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A duplex PCR for the rapid and simultaneous detection of *Brucella* spp. in human blood samples

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ABSTRACT

Objective: To design a duplex PCR for rapid and simultaneous detection of *Brucella* species. in human blood samples. **Methods:** Fifty-two peripheral bloods samples were collected from suspicious patients with brucellosis. Following DNA extraction, PCR assay were performed, using three primers that could simultaneously identify and differentiate three major species of pathogenic *Brucella* in humans and animals. **Results:** Of the 52 peripheral bloods samples tested, 25 sample (48%) showed positive reactions in PCR. Twelve samples were positive for *Brucella abortus* (*B. abortus*) (23%), 13 for *Brucella melitensis* (*B. melitensis*) (25%) and 0 for *Brucella ovis* (*B. ovis*) (0%). **Conclusions:** This work de=monstrates that in case where specific primers were utilized, duplex PCR has proved to be a simple, fast, and relatively inexpensive method for simultaneous detection of important species of *Brucella* in clinical samples.

1. Introduction

Infection with *Brucella* species (Brucellosis) is worldwide in distribution and become recognized as a zoonosis[1]. *Brucella* is small, aerobic, nonfermenting, nonmotile, noncapsules, nonspore, and facultative intracellular, Gram-negative coccobacillus[2]. The genus *Brucella* consists of 10 species, of which *Brucella abortus* (*B. abortus*), *Brucella melitensis* (*B. melitensis*), *Brucella ovis (B. ovis), Brucella canis (B. canis)* and *Brucella suis (B. suis)* are pathogenic for humans. *Brucella microti (B. microti), Brucella inopinata (B. inopinata), Brucella ceti (B. ceti)* and *Brucella pinnipediali (B. pinnipedialis)* are isolated from animals but can occasionally cause disease in man[3,4]. Among these species, the main species in humans are *B. abortus* and *B. melitensis*.They cause brucellosis, also known as undulant fever[1,2]. In most developed countries, this disease well controlled but in South and Central America, Africa, Asia and the Middle East the clinical disease is still common[5,6]. In Iran, two species of *B. melitensis* and *B. abortus* are more common[6]. These species in humans and animals cause forms of the disease which can only be diagnosed through laboratory methods[1,7]. Since the clinical feature of the disease is nonspecific; the most reliable way to diagnose disease is isolation of the bacterium from blood or infected tissues by culture[8]. Factors such as the sample type, sampling time (stage of the disease) and sample preparation equipment for manipulation and isolation technique affected the success rates of culture methods[8,9]. In addition, serological tests that were replaced to culture methods, have little sensitivity, especially in the early stages of the disease that production rate of the antibody is low[7,9-12]. Thus, in different parts of the world, molecular diagnostic techniques with the power to discriminate different species of *Brucella* are currently used

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for diagnosis[13-15].

Duplex PCR assay is a variant of PCR in which two or more loci are simultaneously amplified in the same reaction. Therefore, nowadays, it is used for rapid simultaneous detection of different pathogens, as well as for identification of different genetic disorders and for evaluation the quality and quantity of the samples[16-18]. This study was designed for rapid and simultaneous detection of *Brucella* species in human blood samples by duplex PCR assay.

2. Materials and methods

2.1. Clinical specimens

A total of 52 blood samples were collected from suspicious patients with brucellosis with different ages in Kerman provinces. Three mL blood that mixed with EDTA for extracting DNA was taken from suspected cases. Samples were transferred to laboratory and were kept for 3 weeks at 37 ℃ in incubator.

2.2. Isolation of DNA from clinical blood samples

A modification of the method described by Queipo-Ortuño *et al*[19] was used. Briefly, 0.5 mL of blood with 1 mL of erythrocyte lysis solution [320 mM saccharose, 5 mM $MgCl_2$, 1% Triton ×-100, 10 mM Tris HCl (pH 7.5)], mixed, and centrifuged at 15 000 × *g* for 2 min. The supernatant was discarded, and above steps was repeated for four times until the pellet lost all reddish coloring. Four hundred microliters of nucleic lysis buffer [10 mM Tris-HCl, 1% SDS, 10 mM EDTA, 10 mM sodium acetate (pH 8)] containing proteinase K (10 mg/mL) was mixed and incubated for 30 min at 55 ℃ in shaker incubator. Followed, 100 mL of ammonium acetate (7.5 M) was added and centrifugation at 15 000 × *g* for 10 min. To supernatant, two volumes of absolute ethanol were added, and after centrifuging at 15 000 × *g* for 10 min; the pellets were dissolved in 25 μL of TE buffer (pH 8.0) and stored at 4 ℃ for PCR or at -20 ℃ for long-term storage.

2.3. Primers

After studying the genes and primers which were used so far for detection of *Brucella* species and based on the sensitivity and specificity of the primers and their analysis with BLAST molecular software, suitable primers were selected for simultaneous detection of the species of *B. abortus*, *B. melitensis* and *B. ovis* as follow:

IS711: 5′-TGCCGATCACTTAAGGGCCTTCAT-3′

B1-F: 5′-AAATCGCGTCCTTGCTGGTCTGA-3′

B2-F: 5′-GACGAACGGAATTTTTCCAATCCC-3′

2.4. PCR amplification

Each PCR reaction mixture contained 15 μL Master mix 1 × (Ampliqon Co, Denmark) that contained 1 × PCR buffer, 1.5 mM $MgCl_2$, 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 GenAmp PCR system (Eppendorf, USA)) according to the following program: predenaturation for 5 min at 94 ℃ followed by 35 cycles each containing denaturation at 94 ℃ for 45 s, annealing at 66 ℃ for 45 s and Extension at 72 ℃ for 60 s, followed by final extension at 72 ℃ for 5 min.

Then, The PCR products were analyzed using the electrophoresis technique on 1.5% agarose gel for 1 h at 85 V and 25 mA, stained by SYBERgreen 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 as positive control and suspension containing all of the reagents except template as negative control were used. All PCRs were carried out in duplicate.

2.5. Staistical 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 number (%).

3. Results

Of the 52 suspicious patients tested had a mean age of 31.9 years (SD=9.7) and a mean duration of symptoms before diagnosis of 20 d (SD=5). There was no significant difference between sex and infection by brucellosis. Most of patients were in direct contact with livestock.

In this study, twenty five cases (48%) were positive by PCR method (Figure 1). The isolation rate for *B. melitensis* was 25% (13 cases), *B. abortus* 23% (12 cases) and *B. ovis* 0% (0 cases).

For confirmation of the PCR results, the amplified DNA products were subjected to DNA sequencing as well as restriction endonuclase digestion. The amplified bands pertaining to *B. abortus* and *B. melitensis* were cut with TaqI (92 bp and 402 bp fragments) and RasI (105 bp and 628 bp

fragments), respectively (Figure 2).

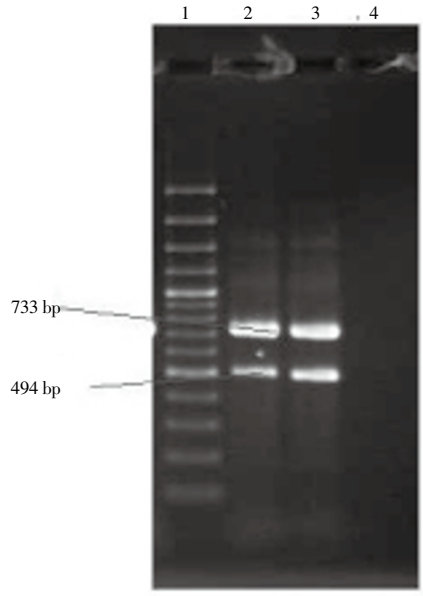

Figure 1. Agarose gel electrophoresis of PCR amplified products generated from DNA samples.
Lane 1 shows DNA size marker (100 bp DNA ladder, SM#333).
Lanes 2 and 3 show 733 bp *B. melitensis* and 494 bp *B. abortus* amplification product. Lane 4 is negative control.

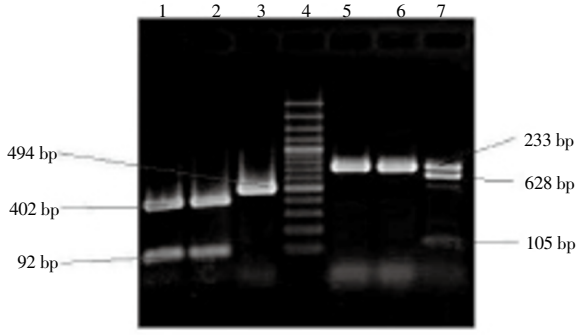

Figure 2. Restriction fragment length polymorphism (RFLP) patterns of PCR products from *B. abortus* and *B. melitensis* after digestion with *Taq*1 and *Rsa* 1 restriction enzyme.
Lanes 1 and 2: PCR-RFLP pattern of *B. abortus* after digestion with *Rsa*1; lane 3: 494 bp-long PCR product from *B. abortus* as the positive control; Lane 4 is DNA size marker (100 bp DNA ladder, SM#333); Lanes 5 and 6: 733 bp-long PCR product from *B. melitensis* as the positive control; Lane 7: PCR-RFLP pattern of *B. melitensis* after digestion with *Taq*1.

4. Discussion

Although brucellosis has been largely eradicated from some parts of the world, but it has not been eradicated from many countries (*eg*. Iran)[1,5,6]. This disease with nonspecific clinical picture is caused by *Brucella* species especially to *B. melitensis* and *B. abortus*[2,3]. Isolation of bacteria from clinical samples (especially human blood samples) with serology or culture methods to isolate microorganisms is very time consuming and costly; on the other hand, rapid diagnosis and differentiation of various bacterial species, especially slow-growing ones, is possible with molecular methods[7,9,13,14]. Therefore, this study was designed for rapid and simultaneous detection of *Brucella* species in human blood samples by duplex PCR assay.

Since duplex PCR is a technique that is able to detect several microorganisms simultaneously in a single amplification reaction within less than 8 h without interference with other microorganisms present in samples[14,18]. This method is done by adding several pairs of primers under standard conditions. Studies have shown that in PCR, especially the duplex PCR, it is important that various concentrations of reaction components (concentrations of $MgCl_2$, dNTP and *Taq* polymerase) be proportionate to obtain the highest efficiency. The concentration should be optimized, since primers may act differently. Also, finding denaturation temperature, denaturation duration, and the annealing temperature are important issues which are costly and time consuming and require expert personnel[13,15]. In this study, unlike studies conducted by Kumar and López-Goñi for simultaneous detection and differentiation of species, particularly *B. abortus*, *B. melitensis* from several pairs of primers (for each microorganism = one primer pair)[16,20], only 3 primers were used for isolation and differentiation of *B. abortus*, *B. melitensis* and *B. ovis* in human blood samples. Using these three primers and through duplex PCR, which is rather similar to conventional PCR, we were able to rapid identify three species of bacteria in a short time (less than 8 h) which seems to reduce the costs and the time of diagnosis.

In this study, like other studies conducted in Iran the isolated rate of *B. melitensis* was higher, but cases of isolated *B. abortus* were considerable[21,22].

The results of the present study, just like those of Queipo-Ortuño *et al* proved that peripheral-blood-based PCR assay is a rapid method, easy to perform, and it is no risks to laboratory personnel and suitable method for *Brucella* species detection from human blood samples[19].

The result of this study is unlike to study of Kazemi *et al* that the isolated rate of *Brucella* 70%, reported[22]. The minute difference in the results of these two studies may be due to the number of subjects, geographical regions under study and the target specificities of the primers used for the amplification reactions.

Despite the fact that more studies are needed in order to elucidate the efficacy and effectiveness of using three primers in duplex PCR assay in clinical samples, the results of this survey clearly indicated that this assay system was simple, fast, and highly specific for detection of *Brucella* species (especially *B. abortus* and *B. melitensis*) in human blood samples. Since simultaneous, rapid and accurate differentiation of *Brucella* species is very important in patients, the duplex PCR developed in this survey could be

a suitable alternative to other *Brucella* species identification methods such as bacterial culture and serology in human blood samples.

Conflict of interest statement

The authors declare no conflict of interest.

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