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ORIGINAL RESEARCH

Comparison of three primer pairs included: novel primers IS711, universal primers B4 - B5 and 16SrRNA in the diagnosis of human brucellosis in suspected patients in Iran

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Abstract

The genus *Brucella* is a worldwide distributed intracellular bacteria, which infects animals and human. Currently, this zoonosis has been diagnosed by microbiological and serological laboratory tests. Different PCR protocols with various primer pairs and different target genes have been published for the detection of *Brucella*, but only a few of these primers have been used in human samples. This study aimed to evaluate and compare the sensitivity and specificity of three primer pairs in the PCR technique, each of which separately amplifies three different regions in the *Brucella* genome, to determine which are more comfortable for the detecting of *Brucella* DNA in human clinical samples.

49 clinical serum samples were isolated from suspected patients in different cities in Iran from October 2017 to July 2018. The suspected patients with brucellosis-compatible symptoms were checked. These primers amplified 3 distinctive fragments in BCSP 31 gene (B4/B5), Designed IS711 primers, and a sequence of 16SrRNA of *Brucella melitensis.*

The results showed that the B4/B5 primer pair had the highest sensitivity and specificity for the detection of both positive and negative samples (100%). The designed IS711 primer pair detected 94% of samples, whereas the 16SrRNA primer pair was the least sensitivity, being able to detect only 30.64% of samples.

The specificity of 3 techniques was 100%. The B4/B5 primers were able to detect the smallest number of bacteria 0.05 CFU/reaction whereas IS711 was able to detect 2 CFU/reaction and 16SrRNA was able to detect 2×10^5 CFU/reaction.

Keywords: Brucella melitensis, Human brucellosis, PCR, Blood samples, Serum samples

Introduction

Brucellosis is the second zoonotic disease after rabies (1). Brucellosis has four phases that included: acute, subacute, and chronic and localize. Each phase has its own manifestation such as: joint pain, symptoms of poisoning, chronic spine, fever, sweating, testis or ovarian inflammation, neurological complications and arthritis in acute phase (2).

Brucella species reproduce inside cells and escape from innate and acquired immune system. Also, the member of the genus *Brucella* can replicate within macrophages and cause infection and disease in animals and humans (3). Twelve nomen species: *B.abortus, B.melitensis*, *B. suis, B.canis, B.ovis, B.neotomae*, The first six bacteria are classical species *(*4). *B. pinnipediae* (5), *B. ceti* (6), were separated from aquatic mammals and have a marine origin. *B. inopinata* separated from people (6), *B. microti* isolated from common voles (7). As of late, the separation of *B.papionis* from baboons was depicted, and the last one, which isolated from mandibular lymph nodes of red foxes (Vulpes vulpes) was *B. vulpis.* The genus of *Brucella* has been a member of the alpha - 2 subdivision of the class proteobacteria, which is closely related to *Rochalmia. Rhizobium* and *Agrobacterium* spp. (8, 9). Usually, the detection of this zoonosis is based on microbiological and serological technique tests. Although the gold standard test is the isolation of causative agents and cultured in microbial media such as blood culture, this technique has been needed long incubation periods, however, the sensitivity of this technique is low, 15% to 70% (10,11). The presence of antibodies in the serum does not mean an active case of brucellosis, patients from areas where brucellosis is endemic often show a low serological response, and animal vaccination may have been false-positive results and also cross- reaction may occur between other gram-negative bacteria and smooth *Brucella* spp. (12,13).

For diagnosis of many infectious diseases caused by slower growth as fastidious bacteria amplification of DNA by PCR is used, especially for detecting *Brucella* DNA (14, 15, 16). However, the molecular assay such as DNA – based technique has proved to be fast (<4h) (17).

The present survey compares 3 PCR techniques for detecting *Brucella* DNA from human serum samples of suspected patients with brucellosis clinical symptoms and arbitrates the technique most suitable for use in a diagnostic microbiology laboratory in terms of sensitivity, robustness, and easy to apply. Three primers include B4/B5 primers, the designed primers of IS711 sequences, and 16SrRNA primers.

Materials and Methods

Between October 2017 to July 2018, a transect study was conducted, based on serum and blood sampling of suspected patients from the different provinces in Iran together with an epidemiological survey. The patient samples were gathered for 10 months. Two patients from Babol, 2 from Shiraz, 6 patients from Mashhad, 4 from Borujerd, 4 from Urmia, 3 from Makoo, 24 from Khoy, and 4 from Tabriz. The study was approved by the Faculty of Medicine, Tehran Islamic Azad University of Medical Sciences, Research Ethics Committee, with approval ID: IR. IUA. TMU. REC: 1397. 245.

After informed consent, serum and blood specimens were gathered from 49 suspected cases of patients with brucellosis symptoms who were referred to hospital and diagnostic laboratories in different cities from northern (4.08%), southern (4.08%), western (79.59%) and eastern (12.24%) provinces in Iran, during 10 months. On the same day, that blood samples were collected, serum samples were also prepared. Before blood collection, we prepared the questionnaire form for each of the patients which contain: individual information of patients such as age, genus, job, city, primary clinical symptoms, and end all was an ethical testimonial of each patient.

The isolated serum sample of patients was kept in tubes containing sodium citrate. The DNA was extracted from the serum specimens of 200µL volume, according to the guideline commercial kit (GTP. Tehran, Iran).

The three primer pairs that were chosen amplified regions of three different *Brucella* genes, which used after bioinformatics analysis:

First, B4 (5′-TGG CTC GGT TGC CAA TAT CAA-3′) and B5 (5′-CGC GCT TGC CTT TCA GGT CTG-3′), with a target gene encoding a 31-kDa *B. abortus* antigen which is

a conserved sequence in all species of *Brucella* [21], which amplified 223 bp fragment. The reaction consisted of 12.5µL 2X PCR master mix (Ampliqon, Denmark), 5 µL DNA template, 0.5 µL of each primer, and nuclease-free water up to 25 µL. The thermo-cycler (Touchgene Gradient PCR Machine) was programmed as follows: Initial denaturation at 95°C for 5 min, 35 cycles of template denaturation at 94°C for 1 min, 30 S for primer annealing at 60°C and 60 S for primer extension at 72°C with final extension cycle at 72°C for 7 min.

Second, using primer pair derived from the 16SrRNA sequence of *Brucella melitensis* Rev. 1. F (5′- GAT GTG GTA ACG CAC ACC AA-3′) and R (5′- CGC AGA CAG TGA CCA TCA AA-3′) amplified a 218 bp fragment. The 16SrRNA PCR assay was carried out in a total volume of 25 µL containing the same mixture, which was used for PCR. The gene amplification using the 16SrRNA primer was programmed as same as first one, but 60 S for primer annealing at 59°C.

Third, IS711 specific primer, which was designed, based on the sequence of *B.melitensis* deposited in the GenBank. The IS711 primer pairs were designed by using the Codoncode Aligner software (V.7.1.2). The designed primers, F (5′-CGC TCG CTG CCA TAC TTG CA-3′) and R (5′-CTG AAC AAG CCG GGC CTG AT-3′) amplified a 448 bp fragment, which was a reiterative genetic compenent of IS711 and was special to *Brucella* spp. At least, one copy of this repetitive genetic element may appear as a common locus in all species of *Brucella* (7). The IS711 PCR assay was carried out in a total volume of 25 µL containing the same mixture, which was used for PCR. The PCR machine program of IS711 primer pairs was as same as the others except the primer annealing at 63°C was the 60S.

In each PCR test, a positive control extricated DNA from *B.melitensis* Rev. 1, and *B. abortus* S19, and negative control extricated DNA from *E. coli* (ATCC 35218) were utilized to control the running procedure and the nonappearance of cross-contamination. All the standard items were checked for the prevention of any probable contamination. The tests were carried out twice. After the amplification process, the samples were run on 1.2% agarose gel (Sigma). The gel was stained by 1 µg/ml ethidium bromide and after distaining, the DNA bands were visualized within Gel documentation UV chamber. For some of the amplified products of BCSP31, IS711, and 16srRNA genes, DNA sequencing has been performed after that, DNA sequences were edited by Chromaspro Version 2.1.3 (Technelysium Pty Ltd, Australia) and BioEdit Version 7.0.5.3. Finally, a standard nucleotide blast has been done in NCBI.

Sensitivity assay

In the present study, for colony-forming unit (CFU) calculation, a 48h incubated suspension of *B. melitensis* and *B. abortus* within sterile PBS was utilized for preparing serial dilutions from 10^{-1} to 10^{-10}. 0.1 ml from each dilution was cultured onto the *Brucella* agar medium and incubation process was as follows: 37°C for 72h. Then, the colonies of *B. melitensis* and *B. abortus* were counted and the bacterial concentration was calculated to be about 5 x10^{8} CFU/ml for both *B. melitensis* and *B. abortus*. At that point a serial dilution of extricated refined DNA of *B. melitensis* and *B. abortus* was prepared from 10^{-1} to 10^{-10}. Afterward, five microliters of each dilution were utilized as template in the PCR process. No amplification was detected with the *E. coli* DNA template.

For statistical analysis utilized the chi-square test. The P-value for all variables was less than 0.01 ($P < 0.01$) so the differences were considered statistically significant.

Results

Epidemiologic data

In this research, the geographical distribution of patient samples in different cities was shown in figure 1.

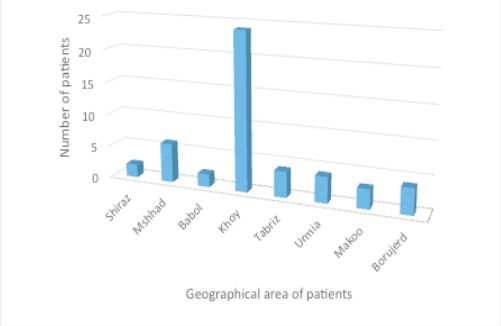

Figure 1. Geographical distribution of studied patients in different cities of Iran.

Amplification with BCSP31-PCR

Detection of the *Brucella* genus with B4 and B5 primers is shown in figure 2. As expected, The BCSP31 gene amplicon size was 223 bp, and all the 49 (100%) serum specimens isolated from patients were positive by B4 and B5 primers.

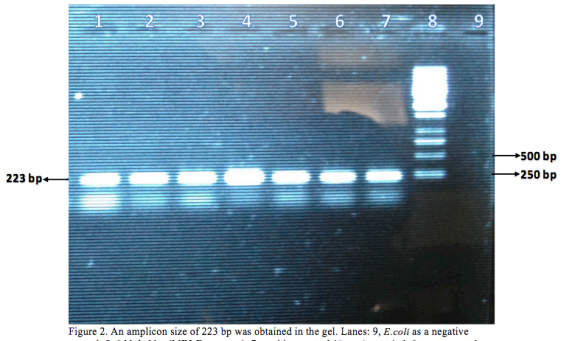

Figure 2. An amplicon size of 223 bp was obtained in the gel. Lanes: 9, *E.coli* as a negative control; 8, 1 kb ladder (MBI-Fermentas); 7, positive control (*B. melitensis*); 1-6, serum samples.

Amplification with 16SrRNA –PCR

16SrRNA primers did detection of Brucella in serum samples. The positive result is shown in figure 3. The amplicon of 16SrRNA is made of 218 bp. Among the 49 serum samples isolated from patients, 15 (30.61%) cases were positive.

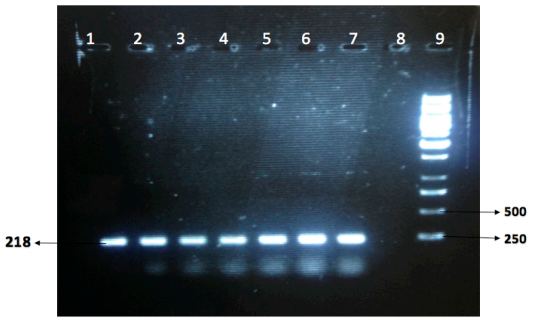

Figure 3. An amplicon size of 218 bp was obtained by PCR using B. melitensis - primers (16SrRNA) and Brucella´s DNA as a template. and template DNA from reference Brucella spp and patients serum. Lanes: 9, 1 kb ladder(MBI-Fermentas);8, E.coli as a negative control; 7, positive control (B.melitensis). 1-6, serum samples.

Amplification with IS711–PCR

Detection of *Brucella* in serum samples was performed by designed IS711 primers, which were specific to the *Brucella* genus. The positive result is shown in figure 4. The amplicon of IS711 is made of 448 bp. 46 (93.87%) cases from 49 serum samples were positive.

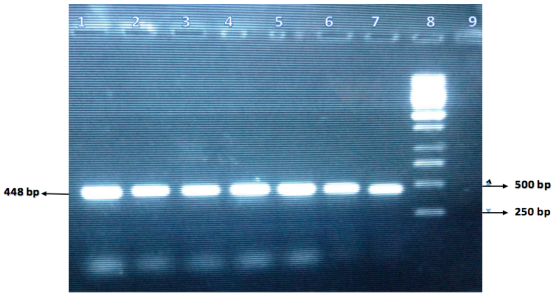

Figure 4. An amplicon size of 448 bp was obtained by PCR using *B. melitensis* - specific primers (IS711) and *Brucella's* DNA as the template. And template DNA from reference *Brucella* spp and patients serum. Lanes: 1-6, serum samples; 7, positive control (*B.melitensis*); 8, 1 kb ladder (MBI-Fermentas); .9, *E.coli* as a negative control.

Testing of the sensitivity

The sensitivities of three primer pairs (B4-B5 and IS711 and 16SrRNA) were evaluated by using serial dilutions of extracted purified DNA molecules of *B. melitensis* and *B. abortus*. The comparative values of related primers are indicated in figures 5, 6, and 7. The designed IS711 primer pairs were able to distinguish bacterial cells with the sum of 2 CFU/reaction for both *B. melitensis* and *B. abortus*, whereas the B4-B5 primers were able to identify bacterial cells with the sum of 0.05 CFU/reaction for both them. And 16SrRNA primers could detect 2×10^5 CFU/reaction. Since it has been estimated that 20 *Brucella* cells are equal with 60 fg of bacterial DNAs (19), It is estimated that B4-B5 primers can detect 1.5 pg of DNA while the novel primers of IS711 can detect the amount of 0.15 ng of DNA and 16SrRNA are able to detect about 15 µg of DNA.

Figure 5. PCR products derived from10- fold serial dilutions of *Brucella* spp. DNA and B4-B5 primers. Lane:1, *E.coli* as a negative control; 2, 1 kb ladder(MBI-Fermentas); 3, positive control (*B.melitensis*); 4-13, diluted DNA from 10^{-1}-10^{-10}.

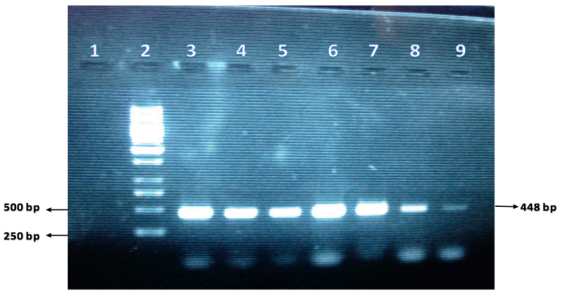

Figure 6. PCR products derived from10- fold serial dilutions of *Brucella* spp. DNA and IS7I1 primers. Lanes: 1, *E.coli* as a negative control; 2, 1 kb ladder (MBI-Fermentas); 3, positive control (*B.melitensis*); 4-9, diluted DNA from 10^{-1}-10^{-6}.

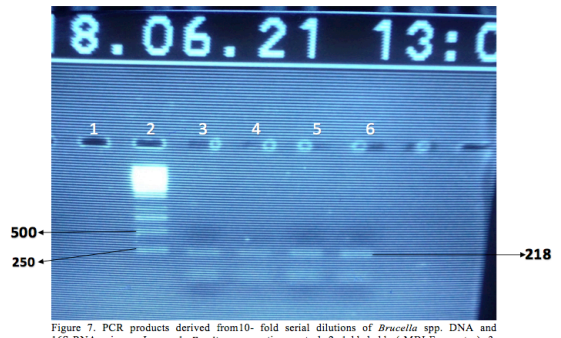

Figure 7. PCR products derived from10- fold serial dilutions of *Brucella* spp. DNA and 16SrRNA primers. Lanes: 1, *E.coli* as a negative control; 2, 1 kb ladder(MBI-Fermentas); 3, positive control (*B.melitensis*); 4-6, diluted DNA from 10^{-1}-10^{-3}.

Discussion

As the clinical symptoms of human brucellosis are not specific (20,21), it is necessary to use laboratory assays, which have high sensitivity and specificity to obtain true and sharp results in a short time. PCR is one of this techniques but PCR needs to be optimized (22, 23). Other techniques such as culture media and serological tests have low sensitivity and specificity (23, 24). PCR is a gold molecular technique for detecting fastidious bacteria such as *Brucella* (18).

In this project, 49 serum samples from suspected patients were gathered from different cities of Iran. For detection of *Brucella* spp. To select the optimum pair of primers which would specifically amplify *Brucella* DNA, we have used to PCR assay with 3 primer pairs including B4/B5, IS711, 16SrRNA, the sensitivity, and specificity of them were compared with each other. So, the sensitivity and specificity of three targets, BCSP 31 and designed IS711 genes and 16SrRNA were compared by PCR. In other experiments, the properties of sensitivity and specificity of different PCR protocols in association with the target gene of BCSP31 for detection of *Brucella* DNA in human blood or serum samples varies between 50% and 100%, respectively (20,24,25,26). The specificity of the B4-B5 PCR assay, as well as the other primer pair assays, was excellent according to previous results (14, 20, 24, 27 and 28). In another survey by Queipo-Ortuno et al. in 1997 used PCR with B4/B5 primers examined 47 peripheral blood samples; the best sensitivity (100%) was reported in comparison with blood culture and serology (70% and 84%, respectively). Our results indicate that: the sensitivity and specificity of B4/B5 primers both of them were 100%. The PCR method with designed primers of IS711 distinguished the huge number of *Brucella* spp. in serum samples. These primers could detect at least 0.2×10^1 CFU/ ml bacteria in the samples and be about 2.5×10^2 times more sensitive than the other IS711 primers, which were used in the detection of this gene by Ciftci in Turkey. They could detect 5×10^2 CFU/ml bacteria by IS711 primers (23). These accomplishments affirm the PCR comes about that were detailed by Khosravi (29) and Elfaki (30). In these studies, a large number of *B. melitensis* DNA was identified by utilizing the IS711 primers. In any case, our discoveries are altogether diverse from the details that come about by Garshasbi. Within the performed think about by Garshasbi, the sensitivity is bellow and a huge number of *B. abortus* DNA was detectable by using the IS711 primers (31).

In this study, the use of diluted *Brucella* DNA (1 to 10 in water) did not improve the overall sensitivity of the B4-B5 or IS711 primer pairs and was not accepted as a routine. Moreover, the detection of *Brucella* is limited to 0.05 CFU/reaction by B4-B5 primers with100%sensitivity, while the detection is limited to 5 CFU/reaction by designed IS711 primer with 93.87% sensitivity, the results suggest that the 3 samples with negative results are caused by the inadequate number of bacteria existing in the serum samples., and the detection is limited to 5×10^5 CFU/reaction by 16SrRNA primer with 30.61% sensitivity. The result of 16SrRNA primers, indicate at least sensitive and needed the highest number of the cells to give a positive bond; all attempts at improving its results were unsuccessful. It is beneficial to specify that diverse sums of template DNA were utilized extending from 1 to 5 µL to dispose of the conceivable response restraint of the template DNA inhibitors. This study aimed to develop a novel molecular method for the detection and identification of *Brucella* spp., which may be the gold standard demonstrative strategy for brucellosis both in creatures and people is still based on the separation of Brucella spp. inside the samples (28).

Conflict of interest

The authors have no conflict of interest to declare.

Acknowledgments

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