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Simultaneous detection and differentiates of *Brucella abortus* and *Brucella melitensis* by combinatorial PCR

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ABSTRACT

Objective: To evaluate simultaneous detection and differentiates of *Brucella abortus(B. abortus)* and *Brucella melitensis (B. melitensis)* through the combinatorial PCR method. **Methods:** This study was designed using three primers that could simultaneously identify and differentiate two major species of pathogenic *Brucella* in humans and animals. Identification and differentiation of each species using the size of the PCR product were determined. To determine the specificity of the method, bacteria close to the genus *Brucella* were used. Finally, to confirm PCR products, In addition to the products sequence, RFLP was performed on PCR products using restriction enzymes. **Results:** The method of optimized combinatorial PCR in this study could simultaneously detect and differentiate *B. abortus* and *B. melitensis* with high specificity and sensitivity in clinical samples. Differentiation of species is based on the resulting bands; therefore, the band 494 bp for *B. abortus* and 733 bp for *B. melitensis* were obtained. RFLP and sequencing results confirmed PCR results. **Conclusions:** The results of this study shows that without routine diagnostic methods such as culture and serology tests, using the molecular method of combinatorial PCR, important species of *Brucella* can be simultaneously identified and differentiated in clinical samples.

1. Introduction

Brucella is small, nonfermenting, aerobic, nonmotile, nonspore, noncapsules, facultative intracellular, Gram-negative coccobacillus. They function as parasites in animals and humans[1,2]. The genus *Brucella* consists of 8 species, of which *Brucella abortus (B. abortus)*, *Brucella melitensis* (*B. melitensis*), *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 pinnipedialis* (*B. pinnipedialis*) are isolated from animals but can occasionally cause disease in man[2,3]. Among these species, the main species in humans are *B. abortus* and *B. melitensis*. They cause brucellosis, also known as Malta fever[4]. This disease remains a major zoonosis and is found worldwide[5]. In most developed countries, it is well controlled but in Asia, Africa, the Middle East, South and Central America, the clinical disease is still common. 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[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, because cultures are not always positive, time consuming and resource-intensive and can delay treatment, serological tests (the most important is Standard Serum Agglutination test (SAT)) replaced culture methods[10]. Unfortunately, these

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methods have little sensitivity, especially in the early stages of the disease that production rate of the antibody is low[7-11]. Also, with these two methods, species cannot be differentiated from each other, thus, in different parts of the world, molecular diagnostic techniques with the power to discriminate different species of *Brucella* are currently used for diagnosis. Various studies have shown that these tests, when compared to culture and serological tests have higher sensitivity and specificity[13,14].

Combinatorial PCR 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[15,16]. This study was designed for simultaneous detection and differentiation of *B. abortus* and *B. melitensis* through the combinatorial PCR method with optimized factors such as annealing temperature and time and magnesium ions.

2. Materials and methods

2.1. Bacteria species

Bacterial strains *B. melitensis* Rev1, *B. abortus* B19, and the standard strains of *Salmonella enteritidis*, *S. infantum*, *S. typhimurium*, *Klebsiella pneumoniae*, *Staphylococcus aureus*, *Yersinia enterocolitica*, *Vibrio cholerae*, *Shigella boydii* and *Escherichia coli* used in this study were obtained from Persian Type Culture Collection of Iranian Research Organization for Science and Technology.

2.2. Test method

After culturing these bacteria in their specific media, all bacteria were killed with 67% methanol and 37% salt. Then, the DNA rate was calculated based on the color sensitivity of Ethidium Bromide. Since the ability of the electrophoresis method in staining with Ethidium bromide to determine DNA is 1 ng in the best conditions, samples purified from the culture medium were diluted serially from 1/2 to 1/80 and then the weakest band was selected.

2.3. Primers

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

Ba-F: 5′-GACGAACGGAATTTTTCCAATCCC-3′,

Bm-F: 5′-AAATCGCGTCCTTGCTGGTCTGA-3′

IS711: 5′-TGCCGATCACTTAAGGGCCTTCAT-3′

2.4. DNA extraction

Extraction of DNA was performed using the salting out method. Briefly, in the beginning, a few colonies of bacteria were added to 1 mL of standard saline and a 0.5 McFarlnd turbidity standard was prepared (In this dilution, there are approximately 0.5×10^8 bacteria). Then, the aforementioned solutions were centrifuged (5 000 rpm for 5 min) and 500 μL of TSB buffer was added to the pellet and mixed gently through inverting. Then, 50 μL of 20% SDS was added to this solution and mixed gently through inverting and the mixture was incubated at 37 ℃ for 24 h. Cell wall debris, denatured proteins, and polysaccharides were removed by precipitation with adding 1/3 volume of 5 mmol/L NaCl and spinning at 12 000 rpm in a microcentrifuge for 10 min at 4 ℃. After that, at least 750 μL of cold 100% ethanol was added to the above-mentioned solutions and followed by storing at -20 ℃ for at least 10 min. Then, the samples were centrifuge for 10 min at the highest speed in a microcentrifuge at 4 ℃. The supernatants were removed as much as possible and 375 μL of cold 70% ethanol was added to pellet and centrifuged for 5 min at 15 000 rpm in a microcentrifuge at 4 ℃. Eventually, the pellet was dissolved in 25 μL of TE buffer (pH 8.0) and stored at 4 ℃ for PCR or at -20 ℃ for long-term storage.

2.5. Optimization of the conditions of PCR amplification

For several times, the PCR process was performed with the genome of standard strains and in each phase, concentrations of primers and DNA template, the annealing temperature and time was used and finally, the appropriate amounts of ingredients were selected to perform the final PCR. Several annealing temperatures between 56-75 ℃ were used. Each PCR reaction mixture contained 15 μL Master mix 1× (Ampliqon Co, Denmark) that contained 1×PCR buffer, 1.5 mmol/L $MgCl_2$, 1 μL template DNA (0.5 μg), 0.15 mmol/L dNTP, 1.25 U Taq DNA polymerase, 20 pmol of each forward and reverse primers and sterile distilled water up to 50 μL. Then, the PCR products were analyzed using the electrophoresis technique on 1.5% agarose gel for 1 h at 85 Volt and 25 mA, stained by SYBERgreen and visualized under UV transilluminator. Finally, amplification products were further evaluated by sequencing and restriction digestion procedures. In the restriction digestion procedure, amplified DNA was digested using two restriction endonucleases depending on the length of the amplicon: TaqI (Fermentas, Leon-Rot, Germany) for products of *B. abortus* and Cac8 I for products *B. Melitensis* (Fermentas, Leon-Rot, Germany).

2.6. Studies of specificity

In order to evaluate the specificity of primers and the

PCR assay, DNA of well-characterized non-*brucella* strains whose phylogenetics and serology were associated with *Brucella* spp. and were employed in the test.

2.7. Sensitivity of PCR based on genomic DNA concentration

In order to evaluate the sensitivity of PCR based on genomic DNA concentration, the concentration of genomic DNA extracted from each of the bacteria was measured as mentioned. Then, for each extracted DNA from bacteria, different dilutions (10^{-1}, 10^{-2}, 10^{-3} and 10^{-4}) were prepared and eventually, using the above-mentioned dilution (final protocol and the optimized thermal profile), the PCR reactions was performed.

3. Results

The results of different temperatures and concentrations in the process of combinatorial PCR Tremocycler of Eppendorf (made in USA) showed that the best product in the following conditions were obtained: pre-denaturation for 5 min at 95 ℃ followed by 30 cycles, each containing denaturation at 94 ℃ for 30 s, annealing at 66 ℃ for 30 s and extension at 72 ℃ for 30 s, followed by final extension at 72 ℃ for 5 min. It should be mentioned that when uniplex-PCR was used, the ability of the primers were such that in the temperature range 56-75 ℃ as the annealing temperature, a single sharp band was obtained. According to optimized reaction conditions of materials and the appropriate thermal cycle, PCR with specific primers was performed and finally, the band 494 bp for *B. abortus* and 733 bp for *B. melitensis* were obtained (Figure 1).

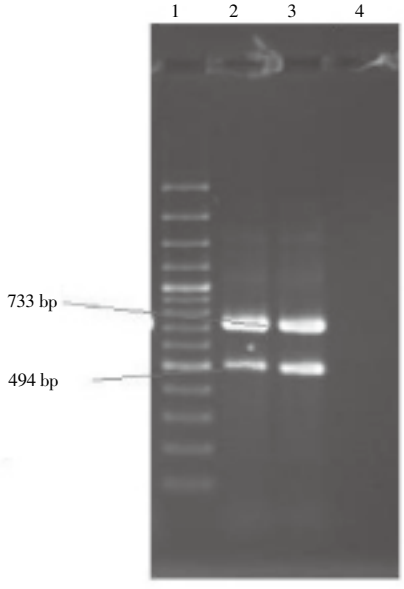

Figure 1. Agarose gel electrophoresis of PCR amplified products generated from DNA samples. Lane 1 shows DNA size marker (100 bp DNA ladder, SM#333). Lanes 2 and 3 show 733 bp *B. melitensis* and 494 bp *B. abortus* amplification product. Lane 4 is negative control.

3.1. Determine the specificity of PCR

Results of determining the specificity of the primers used with the genome of the bacterial species that were listed previously, showed that the primers were quite specific and reacted only with *Brucella* species (Figure 2).

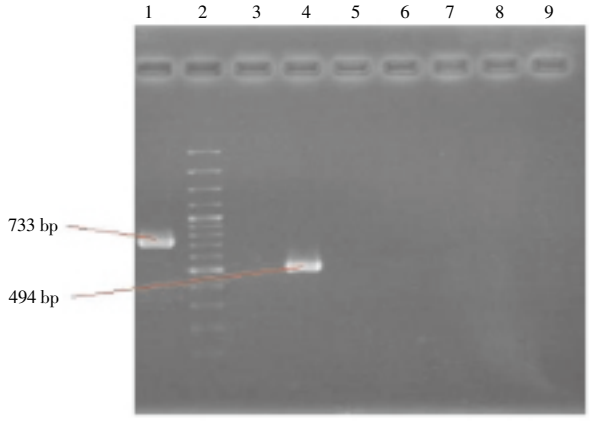

Figure 2. Specificity test of primers.
Lanes 1 and 4: positive control; Lane 2: DNA ladder; Lane 3: *Agrobacterium tumefaciens*; Lane 5: *E. coli* O157; Lane 6: *Vibrio cholerae*; Lane 7: *Salmonella enteritidis*; Lane 8: *Yersinia enterocolitica*; Lane 9: *S. aureus*.

3.2. Determining the sensitivity of PCR

Since the concentration of the genomic DNA in the first sample for bacteria *B. abortus* and *B. melitensis* was 955 ng/μL, several dilutions were prepared to calculate the sensitivity of the reaction, and then these dilutions were used to perform PCR. The results showed that up to the dilution of 10^{-4} of the initial genome product was obtained, but the best product in the dilutions 10^{-2} that the concentration of the genomic DNA in this dilution was 0. 955 ng/μL (Figure 3).

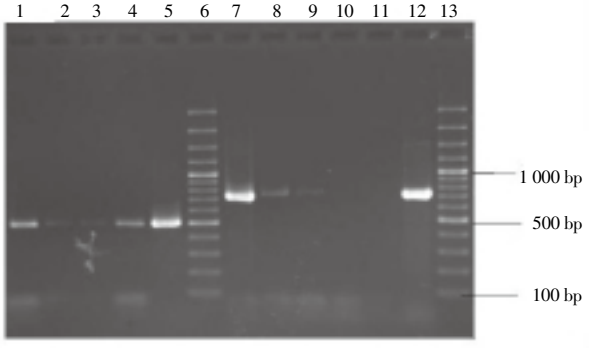

Figure 3. Sensitivity test of primers.
Lane 1: positive control of *B. abortus* genome; Lane 2: 10^{-4} purified *B. abortus* genome; Lane 3: 10^{-3} purified *B. abortus* genome; Lane 4: 10^{-2}purified *B. abortus* genome; Lane 5: 10^{-1} purified *B. abortus* genome; Lane 6: DNA ladder; Lane 7: 10^{-1} purified *B. melitensis* genome; Lane 8: 10^{-2} purified *B. melitensis* genome; Lane 9: 10^{-3} purified *B. melitensis* genome; Lane 10: 10^{-4}purified *B. melitensis* genome; Lane 11: negative control; Lane 12: positive control of *B. melitensis* genome; Lane 13: DNA ladder.

Accordingly, the mean sensitivity of the designed PCR can be determined based on the number of bacterial copies, which can be detected with this technique, and the formula presented in site of genomics and sequencing center by Andrew Staroscik in 2004.

Number of copies = (ng × number/mol) / (bp × ng/g × g/mol of bp)

In this study, initial DNA concentration of *B. abortus* and *B. melitensis* in the dilutions 10^{-2} was 1.89×10^{8} ng/μL and length of bacteria genome is 2.1 kbp, therefore copy number detectable is determine 955.

3.3. Confirmation of the PCR products

By examining and aligning the sequences with the obtained sequencing results of each bacterial agent and the results of PCR-RFLP (Figure 4), it was determined that obtained sequences were exactly related to the desired bacteria.

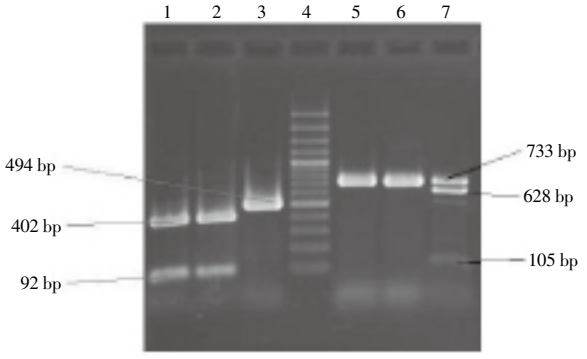

Figure 4. Restriction fragment length polymorphism (RFLP) patterns of PCR products from *B. abortus* and *B. melitensis* after digestion with Taq1 and Rsa1 restriction enzyme.

Lanes 1 and 2: PCR-RFLP pattern of *B. abortus* after digestion with Rsa1; 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 Taq1.

4. Discussion

The isolation of *Brucella* is important since in humans they can cause infections with different clinical features and following late diagnosis, serious complications may ensue. Isolation of bacteria with conventional PCR 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. Therefore, this study was designed for simultaneous detection and differentiates of *B. abortus* and *B. melitensis* through combinatorial PCR[8,10,14,15].

Since combinatorial 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[10,15]. This method is done by adding several pairs of primers under standard conditions. Studies have shown that in PCR, especially the combinatorial 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[15,16]. In this study, unlike studies conducted by Sifuentes-Rincon, Bricker and especially Imaoka for simultaneous detection and differentiation of *Brucella* spp., particularly *B. abortus* and *B. melitensis* from several pairs of primers (for each microorganism = one primer pair)[15,17,18], only 3 primers were used for isolation and differentiation of *B. abortus* and *B. melitensis* in standard samples. Using these three primers and through combinatorial PCR, which is rather similar to conventional PCR, we were able to identify and differentiate two species of bacteria in a short time (less than 8 h) which seems to reduce the costs and the time of diagnosis. It should be mentioned that these three primers can identify and distinguish *B. ovis* from other *Brucella* as well.

The assay described in this report like studies conducted by Bricker, Kumar and García-Yoldi for identification and differentiation of *Brucella* had several advantages. The most important advantage was identification and differentiation of different species of *Brucella* in a short time (*i.e.*, less than a single working day). Identification and differentiate of different species of *Brucella* by conventional methods requires at least several days which can delay treatment in patients. Another major advantage was requiring minimal samples[16,18,19]. This study showed that combinatorial PCR method has could identify 0.9 ng/μL genome in samples. Also, live *Brucella* organisms that are a potential risk for laboratory personnel are not necessary for performing combinatorial PCR; therefore, it can be considered as a safe and reliable technique in clinical laboratories. Finally, this method, due to using specific primers, is a method with high specificity for detection and differentiation of *Brucella* in samples and unlike other methods of routine, is less affected by microbial contaminations that might be present in samples[15].

Also, like other studies such as those conducted by Huber *et al* and Kazemi *et al*, this study showed that PCR was a sensitive and suitable method for detecting *Brucella* species[20-22]. This study showed that the employed PCR method could differentiate different species of *Brucella*. Although the study conducted by Sifuentes-Rincon showed that PCR could detect *Brucella* in clinical samples[17],

RFLP had to be performed on PCR products to differentiate different species.

Despite the fact that more studies are needed in order to elucidate the efficacy and effectiveness of using three primers in combinatorial PCR assay, 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)*. Since simultaneous, rapid and accurate differentiation of *Brucella* species is very important in patients, the PCR developed in this survey could be a suitable alternative to other *Brucella* species identification methods such as conventional PCR, bacterial culture or serology.

Conflict of interest statement

The authors declare no conflict of interest.

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