Differentiation of Brucella abortus and B. melitensis biovars using PCR-RFLP and REP-PCR

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Abstract

Brucellosis is one of the most common zoonotic diseases of animal and human beings. This study aimed to differentiate the Brucella spp. and determines the patterns of biovars by using repetitive element palindromic (REP)-PCR and PCR restriction fragment length polymorphism (RFLP) methods. A total of 100 blood specimens suspected of harbouring brucellosis were collected. Conventional culture methods and multiplex PCR were used for the detection of Brucella genus and species; and REP-PCR was used for Brucella spp. differentiation and polymorphisms sequence analysis. In addition, to identify the biovar patterns of REP-PCR, PCR-RFLP was used. Eighty-three samples were identified as harbouring Brucella spp. by the implementation of multiplex PCR, 72 of which were detected as Brucella melitensis and 11 as B. abortus. Also, through analysing the results of PCR-RFLP, it was found that of 72 B. melitensis samples, 69 were B. melitensis biovar 1 and three species were from other biovars. In addition, the obtained patterns for all of the B. abortus samples were from biovars 3, 5, 6 and 9. This study also optimized a test for the detection of Brucella biovar with the REP-PCR method such that Brucella spp. and biovars could be separated in the shortest possible time.

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Introduction

Brucella is one of the most common causes of human and animal diseases. The disease caused by this organism, which can cause some chronic disabling diseases in humans, is a serious problem in developing and some developed countries [1,2]. Although the mortality of brucellosis in human beings is small, it can cause abortion in livestock, consequently leading to economic losses [3,4]. On the basis of the position of Iran in the Middle East region as well as the uncontrolled entry and exit of livestock on its borders, there is always the possibility of Brucella spp. entering this region. The slaughter of infected animals as well as animal vaccination play an important role in controlling the disease, but as a result of the failure of control programmes, a brucellosis outbreak occurred in Mediterranean countries in 2002 [5–7]. To overcome this problem, studies have shown that effective control and vaccination programmes working on the species differentiation and Brucella biovar identification are essential [8]. For this purpose, different phenotypic and molecular methods have been suggested [9]. Recently new molecular methods have been proposed for differentiating and typing bacteria, which have many advantages, including specificity and reliability, as well as the abandonment of the use of nonsensitive and time-consuming phenotypic techniques [10,11]. It has been shown that molecular methods can be applied to a wide range of microorganisms [12].

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Several molecular methods are used for Brucella spp. typing, including PCR restriction fragment length polymorphism (RFLP), repetitive element palindromic PCR (REP-PCR), enterobacterial repetitive intergenic consensus PCR (ERIC-PCR), random amplified polymorphic DNA PCR (RAPD-PCR), amplified fragment length polymorphism (AFLP), arbitrarily primed PCR (AP-PCR) and single-nucleotide polymorphism (SNP) [13–17]. Several studies have demonstrated that the REP-PCR and PCR-RFLP methods could be potentially used for B. abortus and B. melitensis differentiation [13,15].

REP-PCR is based on repetitive extragenic palindromic sequences in bacterial genomics and primers designed for that sequence. Its properties include an extragenic location and highly conserved repetitive reverse sequences. A large number of copies of this sequence are located in complex clusters and have a good repeatability. This technique is simple and desirable, determining the sequence of the genome is not essential, and DNA can be used instead of a bacterial suspension. In addition, it does not use living microorganisms and reduces the risk of bacterial transmission to laboratory personnel [15,18–21].

The PCR-RFLP method consists of analysing a PCR-based multiplication loci. In this method, outer membrane protein (OMP) as an appropriate marker was is for the differentiation of B. abortus and B. melitensis [13,22]. The outer membrane proteins are made by the OMP2a and OMP2b genes, which are homogeneous and have minor variations [23]. After PCR, the product is subjected to appropriate restriction enzymes, and ultimately, on the basis of the limited number of components from the digestive enzyme, the results are analysed by gel electrophoresis [22,23]. Some studies have reported that all Brucella spp. can be differentiated by PCR-RFLP on the basis of the OMP2a, OMP2b, OMP25 and OMP31 genes [13,24,25].

Because it is important to distinguish between Brucella spp. for typing for different purposes, such as monitoring the source of infection and preventing infection, we aimed to distinguish and type Brucella spp. and determine its biovar pattern by using REP-PCR and PCR-RFLP to achieve an optimal REP-PCR method.

Materials and methods

Sample collection

The differentiation of B. abortus and B. melitensis species, as the main factors of brucellosis, was investigated. In this descriptive cross-sectional study, 100 specimens consisting of blood samples suspected to harbour human brucellosis (from patients with fever, chills and antibody titre above 1/80 by Wright test) and animal brucellosis (from abortion and dead animals) were collected from slaughterhouses and different treatment centres of Tehran, Guilan and Hamedan provinces of Iran in 2015–2016. The study protocol was approved by the ethics committee of Baqiyatallah University of Medical Sciences.

Cultivating samples

Blood samples (10 mL) were taken from the patients suspected to have brucellosis—that is, they had antibody titre >1/80 by Wright test. EDTA was added to 5 mL of blood and was stored in the freezer to extract the DNA. Then another 5 mL of blood sample was insemminated in a vial containing brain–heart infusion BHI (broth medium (Merck) and sent to the microbiology laboratory of Iran University of Medical Sciences, Tehran, Iran. The vial containing BHI was incubated at 37°C for 7 to 28 days in the presence of 5% CO2. After that, 1 mL of BHI was transferred to Brucella broth medium (Merck) and incubated at 37°C for 24 hours in the presence of 5% CO2. Finally, human specimens from broth medium as well as animal specimens from lymph node samples and blood samples with suspected brucellosis were cultured on a Brucella agar medium (Merck) containing 5% sheep’s blood, then incubated at 37°C in anaerobic conditions containing 5% CO2 for 3 days. Brucella S19 and Brucella M16 standard strains were used as positive controls. Finally, biochemical tests such as growth in 2% thionin and H2S production were used to confirm the Brucella spp. [26]. It should be mentioned that the positive culture time for growing colonies was about 3 days, and Brucella agar plates were considered negative after 10 days without any evidence of colony growth.

DNA extraction

According to previous studies, bacterial genomic extraction was performed using the phenol–chloroform method. The obtained DNA was examined by quantitative (agarose gel) and qualitative (spectrophotometry) methods [23]. A genomic DNA extraction kit (Bioneer) was also used to extract DNA from blood samples.

PCR

Brucella genus identification with multiplex PCR. To identify B. abortus and B. melitensis strains, three specific primers of Brucella spp. were used (Table 1). The solution required to conduct a 25 μL PCR comprised the following: 12 μL of master mix (1× PCR buffer, 2.5 U Taq DNA polymerase, 2 mM MgCl2, 0.15 mM deoxyribonucleotide triphosphate (dNTP)), 1 μL of primer (concentration, 10 pmol), 1 μL template DNA (10 ng) and 11 μL distilled water. Amplification was carried out in a Jena Analytik (Thuringia) device as follows: initial denaturation...
μ2.5 U Taq DNA polymerase, 2 mM MgCl2 and 0.15 mM dNTP), consisting of the following: 12 morphism sequence (Table 1). We used a 25 s

at 95°C for 5 minutes, followed by 35 cycles of 40 seconds for denaturation at 90°C, 40 seconds for annealing at 66°C and 40 seconds for primer extension at 72°C, followed by final extension at 72°C for 7 minutes. Electrophoresis of PCR products was performed on 1% agarose gel using SYBR Safe DNA Gel Stain (Invitrogen). The stained gels were viewed on a UV transilluminator (Bio-Rad). Direct PCR was also performed on DNA extracted from blood samples.

REP-PCR. To perform the REP-PCR and distinguish between Brucella spp., specific primers were used for the REP polymorphism sequence (Table 1). We used a 25 μL PCR solution consisting of the following: 12 μL of master mix (1× PCR buffer, 2.5 U Taq DNA polymerase, 2 mM MgCl2 and 0.15 mM dNTP), 1 μL of primer (concentration, 10 pmol), 1 μL of DNA template (10 ng) and 11 μL distilled water. Amplification was carried out by a Jena Analytik (Thuringia) device as follows: initial denaturation at 95°C for 5 minutes, followed by 30 cycles of 60 seconds for denaturation at 95°C, 2 minutes for annealing at 58°C and 2 minutes for primer extension at 72°C, followed by terminal extension at 72°C for 10 minutes. Electrophoresis of PCR products was performed on 1% agarose gel using SYBR Safe DNA Gel Stain (Invitrogen). The stained gels were viewed on a UV transilluminator (Bio-Rad). Then, by observing the bands of the OMP2a and OMP2b genes, the resulting products were digested by FastDigest PstI (Thermo Fisher Scientific). On the basis of the manufacturer’s instructions, 7 μL of the PCR product was mixed with 1.7 μL enzyme buffer, 0.3 μL of enzyme and 6 μL distilled water to reach a final volume of 15 μL; then the solution was placed at 37°C for 1 hour. After that, the entire solution was tested on 2% gel electrophoresis.

Analysis of pattern results
Finally, the relationships between the patterns obtained from REP-PCR and the biovars were evaluated by the PCR-RFLP technique. First the biovars obtained from PCR-RFLP were isolated as a group, and then each same REP-PCR pattern of biovar was placed in that group. Results were expressed as percentages.

Results

Demographic results
Forty human samples from 100 suspected cases of brucellosis were collected from Tehran, Gilan and Hamedan treatment centres. In addition, 60 animal samples were collected from slaughterhouses in these provinces (Table 2).

Microbiology results
By using a specific culture method, 65 samples were confirmed to harbour Brucella spp. Of 65 Brucella samples, 22 human and

| Method         | Primer sequence                                                                 |
|----------------|---------------------------------------------------------------------------------|
| PCR IS711      | F: 5′-GACGACGGAAATTCTCCCACTCC-3, R: 5′-TGCCGATCTTAAGGCTCTTT-3                  |
| REP-PCR        | F: 5′-TIIICGGGGGACCACGACG-3, R: 5′-AACGGTTTACCAAGGCTTAC-3                     |
| PCR-RFLP       | F: 5′-GGCTATTCAAAAATTCGCGG-3, R: 5′-ATCGATTCTCACGCTTTCGT-3                   |

| Method         | Primer sequence                                                                 |
|----------------|---------------------------------------------------------------------------------|
| PCR IS711      | F: 5′-TTCCGAGACGAAATTCTCCCACTCC-3, R: 5′-TGCCGATCTTAAGGCTCTTT-3                |
| REP-PCR        | F: 5′-TIIICGGGGGACCACGACG-3, R: 5′-AACGGTTTACCAAGGCTTAC-3                     |
| PCR-RFLP       | F: 5′-GGCTATTCAAAAATTCGCGG-3, R: 5′-ATCGATTCTCACGCTTTCGT-3                   |

| Characteristic | Human sample | Animal sample | Positive result | Bm | Ba | Bm biovar | Ba biovar | Total |
|---------------|--------------|---------------|----------------|----|----|-----------|-----------|-------|
| Sample collection | 40 | 60 | — | — | — | — | — | 100 |
| Culture and biochemistry tests | 22 | 43 | 65 | 59 | 6 | — | — | 100 |
| PCR IS711 | 28 | 55 | 83 | 72 | 11 | — | — | 100 |
| REP-PCR | 28 | 55 | 83 | 72 | 11 | — | — | 100 |
| PCR-RFLP | 28 | 55 | 83 | 72 | 11 | Biovar 1: 69; other biovars: 3 | Biovars 3, 5, 6, 9: 11 | 83 |
43 animal samples were reported. Also, by the implementation of standard biochemical and microbiologic tests, six of the samples were found to contain \( B. \) \textit{abortus} and 59 \( B. \) \textit{melitensis} (Table 2).

**Multiplex PCR results**

Molecular analysis was performed on all of the collected samples. As can be seen in Fig. 1, the PCR confirmed that 65 samples were positive in culture. In addition, 18 samples of the negative culture cases were positive by direct PCR on the blood samples. Overall, a total of 83 \textit{Brucella} samples were identified by this method, 55 of which were from animals. Furthermore, 11 samples of the confirmed \textit{Brucella} cases were detected as \( B. \) \textit{abortus} and 72 as \( B. \) \textit{melitensis} (Table 2).

**REP-PCR results**

Eighty-three extracted DNA of \textit{Brucella} isolates (cultured specimens and direct blood samples) were used for REP-PCR. By using the gel electrophoresis technique, nine to ten pieces from 200 to 1200 bp in length were obtained for each standard strain (Fig. 2). To confirm the results of REP-PCR, the patterns of collected samples (Fig. 3) were compared to the patterns of \( B. \) \textit{abortus} and \( B. \) \textit{melitensis} standard strains, by which, according to the obtained patterns, 11 cases of \( B. \) \textit{abortus} and 72 cases of \( B. \) \textit{melitensis} infection were detected. In addition, some isolates have different patterns of standards. To determine whether the different patterns (lanes 3 and 5 in Fig. 3) belong to other \textit{Brucella} spp., or because of differences in the biovar, PCR products were sequenced, and PCR-RFLP was used to determine their biovars. On the basis of the sequencing results (Fig. 3), samples 3 and 5 were identified as \( B. \) \textit{melitensis} and \( B. \) \textit{abortus} respectively, which were similar to the culture and PCR results.

**Biovar detection with PCR-RFLP**

After identifying different patterns and species, PCR-RFLP was performed. On the basis of the results obtained from PCR-RFLP (Fig. 4), 69 of 72 were \( B. \) \textit{melitensis} biovar 1. The REP-PCR pattern of this biovar is shown in well no. 2 of Fig. 3. Three samples from other \( B. \) \textit{melitensis} biovars were also identified, which is shown in well no. 3 of Fig. 3. The obtained patterns of 11 \( B. \) \textit{abortus} samples were the same; all belonged to biovars 3, 5, 6 and 9. The REP-PCR pattern of these four biovars is shown in wells 4 and 5 of Fig. 3. The difference between \( B. \) \textit{abortus} and \( B. \) \textit{melitensis} patterns of REP-PCR is due to

![FIG. 1.](image1.png)

![FIG. 2.](image2.png)
differences in their biovar. The results of the OMP2b gene enzymatic cutting showed that the product produced by this gene was not cut by the Ps t1 enzyme (Fig. 4). Finally, this study was able to find a meaningful relationship between these patterns by examining the relationship between REP-PCR and PCR-RFLP patterns. In fact, the patterns obtained from REP-PCR and PCR-RFLP had the same results in determining the Brucella species. By using the PCR-RFLP method, different patterns of biovars in REP-PCR were determined for Brucella spp. The REP-PCR test was optimized so its patterns could be used as standard patterns. Also, the results of this study showed that B. melitensis biovar 1 was the dominant form of Brucella in human and animal samples from Tehran, Gilan and Hamedan provinces.

**Discussion**

REP-PCR is a simple and repeatable method which is commonly used to type and identify Brucella species. Various primers were used for the same genetic purposes in the REP-PCR method for differentiating between Brucella species. Some studies have investigated Brucella differentiation through the REP-PCR technique [15,18,20,21]. The PCR-RFLP is also used for identifying the Brucella species. The advantages of this method are its applicability and easy interpretation. This method was performed by Vizcaino et al. [27] on the OMP31 gene. One study used a number of restriction enzymes to cut the polymorphism regions in the OMP2a locus [28]. Furthermore, Cloeckaert et al. [28], using PCR, replicated the OMP2a and OMP2b genes; the products were then cut with restriction enzymes, which permitted determination of more biovars than in previous studies. In Iran this technique is commonly used to type Brucella species [8,23].

The aim of this study was to use REP-PCR and PCR-RFLP techniques to identify Brucella spp. and find the best REP-PCR method. In this study, multiplex PCR was initially used to isolate B. abortus and B. melitensis and to confirm the results of culture. The results showed that multiplex PCR can provide different patterns for Brucella spp., but isolates inside a biovar cannot be separated [18,29]. After that, the REP-PCR technique was used. On the basis of the results, several different patterns were obtained for each species of B. abortus and B. melitensis.

FIG. 3. PCR product derived from proliferation of polymorphism sequence. Lane 1, DNA Ladder (100 bp); Lanes 2 and 3, B. melitensis pattern; Lanes 4 and 5, B. abortus; Lane 6, negative control (vaccine strain).

FIG. 4. PCR-RFLP product obtained from OMP genes and restriction maps of omp2a and omp2b of Brucella melitensis and B. abortus. Lane 1, OMP2a; lane 2, OMP2b; lane 3, DNA ladder (100 bp); lane 4, B. melitensis biovar 1 (restriction maps of omp2a); lane 5, B. abortus biovar (related to biovars 3, 5, 6 and 9 (restriction maps of omp2a)); lane 6, negative control (vaccine strain); lanes 7 and 8, PCR product of OMP2b.

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The numbers of each template bands were between nine and ten, their size ranged between 200 and 1200 bp and the polymorphism sequence was in the region of 450 to 850 bp. The difference between the obtained patterns with previous studies was due to optimized reaction conditions and using different primers for amplification. It should be noted that REP-PCR is highly influenced by the reaction conditions.

On the basis of the results of REP-PCR, each species of B. abortus and B. melitensis has two different patterns. For confirmation and interpretation of the results, the obtained patterns from the REP-PCR were compared to the standard patterns. Multiplex PCR results were also used to confirm and interpret the results. On the basis of a comparison with the standard patterns, samples 3 and 5 (Fig. 3) had different patterns. To find out the difference between them, the patterns of samples 3 and 5 were sequenced and the results described. In addition, the PCR-RFLP technique was used to determine species and biovars. Finally, it was found that the differences between the patterns of some samples with the standard pattern were due to differences in their biovars. Also, the REP-PCR technique was optimized, and new patterns were created that were different from the standard patterns.

On the bases of the results of this study as well as findings of previous studies, the REP-PCR technique is able to differentiate between Brucella spp.; it also succeeded in differentiating between Brucella biovars. However, according to previous studies, it is not able to differentiate between B. canis and B. suis [15,18]. The relationship between the PCR-RFLP and REP-PCR patterns was evaluated; we found that 69 samples of B. melitensis had sample no. 2 pattern and three samples had pattern no. 3 (Fig. 3). Sample no. 2 pattern showed B. melitensis biovar 1, and the other patterns of B. melitensis were related to other biovars. Also, in the case of B. abortus, 11 samples confirmed this isolate, which has two different patterns in REP-PCR. It was found that different patterns of B. abortus were related to biovars 3, 5, 6 and 9.

According to Table 2, and comparing different methods, it can be concluded that PCR-based methods could provide better identification than culture and biochemical studies. In addition, the time spent on PCR-based reactions (according to the use of PCR on direct blood samples) is less than culture methods [29]. On the basis of the results of this study, the predominant species was B. melitensis biovar 1. Additionally, the predominant biovars of B. abortus were 3, 5, 6 and 9, which are similar to the results of previous studies in Iran. This similarity may be due to the circulation of these biovars in Iran [22,23]. According to the results obtained from typing Brucella spp. in Eastern Mediterranean countries [30,31], B. melitensis biovar 3 is the predominant one, which is different from Iran. This indicates that the source of contamination is different in these areas.

According to the results of this study, to create optimal reaction conditions using specific primers, different polymorphism genes and more restriction enzymes should be taken into account. Primers and enzymes used in the PCR-RFLP method are able to detect biovars of B. melitensis and B. abortus species identified by REP-PCR. Similarly, the patterns obtained in REP-PCR can be used as standard templates for differentiation between Brucella spp. and biovars. Additionally, the use of these methods can save time and money; they can also be suitable for quickly monitoring Brucella species. In addition, an optimized REP-PCR technique can be used in diagnostic and medical laboratories to detect Brucella in suspected cases.

Conflict of interest

None declared.

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