ORIGINAL ARTICLE

# 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.


**Keywords:** *Brucella abortus*, *B. melitensis*, Polymorphisms, REP-PCR, PCR-RFLP
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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 inseminated 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% $CO_2$. 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% $CO_2$. 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% $CO_2$ 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 $H_2S$ 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 $MgCl_2$, 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

**TABLE 1. Primer sequences**

| Method | Primer sequence |
|---|---|
| PCR IS711 | Ba-F: 5′-GACGAACGGAATTTTTCCAATCCC-3′<br>Bm-F: 5′-AAATCGCGTCCTTGCTGGTCTGA-3′<br>IS711: 5′-TGCCGATCACTTAAGGGCCTTCAT-3′ |
| REP-PCR | Rep-1: 5′-IIIGCGCCGICATCAGGC-3′<br>Rep-2: 5′-ACGTCTTATCAGGCCTAC-3′ |
| PCR-RFLP | *omp2a* F: 5′-GGCTATTCAAAATTCTGGCG-3′<br>*omp2a* R: 5′-ATCGATTCTCACGCTTTCGT-3′<br>*omp2b* F: 5′-CCTTCAGCCAAATCAGAATG-3′<br>*omp2b* R: 5′-GGTCAGCATAAAAAGCAAGC-3′ |

Ba, *Brucella abortus*; Bm, *B. melitensis*; F, forward; R, reverse; REP, repetitive element palindromic; RFLP, restriction fragment length polymorphism.

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). *Brucella* S19 and M16 were used as positive controls. 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 $MgCl_2$ 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 96°C for 7 minutes, followed by 35 cycles of 30 seconds for denaturation at 92°C, 60 seconds for annealing at 40°C and 8 minutes 8 for primer extension at 63°C, followed by terminal extension at 72°C for 14 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.

*PCR-RFLP.* To determine different patterns obtained from REP-PCR, a PCR-RFLP reaction with specific primers for the sequence of the OMP polymorphism was used (Table 1). Direct PCR-RFLP was also performed on blood samples. The solution required to perform a PCR of 25 µL included the following: 12 µL of master mix (1× PCR buffer, 2.5 U Taq DNA polymerase, 2 mM $MgCl_2$ and 0.15 mM dNTP), 1 µL of primer (10 pmol), 1 µL of DNA template (10 ng) and 11 µL distilled water. Amplification for the OMP sequence was carried out in 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 *Pst*I (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

**TABLE 2. Evaluation of results of *Brucella* spp. using phenotypic and genotypic methods**

| 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 | — | — | 83 |
| PCR-RFLP | 28 | 55 | 83 | 72 | 11 | Biovar 1: 69; other biovars: 3 | Biovars 3, 5, 6, 9: 11 | 83 |

Bm, *Brucella melitensis;* Ba, *B. abortus;* REP, repetitive element palindromic; RFLP, restriction fragment length polymorphism.

43 animal samples were reported. Also, by the implementation of standard biochemical and microbiologic tests, six of the samples were found to contain *B. abortus* and 59 *B. 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 *Brucella* samples were identified by this method, 55 of which were from animals. Furthermore, 11 samples of the confirmed *Brucella* cases were detected as *B. abortus* and 72 as *B. melitensis* (Table 2).

### REP-PCR results

Eighty-three extracted DNA of *Brucella* isolates (cultured specimens and direct blood samples) were used for REP-PCR. By using the gel electrophoresis technique, nine to ten pieces 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. abortus* and *B. melitensis* standard strains, by which, according to the obtained patterns, 11 cases of *B. abortus* and 72 cases of *B. 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 *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. melitensis* and *B. 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. melitensis* biovar 1. The REP-PCR pattern of this biovar is shown in well no. 2 of Fig. 3. Three samples from other *B. melitensis* biovars were also identified, which is shown in well no. 3 of Fig. 3. The obtained patterns of 11 *B. 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. abortus* and *B. melitensis* patterns of REP-PCR is due to

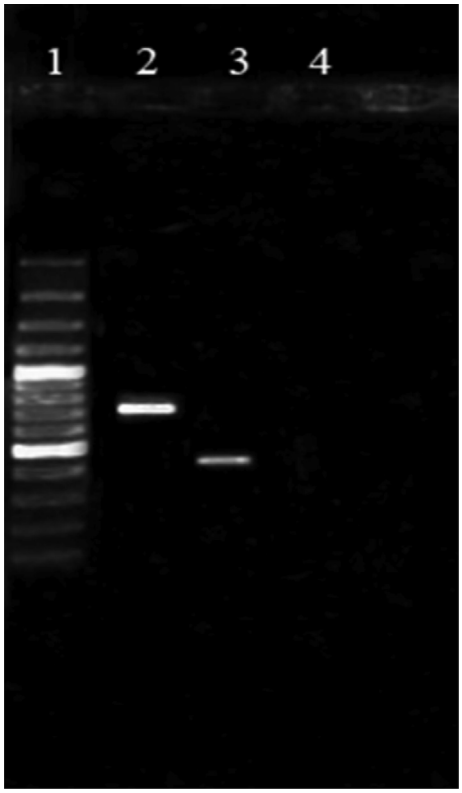


FIG. 1. Multiplex PCR product derived from specific primers for approving cultured *Brucella* spp. Lane 1, ladder (100 bp); lane 2, *B. melitensis;* lane 3, *B. abortus;* lane 4, negative control (vaccine strain).

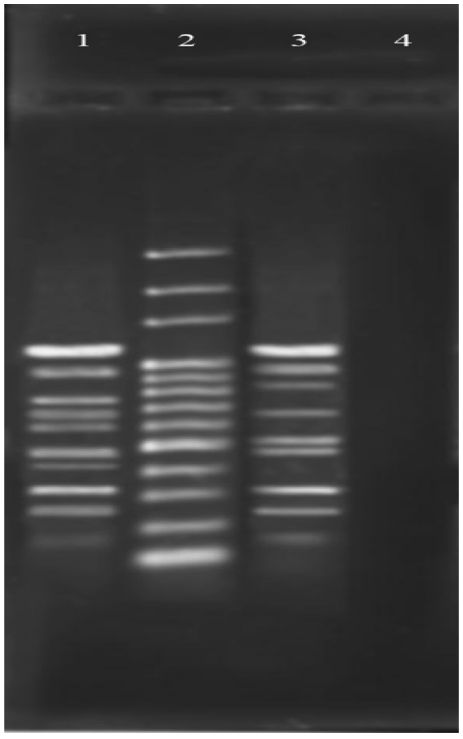


FIG. 2. PCR products derived from proliferation of polymorphism sequence *Brucella melitensis* M16 and *B. abortus* S19. Lane 1, M16 (standard *B. melitensis* species); lane 2, DNA ladder (100 bp); lane 3, S19 (standard *B. abortus* species); lane 4, negative control (vaccine strain).

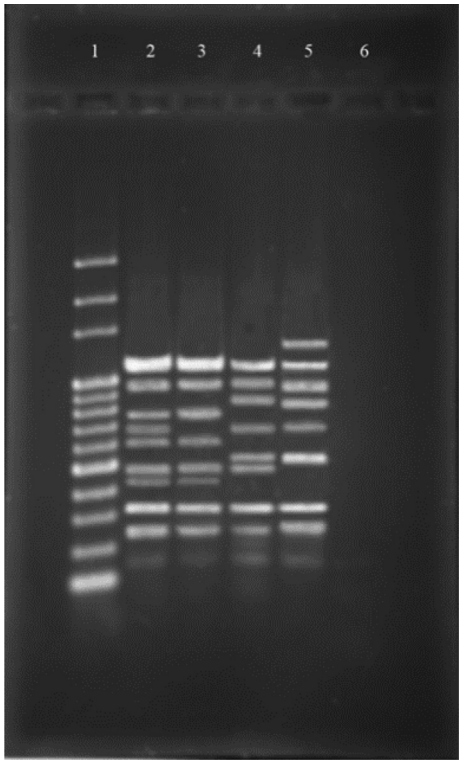


FIG. 3. PCR product derived from proliferation of polymorphism sequence. Lane 1, DNA Ladder (100 bp); Lanes 2 and 3, *B. melitensis* pattern; Lanes 4 and 5, *B. abortus;* Lane 6, negative control (vaccine strain).

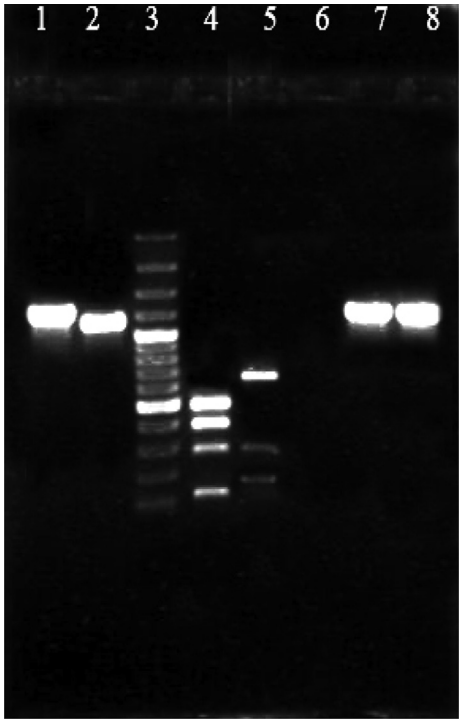


FIG. 4. PCR-RFLP product obtained from OMP genes and restriction maps of *omp*2a and *omp*2b of *Brucella melitensis and B. abortus.* Lane 1, *OMP2a*; lane 2, *OMP2b*; lane 3, DNA ladder (100 bp); lane 4, *B. melitensis* biovar 1 (restriction maps of *omp*2a); lane 5, *B. abortus* biovar (related to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

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 *Pst*1 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.*

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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## References

[1] Mirnejad R, Mohamadi M, Piranfar V, Mortazavi SM, Kachuei R. A duplex PCR for rapid and simultaneous detection of *Brucella* spp. in human blood samples. Asian Pac J Trop Med 2013;6:453–6.

[2] Leylabadlo HE, Bialvaei AZ, Samadi Kafil H. Brucellosis in Iran: why not eradicated?. Clin Infect Dis 2015;61:1629–30.

[3] De Santis R, Ciammaruconi A, Faggioni G, D'Amelio R, Marianelli C, Lista F. Lab on a chip genotyping for *Brucella* spp. based on 15-loci multi locus VNTR analysis. BMC Microbiol 2009;9:66.

[4] Piranfar V, Sharif M, Hashemi M, Vahdati AR, Mirnejad R. Detection and discrimination of two *Brucella* species by multiplex real-time PCR and high-resolution melt analysis curve from human blood and comparison of results using RFLP. Iran J Basic Med Sci 2015;18:909–14.

[5] Osman MA, Abdelgadir A. Detection of brucellosis in sheep intended for export and local slaughter in Khartoum State, Sudan. Afr J Microbiol Res 2012;6:6805–10.

[6] Nagalingam M, Shome R, Balamurugan V, Shome BR, NarayanaRao K, Vivekananda, et al. Molecular typing of *Brucella* species isolates from livestock and human. Trop Anim Health Prod 2012;44:5–9.

[7] Zowghi E, Ebadi A, Yarahmadi M. Isolation and identification of *Brucella* organisms in Iran. Iran J Clin Infect Dis 2008;3:185–8.

[8] Pishva E, Salehi R, Hoseini A, Kargar A, Taba FE, Hajiyan M, et al. Molecular typing of *Brucella* species isolates from human and livestock bloods in Isfahan province. Adv Biomed Res 2015;4:104.

[9] Al Dahouk S, Le Flèche P, Nöckler K, Jacques I, Grayon M, Scholz HC, et al. Evaluation of *Brucella* MLVA typing for human brucellosis. J Microbiol Methods 2007;69:137–45.

[10] Hasani SM, Mirnejad R, Amani J, javad Vafadar M. Comparing rapid and specific detection of *Brucella* in clinical samples by PCR-ELISA and multiplex-PCR method. Iran J Pathol 2016;11:144.
[11] Sidamonidze K, Ramishvili M, Kalandadze I, Tsereteli D, Nikolich M. Epidemiology and molecular typing of *Brucella* strains circulating in Georgia. Georgian Med News 2015;247:58–63.
[12] Ben-Tekaya H, Gorvel JP, Dehio C. *Bartonella* and *Brucella*—weapons and strategies for stealth attack. Cold Spring Harb Perspect Med 2013;3:a010231.
[13] Al Dahouk S, Tomaso H, Prenger-Berninghoff E, Splettstoesser WD, Scholz HC, Neubauer H. Identification of *Brucella* species and biotypes using polymerase chain reaction–restriction fragment length polymorphism (PCR-RFLP). Crit Rev Microbiol 2005;31:191–6.
[14] Gopaul KK, Sells J, Bricker BJ, Crasta OR, Whatmore AM. Rapid and reliable single nucleotide polymorphism–based differentiation of *Brucella* live vaccine strains from field strains. J Clin Microbiol 2010;48:1461–4.
[15] Mercier E, Jumas-Bilak E, Allardet-Servent A, O'Callaghan D, Ramuz M. Polymorphism in *Brucella* strains detected by studying distribution of two short repetitive DNA elements. J Clin Microbiol 1996;34:1299–302.
[16] Tcherneva E, Rijpens N, Jersek B, Herman LM. Differentiation of *Brucella* species by random amplified polymorphic DNA analysis. J Appl Microbiol 2000;88:69–80.
[17] Whatmore AM, Murphy TJ, Shankster S, Young E, Cutler SJ, Macmillan AP. Use of amplified fragment length polymorphism to identify and type *Brucella* isolates of medical and veterinary interest. J Clin Microbiol 2005;43:761–9.
[18] Dong Kyun S. DNA fingerprinting of *Brucella abortus* isolated from bovine brucellosis outbreaks by repetitive element sequence (rep)-PCR. Korean J Vet Res 2005;45:199–205.
[19] Tcherneva E, Rijpens N, Naydensky C, Herman L. Repetitive element sequence based polymerase chain reaction for typing of *Brucella* strains. Vet Microbiol 1996;51:169–78.
[20] Healy M, Huong J, Bittner T, Lising M, Frye S, Raza S, et al. Microbial DNA typing by automated repetitive-sequence-based PCR. J Clin Microbiol 2005;43:199–207.
[21] Candan E, Idil N, Bilkay IS. Usefulness of REP and ERIC-PCR combination for tracking the spread of *Staphylococcus aureus* strains. Minerva Biotecnol 2013;25:245–50.
[22] Helmy NM, Zaki HM, Adawy SS. Identification and differentiation of *Brucella melitensis* Rev. 1 vaccine and *B. melitensis* biovar 3 field isolates in Egypt by serological and PCR-RFLP techniques. J Appl Sci Res 2007;3:841–7.
[23] Mirnejad R, Mohammadi M, Majdi A, Taghizoghi N, Piranfar V. Molecular typing of *Brucella melitensis* and *B. abortus* from human blood samples using PCR-RFLP method. Jundishapur J Microbiol 2013;6(6).
[24] Cloeckaert A, Grayon M, Grepinet O. Identification of *Brucella melitensis* vaccine strain Rev. 1 by PCR-RFLP based on a mutation in the *rpsL* gene. Vaccine 2002;20:2546–50.
[25] Yu WL, Nielsen K. Review of detection of *Brucella* sp. by polymerase chain reaction. Croat Med J 2010;51:306–13.
[26] Tille P. Bailey and Scott's diagnostic microbiology. Amsterdam: Elsevier Health Sciences; 2013. E-book.
[27] Vizcaino N, Verger JM, Grayon M, Zygmunt MS, Cloeckaert A. DNA polymorphism at the omp-31 locus of *Brucella* spp.: evidence for a large deletion in *Brucella abortus*, and other species-specific markers. Microbiology 1997;143:2913–21.
[28] Cloeckaert A, Verger JM, Grayon M, Grepinet O. Restriction site polymorphism of the genes encoding the major 25 kDa and 36 kDa outer-membrane proteins of *Brucella*. Microbiology 1995;141:2111–21.
[29] Mirnejad R, Vahdati AR, Ahmadi A, Mortazavi SM, Piranfar V. Comparison of culture and multiplex PCR technique for detection of *Brucella abortus* and *Brucella melitensis* from human blood samples. Zahedan J Res Med Sci 2013;15:5–8.
[30] Guler S, Kokoglu OF, Ucmak H, Gul M, Ozden S, Ozkan F. Human brucellosis in Turkey: different clinical presentations. J Infect Dev Ctries 2014;8:581–8.
[31] Yumuk Z, O'Callaghan D. Brucellosis in Turkey—an overview. Int J Infect Dis 2012;16:e228–35.