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Evaluation of Different Primers for Detection of *Brucella* by Using PCR Method

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Abstract

Introduction: Brucellosis is a worldwide zoonosis and a significant cause of loss of health in humans and animals. Traditionally, classic diagnosis is carried out by isolation of *Brucella*, which is time-consuming, technically challenging and potentially dangerous. The aim of this study was to expand a molecular test that would be used for the develop detection of *Brucella* in a single reaction with high sensitivity and specificity, by targeting IS711element.

Methods: This study was carried out from 2015 to 2016 at the Ayatolla Rohani hospital in Babol, Iran. The present study was designed to develop PCR assay, based on IS711 gene for rapid diagnosis of *Brucella spp*. and immediate detection of *Brucella*, with high sensitivity and specificity. Four pairs of oligo-nucleotide primers with sizes of 547, 403, 291 and 127bp respectively, were planned to exclusively amplify the targeted genes of Brucella species.

Results: Our results show that, five PCR primers set up, would be helpful in amplifying the DNAs from the genus *Brucella* with high specificity and sensitivity so it can be 12 fg, for *Brucella* species to provide a valuable tool for diagnosis.

Conclusion: This method can be more useful than serological and biochemical tests and in addition, this reduces the number of required tests more rapidly and economically.

Keywords: Brucellosis, Diagnosis, Polymerase chain reaction, IS711gene

1. Introduction

Brucella species are facultative intracellular gram-negative bacteria, which is very common worldwide, causing zoonosis infection disease particularly in domestic animals, and is transmittable to humans (1, 2) The genus *Brucella* consist of 10 species which are isolated from animals, but among these species, *B. abortus* and *B. melitensis* are generally causative agents for humans (3, 4). Since the clinical feature of brucellosis has overlap with an extensive range of infectious and non-infectious diseases, the most reliable way to diagnose this infection, is through laboratory methods, although it is not always successful (5). Nowadays, microbiological culture and serological tests are the most common methods for detection of *Brucella spp.* Although isolation of the bacteria is the "gold standard", microbial culture is often negative and dependent on the culture medium, quantity of circulating bacteria and *Brucella species* (6). Therefore serological tests such as Standard Serum Agglutination test (SAT) seem to be more effective for diagnosis, although, sometimes there can be cross reaction or false positive reaction in samples from areas with subclinical prevalence of brucellosis (7-14). To ensure efficient brucellosis disease prevention and control, a rapid and accurate identification method is required. In recent years, various studies have

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shown that polymerase chain reaction (PCR) can be used routinely to detect DNA from peripheral blood and other tissues as they are rapid, high specific and sensitive. Moreover, various PCR techniques, target genes and primer pairs have been used to detect *Brucella spp.* (15, 16). The most common molecular targets in clinical applications for the detection of *Brucella* spp, are the *IS711* insertion element and the *bcsp31* gene (17, 18). Because of the prevalence of brucellosis in Iran, a specific diagnostic method should be conventional for the control of *Brucella* in this population. The purpose of this study was to expand a molecular test that would be used for the develop detection of *Brucella* in a single reaction, with high sensitivity and specificity by targeting *IS711*element.

2. Material and Methods

2.1. Research design and setting

The present study was conducted from 2015 to 2016 at the Ayatolla Rohani hospital in Babol, Iran. We used *B. melitensis* serotype 1 (strain 16M) and *B. abortus* B19 as the standard strain (provided by the Department of Bacterial Vaccines and Antigens Production, Pasteur Institute of Iran) and the standard strains of *Escherichia coli* ATCC 25922 as the negative control (provided by the Persian Type Culture Collection of Iranian Research), which were phylogenic related with *Brucella spp.* DNA from these strains were isolated using a purification kit (Roche Applied Sciences, Mannheim, Germany) in accordance with the manufacturer's instructions. The DNA pellet was suspended in 10 mMol TE buffer and stored at -20 °C until required for analysis. DNA concentration and purity were assessed by reading NanoDrop 2000c Spectrophotometer A_{260} and A_{280}. To study the influence of DNA template from clinical blood specimens, about 3ml peripheral blood sample was collected and taken for PCR analysis. All samples were aliquot and stored at -20^{oC} until tested. DNA was extracted from whole blood (200 µl) with the QIAamp DNA Blood Mini Kit (Qiagen) in accordance with the manufacturer's instructions.

2.2. Oligonucleotide primers

In this study, we designed four novel primers by extensive literature and nucleotide sequence searches in the NCBI databases. The DNA was then subjected to PCR detection by primers specific to the *IS711* genes and this gene is unique within *Brucella spp*. Primer pairs were studied, using the bioinformatics software AlleleID 6. The DNA sequence comparisons with GenBank database were searched and assessed for species or genus assignment using BLAST. A pair of primers EFQ1 was previously reported to amplicon of 251 base pair (bp) that spans a region of the *B. melitensis* genome (GenBank accession number AE009555) and the *IS711*insertion element (19).We have proved that this pair of primers is specific for the genus *Brucella spp*, because when we used these primers for non-*Brucella* species templates, they did not amplify any PCR product. Primer sequences used in this study are shown in Table 1.

Table 1. List of oligonucleotide primers used in this study

Primer name	Forward (5'→3')	Reverse(5'→3')	Size of amplicon (bp)
Nes2	CAAGCCGCTCATATTCAC	CCAAGGTCAATCCAACAC	547
Nes1	CGCTCGCTGCCATACTTGC	CGCTCGCTGCCATACTTGC	291
Det	AGAATAATCCACAGAAGGTAGAG	ATCCAAGGTCAATCCAACAC	403
Mar	GCATTCAATCTGATGGCGTTCC	GATCACTTAAGGGCCTTCATTGC	127
EFQ1	TGTTTCGGCTCAGAATAATCCA	GCATGCGCTATGATCTGGTTAC	251

2.3. Optimization of DNA amplification

Suitable adjustments were set up in the concentration of critical reagents such as primer, $MgCl_2$ and template DNA, also annealing temperature of thermocycling to obtain optimal amplification of targeted genes of standard strains, for several times. The PCR was performed in 25 µl volumes that contained 2.5 µl of 10X buffer, 0.5 mmol/L $MgCl_2$, 0.3 mmol/L dNTPs (Fermentas, GmbH, Germany), 0.5 pmol/L from each primer, 0.2 unit of Taq DNA polymerase enzyme, and 1 µl extracted DNA (For blood sample we used 5 µl of extracted DNA). PCRs were run using the following steps: a primary denaturation for four minutes at 94ºC followed by 30 cycles of denaturation at 94 ºC for 1minute, annealing gradient temperature ranging from 50 ºC to 70 ºC for 1minute and extension at 72 ºC for two minutes. At the end, one cycle for completion of the final extension was at 72 ºC for 12 minutes. Then, 10µL of the PCR was subjected to electrophoresis on 1.5% agarose gel (Cinagene Co, Iran) stained by 0.5µg of ethidium bromide/ml (Sigma, Germany) and the results were evaluated in the presence of 100 bp DNA size marker (Fermentas Co, Ukraine), visualized under UV transilluminator. Finally, amplification products were sequenced by Macrogen Inc, Seoul, Korea.

2.4. Sensitivity of PCR based on genomic DNA concentration

Comparison of sensitivity between five pairs of primers assay (Nes2, Nes1, Det, Mar and EFQ1) was evaluated by using serial dilutions of DNA template (10^{-1}.10^{-4}) of *B. melitensis* and (final protocol and the optimized thermal profile), the PCR reaction was performed. Then sensitivity for each pair of primers was determined by detection of limitations for the number of bacteria in PCR.

3. Results

Our set of five PCR primers could be useful to amplify the DNAs from the genus *Brucella*. Although the best range of concentration of all primers was 10-15 pmol after standardizing on individual PCRs with target genes, but for solution, 10 pmol primers were used in all experiments. Also, the concentration of magnesium chloride was varied from 0.5 to 3 mM. The optimum chloride magnesium concentration for the assay was determined to be 2 mM. As a result, at the low concentrations there were no detectable PCR fragments, while at high chloride magnesium concentration there were amplified nonspecific PCR products so, gradient annealing temperature at 60 °C was found to be optimum. It should be mentioned that when PCR was used, the ability of the primers were such that in the temperature range 50-70ºC as the annealing temperature, a single sharp band was obtained (Figure 1). The result showed that, the developed PCR assay had complete specificity, for not only was any amplified product observed with DNA of closely related organisms, but also could be selectively amplifying the targeted genes when performed on the clinical isolates (Figure 2). The comparison of the sensitivity of primers showed that the most sensitive primers were MAR amplified (12 fg). EFQ1, Nes2 (6 fg) and Nes1, Det (4 fg), respectively, of purified genomic DNA from *B. melitensis* (Figure 3).

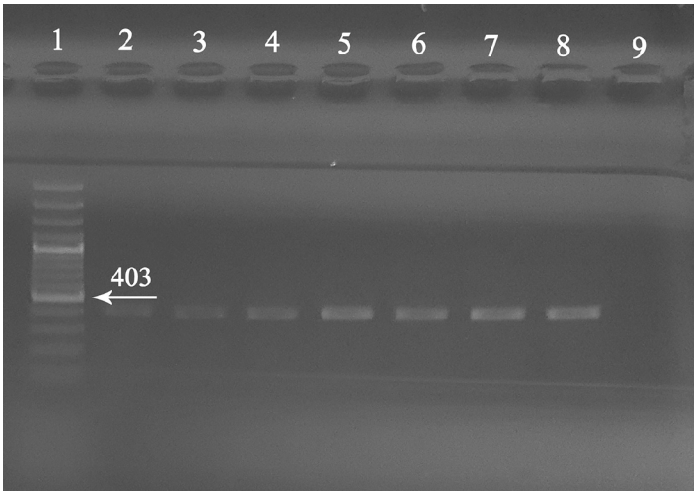

Figure 1. Agarose gel electrophoresis of PCR amplified products. Lane 1: DNA size marker (100 bp DNA ladder), Lanes 2-8: B. melitensis amplification product, Lane 9: negative control.

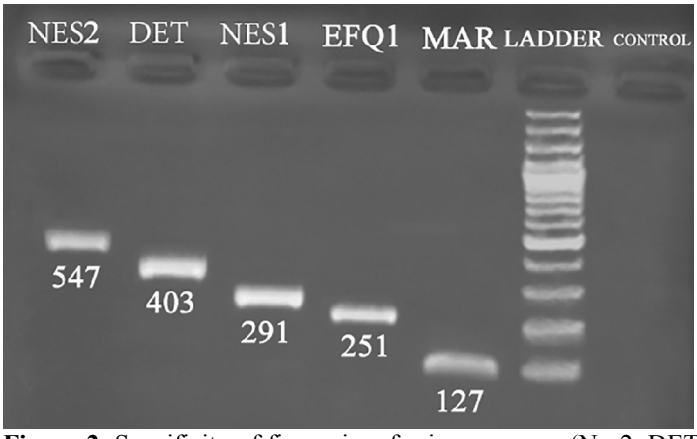

Figure 2. Specificity of five pairs of primers assays (Nes2, DET, Nes1, EFQ1 and Mar). Lanes1-5: positive control of DNA template of B. melitensis, lane 6: DNA size marker (100 bp DNA ladder), lane 7: negative control.

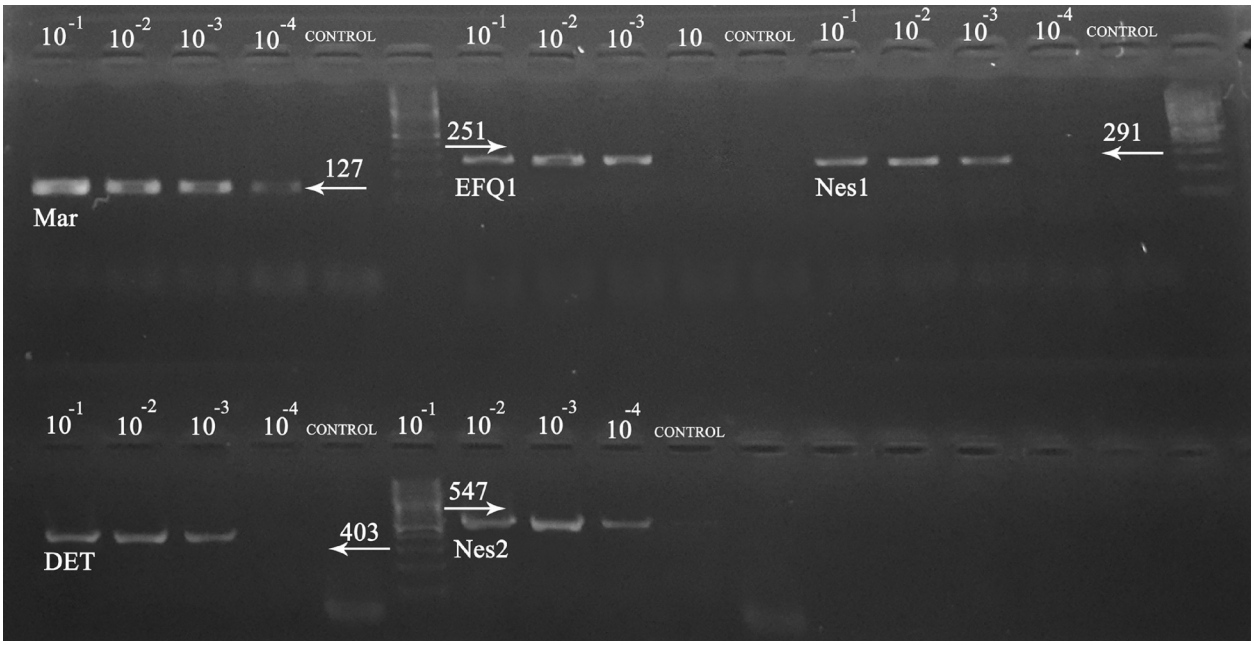

Figure 3. Sensitivity of five pairs of primers assay (Mar, EFQ1, Nes1, DET, and Nes2) with serial dilutions of DNA template of B. melitensis (10-1-10-4) respectively, control negative and DNA size marker (100 bp DNA ladder).

4. Discussion

Detecting *Brucella* species from infected animals and humans has been a public health concern for creating control and preventative policy in the region. Several PCR based methods are presented for *Brucella* diagnosis all over the world (15, 20). Based on our results, the single-stage PCR, using five pairs of primers could detect standard and native strains of *Brucella Spp*. It is important that a variety of 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 subjects which are valuable and time consuming and require expert personnel. In contrast with other studies (20, 21), our study showed difference in sensitivity because of protocol conditions such as concentrations of primers, dNTPs, $MgCl_2$, and Taq polymers. In the study by Da Costa *et al*, the specificity of primers on the 98 non-*Brucella* bacteria, mentioned that all organisms were negative for amplification of the *BCSP31* gene (22). In this study the specificity of each primer pairs that were tested, the exact specific amplification was shown as the correct predicted product size. Moreover, the specificity of the primers has been tested against closely related bacterial species, and indicates that this assay is highly specific for *Brucella*. Some researches stated that during the DNA extraction from different tissues such as blood, some unknown inhibiting factors may be confounded on PCR product and could especially be affected on the sensitivity of it. (23, 24). Interestingly, these primers which were examined in this paper did not reduce the capability of the PCR to detect specific target genes of the *Brucella.spp*. Therefore it is suggested that these primer pairs can also be useful for the direct detection of Brucella species in tissue samples. 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 (25, 26). More studies will be needed to improve the specificity and sensitivity of molecular methods before recommending routine laboratory detection.

5. Conclusions

In the recent study the PCR improved to detect *Brucella* simultaneously in a single reaction. The assay shows that the PCR method has high specificity and sensitivity. This method can be more useful than microbiological and serological tests and in addition, this aspect reduces the number of required tests. Therefore, we are recommending that the PCR can be used to test tissue and blood specimens as well as bacterial culture.

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Conflict of Interest:
There is no conflict of interest to be declared.

Authors' contributions:
All authors contributed to this project and article equally. All authors read and approved the final manuscript.

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