseases (Golshani and Buozari, 2017; Deng et al., 2019). *Brucella* as a Gram-negative intracellular pathogen can infect a wide range of animals and humans (DelVecchio et al., 2002). The most common species of human brucellosis include *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 (Esmaeili, 2015; Golshani and Buozari, 2017; Djalalinia et al., 2019).

Human brucellosis is commonly recognized based on such symptoms as headache, myalgia, high fever, malaise, chills, and even arthralgia of the large joints (Fanni et al., 2013). Based on clinical manifestation time, brucellosis is classified as acute (0-2 months), sub-acute (2-12 months), and chronic (>12 months) (Hasanjani Roushan et al., 2016; Jia et al., 2017). The early diagnosis of brucellosis is critical for the 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 the gold standard for the identification of *Brucella*, it is time-consuming and unsuccessful for chronic brucellosis. Moreover, this method can increase the risks of handling the pathogen in the laboratory (Al Dahouk and Nockler, 2011; Vafaei et al., 2019).

The serological tests are cost-effective, rapid, and high sensitive; nonetheless, the detection of antibodies does not always indicate active brucellosis, and people from endemic areas generally show weak serological responses (Wang et al., 2014; de Glanville et al., 2017). Accordingly, in the endemic areas for *Brucella spp.* in animals, the diagnostic titer of a single serum agglutination test (SAT) depends on levels of endemicity (ranging from 1:80 to 1: 320) (de Glanville et al., 2017).

Among the serological tests, the Rose Bengal test (RBT) and SAT are the most commonly used methods for the detection of brucellosis (Rajaii et al., 2005; Koroglu et al., 2016). Nonetheless, there are limitations to using the mentioned serological tests for the detection of incomplete/blocking antibodies in chronic patients. In such cases, the human globulin Coombs test (Coombs Wright test) is performed by the addition of anti-human globulin (Coombs antibody) to the SAT to eliminate false-negative results. In this respect, the 2- mercaptoethanol (2-ME) test is suitable for the prediction of the course of disease (Mitka et al., 2007; Dias and Dias, 2015).

Polymerase chain reaction (PCR)-based assays have been recently considered for the 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 et al., 2008; Hasanjani Roushan et al., 2016; Tabibnejad et al., 2016). In light of the aforementioned issues, the present study aimed to evaluate the sensitivity and specificity of nested PCR for rapid diagnostic of brucellosis.

2. Material and Methods

2.1. Clinical Specimens

A total of 120 blood specimens were obtained from patients aged 5-60 years with clinical symptoms of brucellosis admitted to the Central Laboratory of Tabriz, Iran. Demographic characteristics of patients are presented in Table 1.

2.2. Microbiological Methods

A 10 ml blood specimen was obtained from hospitalized patients at the time of fever, followed by the conventional blood culture method as described (Mangalgi and Sajjan, 2014). In brief, the blood samples were aseptically inoculated into Castaneda's medium, incubated at 37°C under 10% CO_2, and monitored for 28 days. The isolated bacteria were identified based on microbiological methods, including gram staining, biochemical tests, such as urease, oxidase, and catalase (Table2) (Mangalgi and Sajjan, 2014).

Table 1. Epidemiological data and serological tests results of 120 patients with brucellosis symptoms

Study group(n=120)	Values
Gender	
Female	59 (49.16%)
Male	61 (50.83%)
Age	
5-15	10 (8.33%)
15-30	28 (23.33%)
31-45	36 (30%)
46-60	19 (15.83%)
>60	18 (15%)
SAT ≥ 1/160	66 (55%)
C-SAT	73 (60.83%)
2-ME≥ 1/80	43 (35.83%)
Serum nested PCR	45 (37.5%)
Blood nested PCR	55 (45.83%)
Clinical symptoms	
Fever	98 (81.66%)
Fatigue	90 (75%)
Headache	68 (56.66%)
Chills	54 (45%)
Nocturnal diaphoresis	46 (38.33%)
Weight loss	24 (20%)

Table 2. Phenotypic properties of *Brucella spp* isolates recovered from blood samples

	Gram Staining	Colony Morphology	Incubation Period	Catalase	Urease	Oxidase	Motility
Brucella spp.	Gram negative	non-pigmented, non-hemolytic and small grey	Up to six weeks Slow growing	+	+	+	Non-motile

2.3. The Serological Tests

The serological tests, including SAT, Coombs Wright test (C-SAT), and 2ME, were performed on the sera samples for the detection of *Brucella* antibodies 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. Following that, each sample was incubated at 37° C for 20 h 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 the determination of antibody titer. To eliminate false-negative results in sera, the C-SAT test was also performed as described (Hasanjani Roushan et al., 2016). Furthermore, the 2ME test was performed to eliminate the cross-reacting IgM antibodies and detect *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 (Hasanjani Roushan et al., 2016).

2.4. DNA Extraction from Blood Samples

To this end, lymphocytes were separated from blood using lysis buffer (10 mM $NaHCO_3$, 150 mM NH_4Cl, 1mM EDTA, pH 7.4) (Ghatak et al., 2013). Subsequently, the cells were resuspended in TE buffer (Tris 1M and EDTA 0.5M) containing 10% SDS and 10µL proteinase K and incubated overnight at 42ºC. The extraction of DNA from blood and serum samples was performed by the phenol-chloroform method as described (Ghatak et al., 2013). The quality and quantity of extracted DNA were determined via agarose gel electrophoresis and spectroscopy.

2.5. Detection of *Brucella* by Nested PCR

The existence of *Brucella* DNA in serum and blood samples of patients was examined by nested PCR. The specific primers for nested PCR were designed based on a sequence on the *Brucella* genome nominated as 31-KDa cell surface protein conserved among *brucella* spp (Kumar et al., 2007). The first-round PCR reaction was performed using F1/R1 primers for the detection of a 390bp conserved fragment in the chromosome of four prevalent species in humans, including *B.melitensis*, *B.abortus*, *B.suis*, and *B. canis* (Hasanjani Roushan et al., 2014). Thereafter, the 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'-AAAAGCGTTCTGCGCCGGGA-3' in a 25 µl reaction for 35 cycles (94ºC for 1min, 60ºC for 1min, 72ºC for 1min) after an initial denaturation at 94ºC for 4 min. The final extension was 72ºC for 5 min. The nested PCR protocol was performed on PCR products of the primary reaction by F2/R2 primers; Forward: 5'-CTTTGTGGGCGGCTATCC-3' and Reverse: 5'-CGCACTATCGAGCTTGATGAG-3 with the annealing temperature 59ºC in order to detect a 319bp gene fragment in the four prevalent species. The

products were examined by agarose gel electrophoresis. The positive PCR products were confirmed by sequencing.

2.6. Statistical Analysis

Statistical analysis was carried out using an independent t-test and chi-square ($P< 0.05$). The efficiency of the nested PCR was evaluated by the measurement of sensitivity and specificity of nested PCR, as compared to conventional diagnostic methods. The sensitivity and specificity of the tests were evaluated as follows:

% sensitivity= [true positives/ true positives + false negatives] × 100

%Specificity= [true negatives/ (true negatives+false positives)]×100

3. Results

3.1. Results of Microbiological Test

Out of 120 blood samples obtained from patients with brucellosis symptoms, only 10 (8.33%) cases were positive in blood cultures as non-pigmented, non-hemolytic, and small grey colonies (Table2) which were also positive in both PCR and SAT tests.

3.2. Detection of Brucella Antibodies by SAT, 2ME, and C-SAT Tests

Out of 120 enrolled patients, 73 (60.83 %) cases tested positive for brucellosis based on serological tests, and 66(55%) subjects were positive in both SAT and C-SAT. In addition, seven cases with SAT were positive in 1:80 C-STA indicating chronic brucellosis and false-negative results in SAT test. Out of these 66 patients, 32 (48.48 %) cases were positive in all three tests and 7 subjects were positive in both STA and 2ME tests. Moreover, six SAT positive cases were negative in the 2ME test. Out of 66 SAT positive samples, the SAT titer was four-fold higher than the 2ME titer in 19 (28.78 %) cases. These findings are summarized in Table1.

3.3. Diagnosis of Brucellosis Using Nested PCR

In total, out of 120 samples, 60 (50%) cases were positive in nested PCR performed on blood and serum samples (Figure1).

Out of 66 SAT positive samples, 45 (68.18 %) and 37 (56.06%) cases were positive in blood nested PCR and serum nested PCR (Table 3). Moreover, 49 (81.66 %) SAT positive cases were positive in blood and serum nested PCR. Furthermore, there were seven nested-PCR positive cases with SAT titer 1:80 but positive in C-SAT≥1:160.

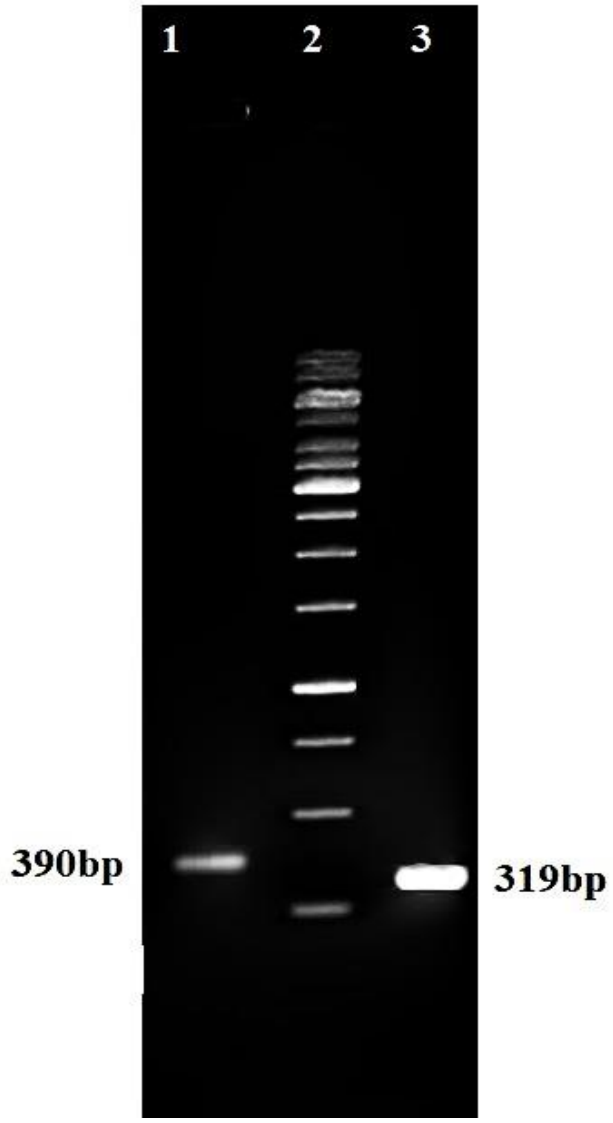

Figure 1. Results of nested Polymerase chain reaction (PCR) for the identification of Brucella clinical isolates, Lane1: the primary PCR reaction with F1R1 primers as a band of 390bp displayed in electrophoresis gel, lane 2: 1kb DNA marker, Lane 3: nested PCR reaction using pair F2R2 primers as a sharp band of 319bp displayed in electrophoresis gel

Table 3. Comparison of results of different diagnostic methods for human brucellosis, Serum agglutination test (SAT), Coombs Wright test (C-SAT), 2- mercaptoethanol (2-ME), nested Polymerase chain reaction, and blood culture

Titer	SAT (%)	C-SAT (%)	2-ME (%)	Serum Nested PCR (%)	Blood Nested PCR (%)	Blood culture (%)
<1:80	46 (38.3)	46 (38.3)	3 (2.5)	0	0	0
1:80	9 (7.5)	4 (3.33)	18 (15)	7 (5.83)	7 (5.83)	0
1:160	31 (25.8)	20 (16.66)	2 (1.66)	17 (14.16)	18 (15)	2 (1.6)
1:320	20 (16.66)	16 (13.33)	3 (2.5)	10 (8.33)	11 (9.16)	2 (1.6)
1:640	9 (7.5)	7 (5.83)	3 (2.5)	1 (0.83)	4 (3.33)	3 (2.5)
1:1,280	9 (7.5)	10 (8.33)	1 (0.83)	3 (2.5)	5 (4.16)	3 (2.5)

3.4. Assessment of Specificity and Sensitivity of Nested PCR

The sensitivity and specificity of the methods were calculated to make a comparison between the efficiency of the nested PCR and the results obtained from blood culture and SAT ≥ 1:160 methods in the detection of *Brucella* in serum/blood samples. In general, 60 (90.90%) nested PCR samples were positive in SAT test. In comparison with the 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 calculated at 100%, compared to blood culture and SAT ≥1:160 methods. It is noteworthy that the sensitivity of blood nested PCR was more than serum nested PCR, SAT, and blood culture (P<0.001).

4. Discussion

The serologic methods are the most commonly used procedures for the detection of human brucellosis; nonetheless, there are several limitations such as low specificity and sensitivity due to serological cross-reactivity or weak immune responses (Park et al., 2012). Moreover, the serological tests are not able to differentiate between chronic and acute patients. Although the blood culture is suitable for isolation and identification of brucellosis, the sensitivity of this method is low, reliant on Brucella species, the

stage of disease, and type of culture medium (Pabuccuoglu et al., 2011; Dadar et al., 2019).

The present study evaluated the sensitivity and specificity of nested PCR, as compared to conventional diagnostic methods. Out of 120 patients, the *Brucella* antibody was detected in 73 (60.83%) cases with clinical symptoms of brucellosis using the methods of STA, C-STA, and 2ME. 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 a study conducted by Roushan et al. who reported 10% positive blood culture (Zerva et al., 2001; Hasanjani Roushan et al., 2016).

In the present study, when the blood culture method was considered the gold standard, almost 84% of positive brucellosis samples were ignored. In line with the results obtained in previous studies, the findings of the current research indicated that the culture method may be negative, while other tests are positive (Pabuccuoglu et al., 2011; Hasanjani Roushan et al., 2016; Tabibnejad et al., 2016). Moreover, in agreement with previous reports, the sensitivity and specificity of nested PCR was 100%, as compared to blood culture (Hekmatimoghaddam et al., 2013; Hanaa et al., 2016). The high sensitivity of nested PCR suggested that PCR is more reliable than blood culture for rapid diagnosis of acute and chronic brucellosis. Furthermore, seven cases with negative SAT and positive C-SAT test diagnosed as chronic brucellosis were positive in nested PCR.

Consistent with several previous studies, the sensitivity and specificity of nested PCR were reported as 79% and 100%, respectively, in comparison with SAT (Khosravi et al., 2005; Hassanain and Ahmed, 2012; Masallat et al., 2013). In the current study, the sensitivity of the blood nested PCR was higher than serum nested PCR. This observation was in agreement with the findings obtained by Keid et al. (2010). As mentioned earlier, the serum-nested PCR is more rapid than blood nested PCR due to an easier DNA extraction process; however, the blood nested PCR has higher sensitivity (Yazew et al., 2009; Alikhani et al., 2012).

In the present study, the nested PCR was able to identify *Brucella* DNA even in SAT negative cases but positive-CSAT indicating the ability of nested PCR to identify chronic brucellosis (Gemechu et al., 2011; Asaad and Alqahtani, 2012). These findings were consistent with previous reports which put an emphasis on the advantages of nested PCR for the diagnosis of brucellosis in both early-stage and chronic diseases (Zamanian et al., 2015; Hasanjani Roushan et al., 2016). Nevertheless, the nested PCR is not applicable when *Brucella* is negative in blood.

5. Conclusion

As evidenced by the results of the present study, the nested PCR showed higher efficiency for rapid diagnosis of human brucellosis, compare to the blood culture method.

Abbreviations

SAT: Serum Agglutination Test, 2-ME: 2-Mercaptoethanol

Authors' Contribution

Study concept and design: S. F.
Acquisition of data: N. S.
Analysis and interpretation of data: L. R.
Drafting of the manuscript: L. R. and N. S.
Critical revision of the manuscript for important intellectual content: B. N.
Statistical analysis: L. R.
Administrative, technical, and material support: S. F.

Ethics

We hereby declare all ethical standards have been respected in preparation of the submitted article.

Conflict of Interest

The authors declare that they have no conflict of interest.

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