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Molecular Typing of *Brucella* Species Isolated from Humans and Animals Using Polymerase Chain Reaction-Restriction Fragment Length Polymorphism Technique

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Abstract

Background: Brucellosis is a zoonotic disease that causes major economic and public health problems. It is one of the most important diseases in humans and domestic animals. Hence, the exact identification of *Brucella* spp. is important for strategies of treatment and control. Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) is one of the molecular techniques characterized by amplification of deoxyribonucleic acid (DNA) sequence and restriction enzyme digestion.
Objectives: This study aimed at identifying genetic polymorphisms of *omp2a* genes among 90 *Brucella* isolated from humans and animals, using the PCR-RFLP method.
Methods: Ninety *Brucella* spp. isolated from humans and animals in two different regions of Iran were used in this study. Biochemical tests and the *Brucella omp2a* (1100 bp) gene-PCR was used for identification of *Brucella* isolates. Polymerase Chain Reaction products were digested by restriction endonuclease enzyme *pstI* and gene sequencing analysis was carried out for molecular typing of *Brucella* strains. Therefore, genetic relatedness was revealed by a dendrogram.
Results: Analysis of the 90 *Brucella* strains by biochemical tests, PCR, and PCR-RFLP methods with *PstI* enzyme and *omp2a* sequencing showed four unique RFLP Profiles (P1-P4). Seventy-nine (87.8%) of the *Brucella* isolates belonged to *B. melitensis* strain 20236. From 30 animal isolates, nine (30%) belonged to *B. melitensis* biovare1 and two (6.6%) to *B. abortus* strain. According to the RFLP dendrogram, group 1 and 2 had higher genetic relatedness similarity.
Conclusions: The results showed *B. melitensis* strain 20236 was the predominant strain among human and animal *Brucella* isolates. Likewise, according to dendrogram results, the PCR-RFLP technique was not able to separate human and animal species of *B. melitensis* from *B. abortus*.

Keywords: Omp2, Pst1, PCR, *Brucella*,

1. Background

Brucella is a Gram-negative facultative intracellular bacillus that can infect many human and animal species. Conventionally, they are classified to seven species, each of them comprised of several biovars (1). Annually, more than 500,000 human cases of brucellosis are reported to the world health organization (2). The prevalence of brucellosis in Iran was reported as 0.5% to 10.9%, in different provinces (3). Incidence rate of brucellosis was about 66 to 100 per 100,000 population in the province of Hamadan, Iran (4). Brucellosis still remains an unrestrained problem in many regions, such as the Middle East, Mediterranean countries, Latin America, Africa, and some parts of Asia (5). The disease is endemic and previous studies have reported that *B. melitensis* biotype 1 is the predominant strain in human and animal isolates in Iran (1, 6).

Distinction between species and biovars of *Brucella* spp. is currently based on differential tests, such as phenotypic characterization of lipopolysaccharides (LPS) antigens, dye-sensitivity, H_2S production, phage typing, CO_2 requirement, and metabolic properties (7). However, phenotypic tests are less sensitive and not always reliable (8). While, some species of *Brucella* may be characterized by serological tests, they are not able to discriminate between

species and biovars (9). Homology of DNA for all species in the genus of *Brucella* is more than 90% and probable new biovars and strains of *Brucella* enter the area. Consequently, investigations about the epidemiology of brucellosis, pathogenicity, and ecological differences between species and biovars of *Brucella* may help choose the correct vaccine, plan epidemiological studies, and control and eradicate the condition (10, 11). Molecular techniques have shown accurate typing of *Brucella* spp. based on specific identification of *Brucella* nucleotide sequences associated with the genus, species, and biovars. Therefore, these methods are important tools for diagnosis in epidemiologic studies (12).

Furthermore, PCR-RFLP is a molecular technique applied by amplification of DNA sequence and restriction enzyme digestion. However, RFLP analysis is widely used for the identification of bacterial species and biovars of the genus *Brucella* (13). The *omp2* locus contains two gene copies (*omp2a* and *omp2b*) coding for porin proteins that are used for molecular typing and identification of *Brucella* at the species, biovar, or strain level (14). The point mutations may effect the sequence amplified and change the diagnosis sites of specific restriction enzymes (15). The PCR-RFLP method has several advantages in comparison to bacteriological techniques. It is considered rapid and easy to performed, cost effective, easy to fit training into laboratory schedules, and does not need advanced tools and live specimens. Disadvantages include need for specific restriction enzymes and failure to identify variations in the nucleic acid sequence analyses (16). This study was the first report to determine the molecular typing of *Brucella* species by PCR-RFLP in Hamadan, west of Iran. The aim of this study was to analyze the epidemiological correlation and molecular typing of *Brucella* spp isolated from human and animals using the PCR-RFLP method.

2. Objectives

This study aimed at identifying genetic polymorphisms of *omp2a* genes among 90 *Brucella* spp. isolated from human and animals using the PCR-RFLP method. Also, it evaluated the genetic diversity and relationship among *Brucella* strains using PCR – RFLP and gene sequencing.

3. Methods

3.1. Samples Collection

In total, 90 *Brucella* strains were isolated from different sources of humans and animals (cattle, sheep and goat) in two regions of Iran (Tehran and Hamadan). Sixty *Brucella* spp were isolated from blood cultures collected from patients, who were referred to Sina Hospital of Hamadan with clinical symptoms, including malaise, arthralgia, myalgia, weakness, weight loss, splenomegaly, lymphadenopathy, and serum antibody titers $\geq$ 1/160. Ten milliliters of blood from each patient was transferred to the BACTEC blood culture system (9050 BD Company, U.S.A) and incubated at 37°C for seven day (17). Thirty animal *Brucella* isolates were obtained from the Department of Microbiology of the Faculty of Medicine, Iran University of Medical Sciences, Tehran, Iran. These bacteria were isolated from blood, retropharyngeal, and lymph nodes of animals (cattle, sheep, and goat) that had seropositive tests for brucellosis.

3.2. Bacteriological Identification

Brucella strains were grown on 5% sheep *Brucella*-agar (Merck, Germany) plates and incubated at 37°C in the presence of 5% to 10% CO_2 for 72 hours. Morphology and biochemical reactions including catalase, oxidase, urease hydrolysis tests, CO_2 requirement and H_2S production was considered (7). All isolates were cultured on *Brucella* broth media (Merck, Germany) with 20% glycerol and stored at -80°C in the department of microbiology of Hamadan University of Medical Sciences, Hamadan, Iran.

3.3. Polymerase Chain Reaction Assay

DNA was extracted by a modification of the method described by Queipo-Ortuno (18). The *Brucella* isolates were examined for the presence of *omp2a* gene by PCR amplification (19). Polymerase chain reaction for *omp2a* was performed in the thermal cycler (Bio-Rad, USA). The Primer sequences and condition of PCR is shown in Tables 1 and 2.

Table 1. Polymerase Chain Reaction Mixture

Reaction	Volume, μL	Company
Mastermix 2X	12.5	Pars Tous Co, IRAN
DN A template	2	-
Primer forward 10 pmol	1	Bioneer Co, Korea
Primer reverse 10 pmol	1	Bioneer Co, Korea
DDH_2O	8.5	-
Total volume	25	-

The PCR products were analyzed using the electrophoresis technique on 1.5% agarose gel in 1x Tris-Boric-EDTA (TBE) buffer. Amplified products were stained by syber-safe and visualized under UV transilluminator. *Brucella* abortus S19 strain was used as a positive control and sterile distilled water was used as a negative control. A 1-kb plus ladder (Bioneer Co., Korea) was used as the molecular size marker (20).

Table 2. Primers Sequences, Temperature Program, and Cycling Conditions for *Omp2a* –Polymerase Chain Reaction

Primer Sequences (5′-3′)	Amplified Size, bp	Primary Denaturation	Cycling Conditions (35 cycles)			References
			Secondary Denaturation	Annealing	Extension	
F- GGC-TATTCAAAATTCTG-GCG	1100	95°C/5 min	95°C/ 45 s	53°C/45s (human); 56°C/45s (animal)	72°C/60 s	(10, 19)
R- ATCGATTCT-CACGCTTTCGT						

3.4. Digestion of the Polymerase Chain Reaction Products

Polymerase chain reaction-RFLP was used to differentiate all strains of *Brucella*. The PCR products were digested using Pst I restriction endonuclease enzyme. The *PstI* restriction enzyme was used according to the manufacturer's (Thermo, Fermentase, USA) instructions. Briefly, the digestion mixture included 5 μL of PCR product, 1 μL enzyme buffers, 0.5 μL *PstI* restriction enzyme (10 to 20 U), and sterile distilled water up to 15.5 μL. The microtubes were incubated for four hours at 37°C. The products of RFLP were electrophoresed on 1.5% agarose gel with Syber-safe stain, and gel visualized under UV transilluminator. A 100-bp plus DNA ladder (Bioneer Co., Korea) was used as the molecular size marker (10).

3.5. PCR-RFLP analysis

Analysis of RFLP patterns was conducted using BioNumerics version 7.5 and compared the Dice coefficient and unweighted pair group method using arithmetic averages (UPGMA) groups with 0.5% optimization and a 0.5-% band position tolerance window (21).

4. Results

Ninety *Brucella* isolates, including 60 (66.7%) human strains comprised of *B. melitensis*, and 30 (33.3%) animal strains, including 28 *B. melitensis* and 2 *B. abortus*, were obtained from different sources and investigated. Phenotypic and molecular typing methods were performed to evaluate the species and the biovar of the *Brucella* isolates. The morphology and biochemical tests of the isolates showed that they had small colonies with high transparent and smooth surfaces on *Brucella* agar and gram-negative coccobacilli with Gram stain, were negative for H_2S production, and positive for urease and oxidase without any necessity for the presence of CO_2 and were identified as *B. melitensis*. However, the isolates, which were identified as *B. abortus*, were positive for oxidase, H2S production, urea hydrolysis, and growth in the presence of CO_2.

4.1. Polymerase Chain Reaction Results

After identification of *Brucella* strains by biochemical standard methods, the isolates were confirmed by the PCR technique with obtained bands of 1100 bp for *omp2a* gene (Figure 1).

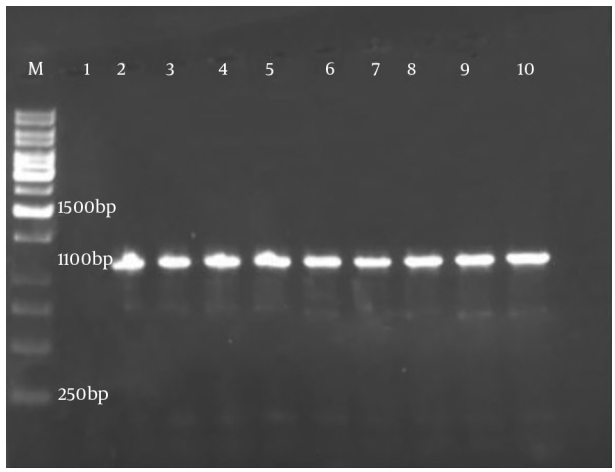

Figure 1. The result of *omp2a* gene amplification in human and animal isolates. Lane M: molecular ladder 1 kb, lane 1: negative control, lane 2 - 5: animal isolates of *Brucella*, lane 6 - 9: H human isolates of *Brucella* and lane 10: positive control (*Brucellaabortus* vaccine strain S19).

4.2. PCR-RFLP

According to RFLP dendrogram, four groups (G1 to G4) and four patterns included P1 (five bands), P2 (four bands), P3 (four bands), and P4 (three bands), based on the host sources, were obtained (Figure 2). The created bands ranged from 60 to 500 bp. The fragments created in each pattern and the size of their bands is shown in Table 3. Pst1 digestion of the PCR products produced four distinct RFLP patterns for animal strains (P1 to P4) and two RFLP patterns (P1 to P2) for human strains (Figure 3).

The *omp2a* gene sequence of isolates were submitted to GenBank and assigned by accession number: 06043563. The PCR-RFLP analysis of *omp2a* gene is displayed in Table 3.

Table 3. PCR-RFLP Patterns of *Brucella omp2a* Gene Digested by Pst1 Enzyme[a]

RFLP Pattern Fragments, bp	P1 (60, 110, 270, 420, 460)		P2 (60, 110, 300, 450)	P3 (110, 280, 400, 500)	P4 (140, 290, 500)	Total
	B. melitensis strain 20236	*B. abortus*	*B. melitensis* strain 20236	*B. melitensis* Biovar1	*B. melitensis* Biovar1	
Human isolates	24 (40)	-	36 (60)	-	-	60
Animals isolates	8 (26.7)	2 (6.6)	11 (36.7)	6 (20)	3 (10)	30
Total	34 (37.8)	47 (52.2)	6 (6.7)	3 (3.3)	90	

[a]Values are expressed as No. (%).

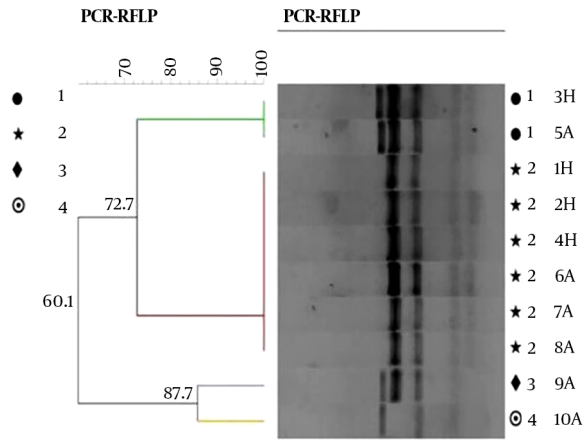

Figure 2. Dendrogram of *omp2a* Gene PCR-RFLP. H: human isolates; A: animal isolates.

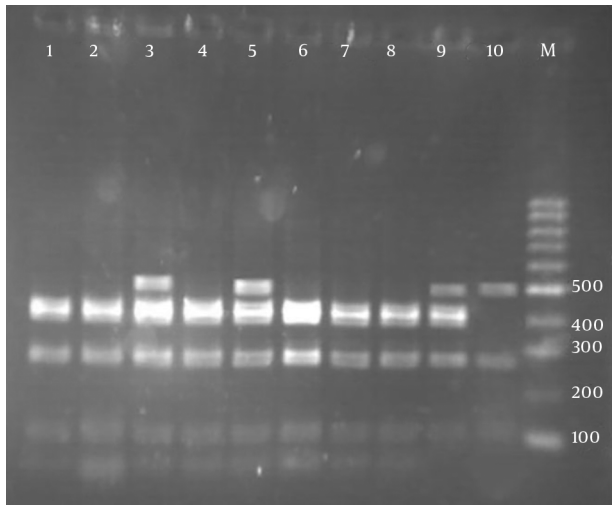

Figure 3. Agarose gel electrophoresis of *pstI* restriction fragments of *omp2a* polymerase chain reaction products. Lanes:1, 2, 4, 6, 7, 8 (fragments size: 60, 110, 300, 450 bp (P2)); lanes 3, 5 (fragments size: 60, 110, 270, 420, 460 bp (P1)); lane: 9 (fragments size: 110, 280, 400, 500 bp (P3)); lane: 10 (fragments size: 140, 290, 500 bp (P4)); lane M: 100 bp molecular weight marker; lanes: 1 - 3 human isolates and lanes: 4 - 10 animal isolates.

By gene sequencing analysis of 60 human strains, 100% of isolates belonged to *B. meltensis* strain 20236 (P1, P2). Out of 30 animal *Brucella* isolates, 19 (63.4%) belonged to *B. meltensis* strain 20236 (P1, P2), nine (30%) belonged to *B. meltensis* biovare1 (P3, P4), and two (6.6 %) *B. abortus* (P1) (Table 3).

The dendrogram groups were defined by a cutoff value of 80% similarity, calculated using the BioNumerics software package (21).

From a total of 90 *Brucella* isolates, 24 (26.6%) of the human strains (*B. melitensis*), 10 (11%) of the animal strains (*B. melitensis* and *B. abortus*), and *B. abortus* vaccine strain (S19) were included in the P1 pattern (Table 3) with whole genetic similarity. Group 2 (P2) contained 36 (40%) of human (*B. melitensis*) and 11 (12.2%) of animal strains (*B. melitensis*) with complete genetic similarity. Group 3 (P3) and group 4 (P4) contained 6 (6.7%) and 3 (3.3%) of animal strains (*B. melitensis*), respectively. Group 2 (52.2%) had the most common patterns in all *Brucella* isolates. The dendrogram results showed a small variation among the isolates.

5. Discussion

Brucellosis is an important bacterial zoonosis, causing health problems in developing countries. *Brucella* are gram negative and facultative intracellular bacteria and are the causative agent of brucellosis that spread in various animal species and humans (22). The current research studied 60 human and 30 animal *Brucella* isolates. After identification of *Brucella* strains by phenotypic and biochemical tests, the isolates were confirmed for the presence of *omp2a* gene by PCR. All strains were *omp2a* gene positive. Recently, the PCR technique has increasingly been performed as a diagnostic and a confirmation method in *Brucella* spp. Identification (23, 24). In this study, detection of polymorphism for all Bruclla isolates by the *omp2a* gene as a target of PCR-RFLP and Pst1 enzyme was carried out.

For detection of polymorphism in *Brucella* spp. *omp2a*

and *omp2b* fragments and several restriction enzymes were used in various studies (10, 25). In the present study, similar to other studies in Iran and different parts ofthe world, amplification of *omp2a* gene from clinical and animal isolates showed different sizes (50 to 500 bp) (1, 26). The study conducted by Unver et al. showed that the sizes of PCR products for clinical isolates of *Brucella* spp. ranged from 77 bp to 1200 bp (26).

In the present study, RFLP analysis of the *omp2a* gene in 90 *Brucella* isolates indicated four distinct RFLP patterns in animal isolates and two RFLP profiles in human isolates (Figure 3). The most common pattern in *Brucella* spp. isolated from human and animals in Hamadan and Tehran was pattern P2. Gene sequencing analysis for *Brucella* strains showed two profiles including, *B. melitensis* strain 20236 with P1 and P2 RFLP patterns in animal and human *Brucella* strains and *B. melitensis* biovar 1 with P3 and P4 RFLP patterns only in animal isolates. These results indicated that PCR-RFLP of *omp2a* gene was not able to accurately discriminate human and animal *B. melitensis* biovars from each other and from *B. abortus*. Also, comparable results were reported by Mirnejad et al. and Pishva et al. (6, 10).

In this study, using gene sequencing, *B. melitensis* strain 20236 was predominant yet in some other studies, *B. melitensis* biovar 1 was the predominant cause of human and animal brucellosis and it was commonly isolated from patients and widely spread in some areas of Iran (1, 6, 27). It has been reported that mixing herds and keeping the animals in shelters is a major risk factor for transmission of the infection (28). Iran's geographical position has always been an important risk factor in the spread of brucellosis, mainly from eastern and western neighbors, such as Afghanistan, Pakistan, and Iraq, and it is possible for new *Brucella* strains to enter the country (29). These events may alter the epidemic genotypes of the disease in Iran and presence of new emerging strains and their source should be continuously considered.

In the current study, according to RFLP dendrogram, group 1 (P1) included *B. melitensis* isolated from humans and animals, and *B. abortus* strains isolated from animals. Group 2 (P2) contained *B. melitensis* isolated from humans and animals. Analysis of obtained patterns indicated low heterogeneity in the omp2 gene of *Brucella* strains, which may be due to common clone and same ancestor of *Brucella* spp. It is also possible that the bacteria had spread from animals to other animals or humans, and isolates were epidemiologically linked and therefore the outbreaks were likely due to the same species clone. Occurrence of these events can be directly due to the situation of brucellosis in animals in relation to the geographical area, and disease mostly involves people, who are in close contact with animals, such as veterinarian, ranchers and those, who are accustomed to eating goat, sheep, and raw milk (29). Therefore, defining the origin of infection in human and animals is also very important.

In this study, according to the results of sequencing, groups 3 (P3) and 4 (P4) isolated from animals belonged to *B. melitensis* biovare1, and another important result indicated complete similarities were not found among the animal isolates in single clones (G3 and G4). Furthermore, the *B. abortus* isolates did not transfer to human through animals, and this is similar to previous reports from Iran (30, 31). The current results showed different patterns of *omp2a* gene in *Brucella* spp. isolated from animals, and polymorphism analysis in *omp2a* gene will be useful in control and prevention of infections caused by *Brucella* strains and must be performed continuously.

5.1. Conclusions

The current findings, like other studies, confirmed that the frequency of *B. melitensis* is higher in human and animal resources and demonstrated that the *B. melitensis* strain 20236 was more predominant. Despite the broad application of PCR-RFLP in determining the polymorphism and understanding the epidemiology of important human pathogens, this technique was unable to differentiate human and animal species of *B. melitensis* from *B. abortus*, yet gene sequencing analysis could discriminate *B. melitensis* biovars.

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Footnotes

Authors' Contribution: Nasrin Bahmani: collection of human *Brucella* strains, performing the experiments, and drafting of the manuscript. Mohammad Yousef Alikhani: study design and supervision of the experiments. Seyed Hamid Hashemi, Mohammad Reza Arabestani and Reza mirnejad: editing the manuscript and critical revision. Faramarz Masjedianjazi: collection of animal *Brucella* strains.

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References

1. Salehi M, Pishva E, Salehi R, Rahmani R. Isolation of Brucella abortus using PCR-RFLP analysis. *Iran J Public Health*. 2006;**35**(4):22–7.
2. Atluri VL, Xavier MN, de Jong MF, den Hartigh AB, Tsolis RM. Interactions of the human pathogenic Brucella species with their hosts. *Annu Rev Microbiol*. 2011;**65**:523–41. doi: 10.1146/annurev-micro-090110-102905. [PubMed: 21939378].
3. Hasanjani Roushan MR, Mohrez M, Smailnejad Gangi SM, Soleimani Amiri MJ, Hajiahmadi M. Epidemiological features and clinical manifestations in 469 adult patients with brucellosis in Babol, Northern Iran. *Epidemiol Infect*. 2004;**132**(6):1109–14. [PubMed: 15635968]. [PubMed Central: PMC2870202].
4. Zeinali M, Shirzadi M, Sharifian J. *National guideline for Brucellosis control*. Tehran: Ministry of Health and Medical Education; 2009. p. 10–7.
5. Diaz Aparicio E. Epidemiology of brucellosis in domestic animals caused by Brucella melitensis, Brucella suis and Brucella abortus. *Rev Sci Tech*. 2013;**32**(1):43–51. 53-60. [PubMed: 23837364].
6. 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. doi: 10.4103/2277-9175.157798. [PubMed: 26261806]. [PubMed Central: PMC4513321].
7. Alton GG, Jones LM, Angus RD, Verger JM. *Techniques for the brucellosis laboratory. 1988*. Paris: Institut National de la Recherche Agronomique; 1988.
8. 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**(4):191–6. doi: 10.1080/10408410500304041. [PubMed: 16417200].
9. Hajia M, Fallah F, Angoti G, Karimi A, Rahbar M, Gachkar L, et al. Comparison of Methods for Diagnosing Brucellosis. *Lab Med*. 2013;**44**(1):29–33. doi: 10.1309/lm4j9mwobipa6rbn.
10. 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). doi: 10.5812/jjm.7197.
11. 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**(1):5–9. doi: 10.1007/s11250-011-9886-1. [PubMed: 21647774].
12. De Massis F, Ancora M, Atzeni M, Rolesu S, Bandino E, Danzetta ML, et al. MLVA as an Epidemiological Tool To Trace Back Brucella melitensis Biovar 1 Re-Emergence in Italy. *Transbound Emerg Dis*. 2015;**62**(5):463–9. doi: 10.1111/tbed.12397. [PubMed: 26194658].
13. Garcia-Yoldi D, Marin CM, Lopez-Goni I. Restriction site polymorphisms in the genes encoding new members of group 3 outer membrane protein family of Brucella spp. *FEMS Microbiol Lett*. 2005;**245**(1):79–84. doi: 10.1016/j.femsle.2005.02.026. [PubMed: 15796983].
14. Pishva E, Salehi M, Gharavi M. Relationship between Brucella immunocomplex and glomerulopathies. *Arch Clin Infect Dis*. 2008;**3**(3).
15. Lukinmaa S, Nakari UM, Eklund M, Siitonen A. Application of molecular genetic methods in diagnostics and epidemiology of food-borne bacterial pathogens. *APMIS*. 2004;**112**(11-12):908–29. doi: 10.1111/j.1600-0463.2004.apm11211-1213.x. [PubMed: 15638843].
16. Berg H. *Restriction Fragment Length Polymorphism Analysis of PCR-Amplified Fragments (PCR-RFLP) and Gel Electrophoresis - Valuable Tool for Genotyping and Genetic Fingerprinting*. InTechopen; 2012. doi: 10.5772/37724.
17. Hashemi SH, Alikhani MY, Naseri Z, Farajnia S, Peeri-Dogaheh H. Diagnosis of human brucellosis by blood culture (BACTEC) and PCR method via whole blood and serum. *Jundishapur J Microbiol*. 2013;**6**(3):248–51.
18. Queipo-Ortuno MI, Morata P, Ocon P, Manchado P, Colmenero JD. Rapid diagnosis of human brucellosis by peripheral-blood PCR assay. *J Clin Microbiol*. 1997;**35**(11):2927–30. [PubMed: 9350761]. [PubMed Central: PMC230089].
19. Bahmani N, Naseri Z, Alikhani MY, Karami M, Kalvandi R. Evaluation of antibacterial effects of Withania coagulans and Cynara cardunculus extracts on clinical isolates of Brucella strains. *Int J Med Res Health Sci*. 2016;**5**(9):90–5.
20. Mullis KB, Faloona FA. Specific synthesis of DNA in vitro via a polymerase-catalyzed chain reaction. *Methods Enzymol*. 1987;**155**:335–50. [PubMed: 3431465].
21. Hunter PR, Gaston MA. Numerical index of the discriminatory ability of typing systems: an application of Simpson's index of diversity. *J Clin Microbiol*. 1988;**26**(11):2465–6. [PubMed: 3069867]. [PubMed Central: PMC266921].
22. Cerekci A, Kilic S, Bayraktar M, Uyanik MH, Yasar E, Esen B. [Comparison of conventional methods and real-time multiplex polymerase chain reaction for identification and typing of Brucella isolates of human origin]. *Mikrobiyol Bul*. 2011;**45**(3):392–400. [PubMed: 21935772].
23. Kaynak-Onurdag F, Okten S, Sen B. Screening Brucella spp. in bovine raw milk by real-time quantitative PCR and conventional methods in a pilot region of vaccination, Edirne, Turkey. *J Dairy Sci*. 2016;**99**(5):3351–7. doi: 10.3168/jds.2015-10637. [PubMed: 26971148].
24. Qasem JA, AlMomin S, Al-Mouqati SA, Kumar V. Characterization and evaluation of an arbitrary primed Polymerase Chain Reaction (PCR) product for the specific detection of Brucella species. *Saudi J Biol Sci*. 2015;**22**(2):220–6. doi: 10.1016/j.sjbs.2014.09.014. [PubMed: 25737656]. [PubMed Central: PMC4336441].
25. Samadi A, Ababneh MM, Giadinis ND, Lafi SQ. Ovine and Caprine Brucellosis (Brucella melitensis) in Aborted Animals in Jordanian Sheep and Goat Flocks. *Vet Med Int*. 2010;**2010**:458695. doi: 10.4061/2010/458695. [PubMed: 21052561]. [PubMed Central: PMC2971571].
26. Unver A, Erdogan HM, Atabay HI, Sahin M, Celebi O. Isolation, identification, and molecular characterization of Brucella melitensis from aborted sheep fetuses in Kars, Turkey. *Revue de medecine veterinaire*. 2006;**157**(1):42.
27. Behroozikhah AM, Bagheri Nejad R, Amiri K, Bahonar AR. Identification at biovar level of Brucella isolates causing abortion in small ruminants of iran. *J Pathog*. 2012;**2012**:357235. doi: 10.1155/2012/357235. [PubMed: 23251805]. [PubMed Central: PMC3515911].
28. Aitken I. *Diseases of sheep*. John Wiley & Sons; 2008.
29. Esmaeili H. Brucellosis in Islamic republic of Iran. *J Med Bacteriol*. 2015;**3**(3-4):47–57.
30. Najafi N, Ghassemian R, Davoody AR, Tayebi A. An unusual complication of a common endemic disease: clinical and laboratory aspects of patients with brucella epididymoorchitis in the north of Iran. *BMC Res Notes*. 2011;**4**:286. doi: 10.1186/1756-0500-4-286. [PubMed: 21834966]. [PubMed Central: PMC3170334].
31. Shareef JM. A Review of Serological Investigations of Brucellosis Among Farm Animals and Humans in Northern Provinces of Iraq (1974?2004). *J Vet Med Series B*. 2006;**53**(s1):38–40. doi: 10.1111/j.1439-0450.2006.01021.x.