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Comparison of PCR-RFLP and PFGE for determining the clonality of *Brucella* isolates from human and livestock specimens

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ABSTRACT

Brucellosis is an important zoonotic disease caused by different species of genus *Brucella* that are pathogenic for humans and a variety of animals. Accurate detection of *Brucella* spp. infection is important for control of disease. The aim of this study was to comparison of molecular genotyping of *Brucella* strains by Pulsed-field gel electrophoresis (PFGE) and polymerase chain reaction -Restriction Fragment Length Polymorphism (PCR-RFLP) techniques.

Twenty- seven *Brucella* spp. were isolated from human and animal samples. The isolates identified by conventional microbiological methods and confirmed using PCR for amplification of *omp2a* gene. Molecular typing of *Brucella* strains carried out by PCR-RFLP after *PstI* and PFGE of chromosomal DNA after *XbaI* enzyme digestion. The *omp2a* gene PCR Products with different patterns of PCR-RFLP were sequenced.

The *omp2a gene* amplification of all human and animal *Brucella* isolates were positive for 1100 bp fragment. By PCR-RFLP analysis two genotypes/patterns for human isolates and four genotypes for animal isolates were obtained. In PFGE analysis totally, 7 common clones/clusters and 3 single clones were obtained.

The results of this study showed the PFGE method is the more reliable and useful assay for molecular typing of *Brucella* strains and is more preferred to PCR-RFLP in determination of genetic similarity among human and animal *Brucella* isolates. The presented data showed PCR-RFLP analysis was not able to differentiate between *B. melitensis* biovars and vaccine strain.

1. Introduction

Brucellosis is one of the most important zoonotic infections in worldwide and caused by a facultative intracellular pathogen which belongs to *Brucella* genus. Brucellosis as a major health problem is usually transmitted by direct contact with infected animals and their products (Bikas et al., 2003). Brucellosis is still a serious problem in many developing countries in Africa, Asia, Southern Europe, Middle East and it is endemic in some parts of Iran. The clinical studies have shown that, the disease is widespread and sometimes creates severe clinical disease with complications (Sofian et al., 2008; Hasanjani et al., 2004). Annually, more than 500,000 cases of human brucellosis, globally are reported to the World Health Organization (Aloufi, et al., 2016). The diagnosis and discrimination between the *Brucella* species and biovars is routinely based on phenotypic and biochemical characteristics which their sensitivity is low and infection risk in laboratory staffs should not be overlooked. Although, the serological tests are suitable to identify the disease but cross-reactions with other bacteria may lead to false positive results.

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Molecular methods for brucellosis detection are faster and more sensitive than traditional methods, but the sensitivity and specificity of PCR tests may be vary among laboratories (Alamian et al., 2017). Therefore, identification and molecular typing of predominant species and biovars of *Brucella* is necessary for consideration of outbreaks, epidemiological studies, and differentiation of the re-infection from human relapses, eradication programs, treatment management for brucellosis and help to find the origin of infections (Benkirane, 2006).

Several Typing techniques such as multiplex PCR, PCR-RFLP, PFGE, MLVA, and Ribotyping have been developed to identify the DNA polymorphism and identification of species and biovars of *Brucella* isolates (Hossain et al., 2013; Georgi et al., 2017). Detection of polymorphisms by PCR-RFLP has several advantages including the rapid and easy application, explanation and use for large numbers of samples (Mirnejad et al., 2013). In the other hand, PFGE has been introduced as the gold standard in bacterial genotyping; survey the whole genome of microorganisms with high resolution, stability, reproducibility and discriminatory power to reveal clonality and identifying strains responsible for disease outbreaks. This technique also identifies the route and possible source of infections (Hossain et al., 2014; Bras and Vet, 2012; Alton et al., 1988).

Several studies confirmed that the *omp2* gene as a locus contains two gene copies (*omp2a* and *omp2b)* with high homology (85%), is useful for identification and molecular typing of *Brucella* at the species, biovar, or strain levels (Whatmore, 2009; Mirnejad et al., 2013; Hanot Mambres et al., 2017).

This study was carried out to investigate the ability of PFGE and PCR-RFLP as molecular typing methods in discriminating of *Bucella* spp. isolated from human and animal samples.

2. Materials and methods

2.1. Bacterial collection and identification

The study was approved by the ethics committee of Hamadan University of medical science, Hamadan, Iran. In this study, 27 *Brucella* strains were collected from human and animals in two different locations in Iran (Hamadan and Tehran provinces). Thirteen human *Brucella* strains were isolated during the period of April 2015 to December 2016 from blood cultures of brucellosis patients who referred to Sina hospital of Hamadan, west of Iran. A total of 13 animal *Brucella* isolates and *B. melitensis* vaccine strain (Rev1) was obtained of Department of Microbiology, Iran University of Medical Sciences, Tehran, Iran. These strains were identified by phenotypic and biochemical standard methods. Additionally the agglutination with mono-specific A and M antisera was performed (Alton et al., 1988).

2.2. DNA extraction and PCR assay

DNA extraction was carried out according to a previous study with some modifications (Queipo-ortuno et al., 1997). Briefly, 0.5 ml of distilled water with 1 loop of bacteria were mixed and centrifuged at 13,000 g for 1 min and the supernatant was removed. Then, 100 μl of 50 mM NAOH was added and put in the water bath at 95 °C for 30 min. Finally 50 μl of 20 mM Tris buffer was added and centrifuged and the DNA obtained was used for PCR.

The *omp2a* gene was detected by PCR using Forward-5′-GGCT ATTCAAAATTCTGGCG-3′ and Riverse-5′-ATCGATTCTCACGCTTTCG T-3′ specific primer pairs (Bahmani et al., 2016). The PCR amplification procedure was performed with 25 μl of master mix containing 0.2 μl of Taq polymerase 5 U/μl, 2.5 μl of 10X PCR buffer along with MgCl2, 1 μl of 10 pM from each primers, 2.5 μl of dNTPs MIX (2 Mm), 3 μl of DNA template, 14.8 μl of DNase and RNase-Free Distilled Water. The oligonucleotides and all reagents for PCRs were synthesized and purchased from Bioneer Co (South Korea). Agarose gel electrophoresis of the PCR product with 100 bp size marker (Fermentas, Korea) was carried out in a 2% agarose gel and stained with syber safe and visualized under UV transilluminator. (Al Dahouk et al., 2005). The *B. melitensis* (biovar 1, ATCC 23456) and distilled water were used as positive and negative controls, respectively.

2.3. PCR-RFLP

The *omp2a* gene amplification products were digested with 10–20 U *pstI* restriction enzyme (Thermo Fisher Scientific, Waltham, MA) according to the manufacturer's procedure and incubated at 37 °C for 4 h. The restricted fragments were separated by 1% agarose gel electrophoresis. The PCR-RFLP patterns were recorded using gel documentation.

2.4. PFGE analysis

Genomic DNA of *Brucella* strains was prepared in agarose plugs and digested by *XbaI* enzyme (Thermo Fisher Scientific, Waltham, MA) as described previously with minor modifications (Ridler et al., 2005). The plugs were placed in lysis buffer (50 mMTris–HCl [pH 8.0], 50 mM EDTA [pH 8.0], 1% Sodium lauroyl sarcosine, 1% SDS and 0.1 mg/ml of proteinase K) and incubated overnight at 55 °C. The plugs were digested with 30 U of *XbaI* overnight at 37 °C and after washing, completely digested genomic DNA were embedded into pre-formed wells of 1% agarose gel electrophoretic. Separation of the DNA genome was done with Rottaphor Biometra electric fields (version 6, Germany) using TBE buffer (45 mM Tris–HCl, 45 mM boric acid, 1.0 mM EDTA, pH 8.0) at 14 °C for 22 h at 120 v and 5 s and 35 s. Lambda Ladder PFGE Marker (NEB: N0340) was used as the molecular reference marker. Dendrograms for PFGE and PCR-RFLP were constructed according to obtained DNA fragment genotypes using BioNumerics total lab software version7.5 (Applied Maths, StMartens-Latem, and Belgium).

The analysis of similarity was calculated using the unweighted pair group average (UPGMA) and the Dice coefficient for cluster analyses. The criteria for related clones or classifications as similar types were considered when 80% or more of the patterns were similar (Tenover et al., 1995).

3. Results

Analysis of 27 *Brucella* isolates by microbiological standard methods, agglutination with monospecific anti-M sera, and *omp2a* gene – PCR with 1100 bp amplification product (Fig. 1) showed that all isolates were identified as *B. melitensis*.

3.1. PCR-RFLP

The PCR-RFLP analysis by agarose gel electrophoresis revealed the presence of 4 patterns/genotypes (P1-P4) for animal isolates and two patterns for human isolates (P1–P2). The P1 and P2 patterns were common in animal and human strains. The Bands of molecular sizes ranged from 60 to 500 bp in human and animal *Brucella* strains (Fig. 2).

The PCR Products with different patterns were ordering for sequencing by Bioneer Co (South Korea) and the *omp2a* gene sequence of the *Brucella* isolates was submitted to Gen Bank and assigned under accession No: 06043563. Gene sequencing and BLAST analysis of *omp2a* gene revealed, among 13 human *B. melitensis* isolates 61.5% pattern 2 (P2) and 38.5% pattern1 (P1)

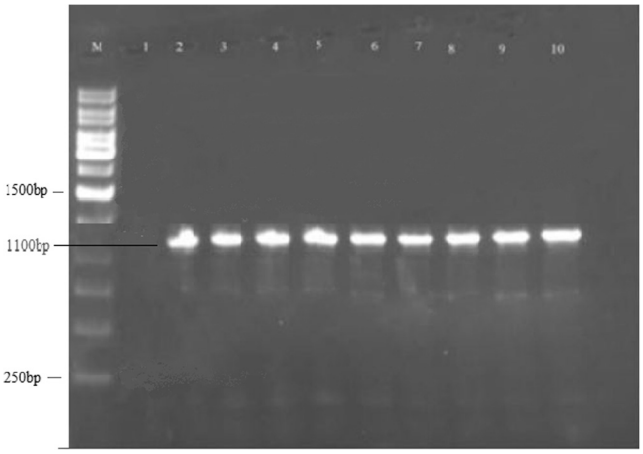

Fig. 1. The result of *omp2a* gene amplification in human and animal *Brucella* isolates. Lane M: 1 kb size ladder, lane1: negative control (distilled water), lane 2–5: positive for human *Brucella* isolates, lane 6–9: positive for animal *Brucella* isolates, lane 10: positive control (*B. melitensis* biovar1).

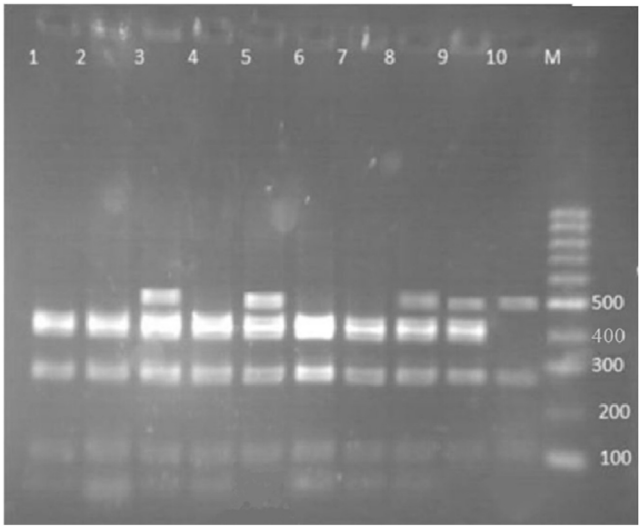

Fig. 2. Agarose gel electrophoresis of *PstI* restriction fragments of *omp2a*-PCR products. Lane 1, 2: human strains (Pattern 2), Lane3: human strain(Pattern 1), lane 4,6,7 animal strains (Pattern 2), lane 5: Positive control (*B. melitensis* biovare1 with Pattern 3), lane 8 animal strain (Pattern1), lane 9 animal strain (Pattern 3), lane 10 animal strain (Pattern 4). Lane M: 100 bp molecular weight marker.

belong to *B. meltensis* strain 20236. From 13 animal *B. melitensis* strains, 30.8% pattern 1(P1) and 38.5% pattern 2 (P2) belong to *B. meltensis* strain 20236 and 23% pattern 3 (P3) and 7.7% pattern 4 (P4) belong to *B. melitensis* biovar 1. The *B. meltensis* vaccine strain (Rev1) calcified in pattern 1. The P2 was the most common pattern among human and animal isolates. In the PCR-RFLP patterns ten *Brucella* strains were selected among 27 animal and human isolates for the instruction of dendrogram. The dendrogram showed 4 groups (G1-G4) and 4 patterns (P1-P4) (Fig. 3). According to the dendrogram, similarities among members of group 1 and 2 were 100%. The PCR-RFLP analysis was summarized in Table 1.

3.2. PFGE

The PFGE analysis was used for typing and genetic relationship between the *B. melitensis* strains isolated from human and animal samples (Fig. 4, Table 2). In this survey, PFGE analysis of 27 *B. melitensis* isolates (13 animal isolates, 13 human isolates and a vaccine strain; *B. melitensis* Rev1) revealed 7 common clones/clusters (I, II, III, IV, V, VI and VII) and 3 single clones (8a, 9a, 10a genotypes). Cluster II with 8 members (29.7%) was predominant. In this cluster, 5 isolates obtained from patients and 3 members isolated from sheep. Also, four isolates had full similarity in their genotypes (2d genotype) which, one of them belongs to animal strain (58A) and the others were human strains (Fig. 4). Likewise, cluster VII included one animal strain (57A) and 2 human strains (50H and 56H) with complete similarity. In cluster 1, there were 2 strains including one animal strain (101A) originated from sheep and another was *B. melitensis* vaccine strain Rev1. Cluster III included 3 animal strains which of them, two strains with complete similarity originated from sheep and another originated from gout. The cluster IV (4a genotype) and V (5a genotype) each have two sheep isolates with full similarity and cluster VI included four human strains with 2 genotypes (6a and 6b). Among the 27 *Brucella* strains, 21(80%) isolates have a similarity of 80–90% (Fig. 4).

4. Discussion

Brucella spp. causes the brucellosis in humans and animals all over the world, especially in countries that uncontrolled animal brucellosis. The results of previous studies indicated that the mean annual incidence of human brucellosis in Iran since 2001–2009 was 29.83/100,000 population and according to the Iranian Ministry of Health and Medical Education in 2008 and 2009, the incidence rate of brucellosis was about 66–100 and 31–41 per 100,000 population, respectively in the province of Hamadan, west of Iran (Rostami et al., 2015; Esmaeili, 2014; Mirnejad et al., 2017).

The typing of microbial pathogens or identifying bacteria at the strain level is important for diagnosis, treatment, and epidemiological surveillance of bacterial infections (Benkirane, 2006). This study is the first report of molecular typing of *B. melitensis* strains isolated from human and animals, simultaneously by PFGE and PCR-RFLP from Iran.

Twenty-seven *B. melitensis* were isolated from patient and animal samples in two provinces of Iran; Hamadan and Tehran. The isolates were successfully identified by the conventional biochemical tests and confirmed by PCR method for the presence of *omp2a* gene. Molecular typing of the isolates were performed by PCR-RFLP and PFGE methods and the results were compared.

In the present study by PFGE analysis following digestion of total genomic DNA with the *XbaI* restriction endonuclease, the numbers of genotypes generated (16 genotypes in human and animal strains) were more than PCR-RFLP method (4 genotypes in human and animal isolates) using the *pst1* restriction enzyme (Figs. 4 and 2). The difference in discrimination results of these methods can be due to high discriminatory potential of PFGE and separation power of enzymes used, therefore, PFGE can show more genetic relatedness and diversity in human and animal isolates of *Brucella* strains. Increasing of different patterns may help to find a new source of infection. According to the previous studies, using the full genome compared to genomic fragments can be much more useful for typing of *Brucella* isolates (Jensen et al., 1999). In a number of studies, researchers have performed PFGE analysis of *Brucella* species and have indicated that this technique is useful for differentiating between species of *Brucella* (Jensen et al., 1999; Allardet-Servent et al., 1988). The results of a current study indicated that genotyping of human isolates of *B. melitensis* by PFGE and PCR-RFLP analysis had higher similarity than animal strains (Figs. 3 and 4). Full or close genetic similarity among these strains might be due to common sources of infection or bacterial common clones. The more diversity in the genotypes of animal isolates of *B. melitensis* can be resulted from polymorphism, point mutation, deletion or insertion in *omp2a* gene.

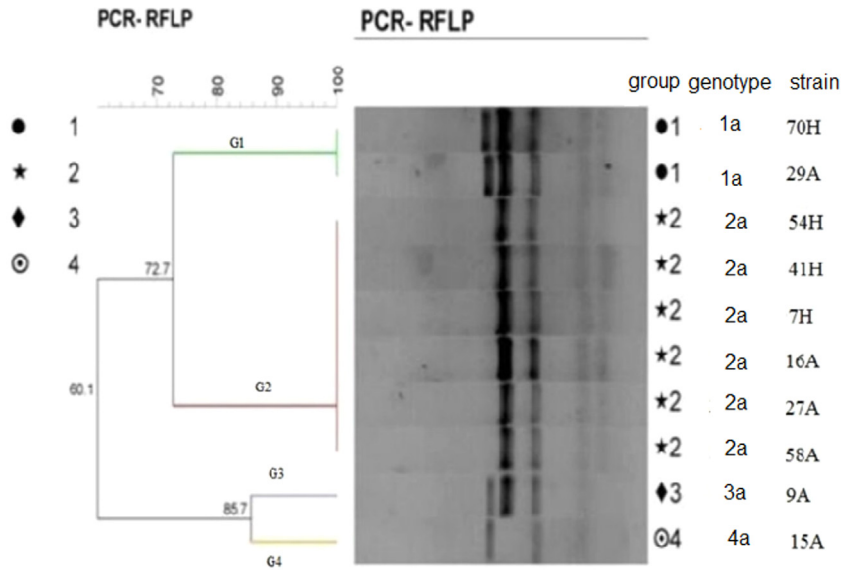

Fig. 3. PCR-RFLP dendrogram following *pst1* digested of *omp2a* gene amplification product. H: human, A: animal.

Table 1
Characterization of *B. melitensis* by PCR-RFLP analysis and *PstI* restriction enzyme.

Strain number	Host	ID	Species	Group	Patterns				Strain	Biovar
					P1	P2	P3	P4		
1	Sheep	30	*B. melitensis*	1	+	–	–	–	20236	
2	Sheep	36	*B. melitensis*	1	+	–	–	–	20236	
3	Sheep	21	*B. melitensis*	1	–	+	–	–	20236	
4	Sheep	29	*B. melitensis*	1	–	+	–	–	20236	
5	Human	70	*B. melitensis*	1	–	+	–	–	20236	
6	Human	53	*B. melitensis*	1	+	–	–	–	20236	
7	Human	55	*B. melitensis*	1	+	–	–	–	20236	
8	Human	51	*B. melitensis*	1	+	–	–	–	20236	
9	Human	52	*B. melitensis*	1	+	–	–	–	20236	
10	Human	41	*B. melitensis*	2	–	+	–	–	20236	
11	Human	54	*B. melitensis*	2	–	+	–	–	20236	
12	Human	56	*B. melitensis*	2	+	–	–	–	20236	
13	Human	50	*B. melitensis*	2	–	+	–	–	20236	
14	Human	17	*B. melitensis*	2	–	+	–	–	20236	
15	Human	22	*B. melitensis*	2	–	+	–	–	20236	
16	Human	7	*B. melitensis*	2	–	+	–	–	20236	
17	Human	49	*B. melitensis*	2	–	+	–	–	20236	
18	Sheep	16	*B. melitensis*	2	–	+	–	–	20236	
19	Sheep	27	*B. melitensis*	2	–	+	–	–	20236	
20	Sheep	58	*B. melitensis*	2	–	+	–	–	20236	
21	Sheep	57	*B. melitensis*	2	–	+	–	–	20236	
22	Sheep	18	*B. melitensis*	3	–	–	+	–		1
23	Sheep	20	*B. melitensis*	3	–	–	+	–		1
24	Sheep	15	*B. melitensis*	3	–	–	+	–		1
25	sheep	101	*B. melitensis*	1	+	–	–	–		
26	Gout	9	*B. melitensis*	4	–	–	–	+		1
27	vaccine		*B. melitensis* Rev1	1	+	–	+	–		

–: negative; +: positive.

In current study in PFGE analysis cluster I had two members including one *B. melitensis* isolated from sheep (101A strain) and the other was *B. melitensis* vaccine strain Rev1 with close patterns similarities (84.6%). It shows the origin of this animal isolate may be due to the vaccine strain or may be a genetic variant of the vaccine strain. *B. melitensis* Rev1 is the vaccine used for the prevention of brucellosis in goats and sheep in endemic regions. There are some reports have shown that the strains used in animal vaccinations may be the source of human and animal brucellosis (Refai, 2002; Motaharinia et al., 2013; Bardenstein et al., 2002). Following the horizontal infection among sheep in South Africa human infection with the vaccine strain Rev.1 was reported. (Pieterson and Pefanis, 1988). In a study reported after *Brucella* vaccination in cattle and sheep with close contact with each other, the Rev-1 vaccine strain was isolated from 2 aborted fetuses (Pishva et al., 2008).

In the present study, PFGE analysis showed two animals isolates (58a and 57a) with complete genotype similarities (100%) with human isolates in cluster II and VII. This indicated these isolates were transmitted and circulating between animals and human in two geographical areas close to each other. Some of the studies reported the incidence of brucellosis in humans is directly associated with the brucellosis of animals in a particular geographic area (Nagalingam et al., 2012).

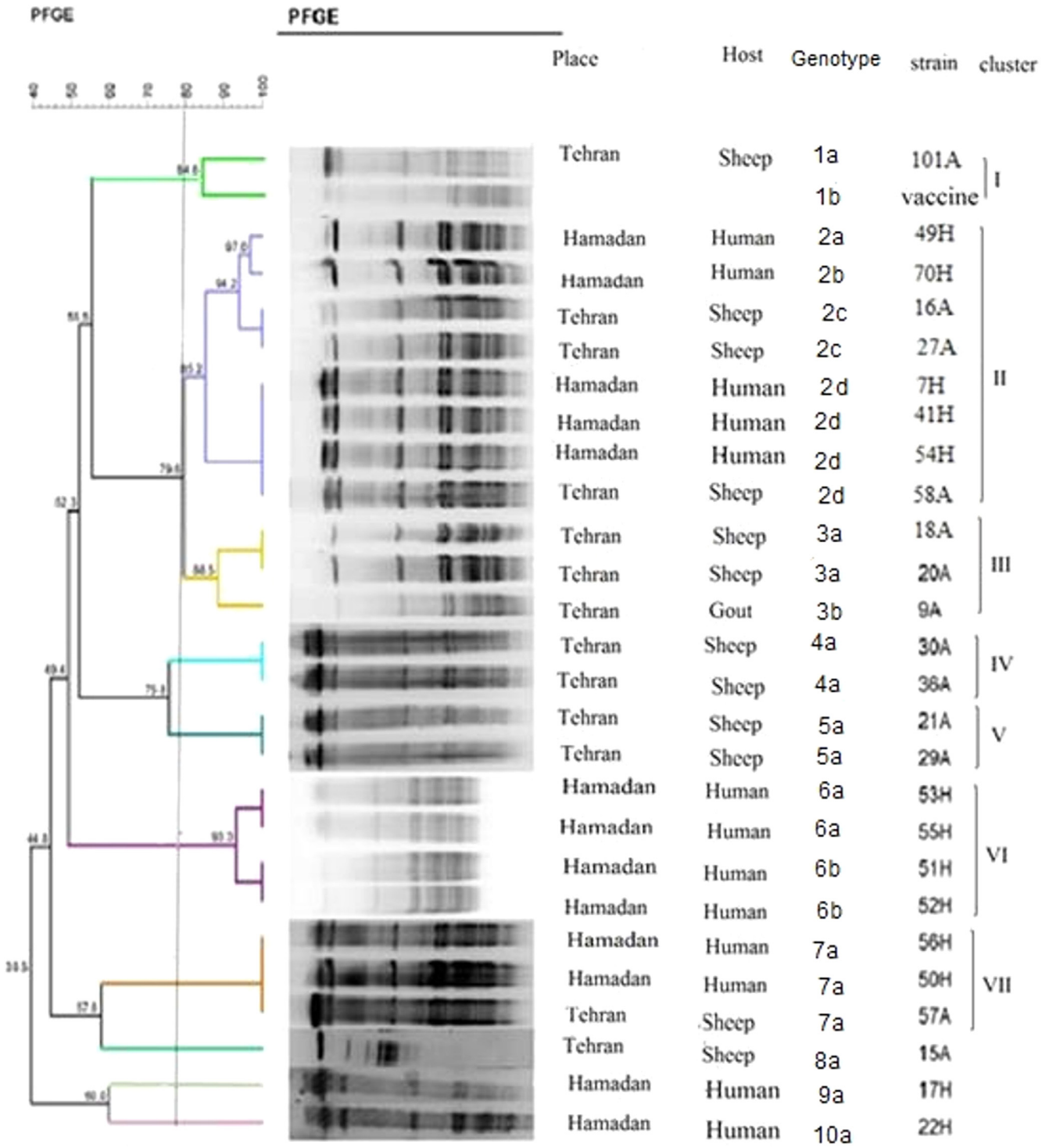

Fig. 4. PFGE dendrogram of human and animal *Brucella* isolates. A: animal; H: human.

In this survey, all human and animal *Brucella* isolates were identified by standard microbiological tests as *B. melitensis* biovar1. The PCR-RFLP showed 4 patterns (P1-P4) for animals and 2 patterns (P1 and P2) for human isolates, while gene sequencing analysis for *Brucella* strains showed two profiles including, *B. melitensis* strain 20236 (P1 and P2) and *B. melitensis* biovar 1 (P3 and P4) only in animal strains. On the other hand, according to dendrogram in PFGE analysis (Fig. 4) cluster II was predominant with 8 strains and 4 genotypes but in PCR-RFLP, these isolates are located in a single clone with one genotype (Fig. 3 and Table 2). These results indicated PCR-RFLP unable to separate human and animal *B. melitensis* biovars from each other and from vaccine strain. The previously studies showed PCR-RFLP was not able to differentiate *B. melitensis* biovars in animal and human samples (Pishva et al., 2008; Mirnejad et al., 2013). The some reports showed that predominant genotypes of *Brucella* strains have a greater capacity for transmission and this confirms the dominant infectious nature (Ridler et al., 2005).

According to gene sequencing analysis the *B. melitensis* 20236 strains was predominant (69.3%) in human and animal isolates.

Table 2
Characterization and distribution of *B. melitensis*in PFGE analysis.

Key	Cluster	Members	Animal	Human
1	I	2(7.4%)	2[a]	
2	II	8(29.7%)	3	5
3	III	3(11. 1%)	3	
4	IV	2(7.4%)	2	
5	V	2(7.4%)	2	
6	VI	4(14.8%)		4
7	VII	3(11.1%)	1	2
8	single clone	3(11.1%)	1	2

[a] One animal strain and one vaccine strain.

These results were different from the reports of other studies in Iran. The results reported from Iran showed the patterns of PCR-RFLP for all of the isolates were similar to pattern of the *B. melitensis* biovar 1 (Pishva et al., 2008; Salehi et al., 2006). These differences indicated the change of epidemic patterns of the *Brucella* spp. in Iran. Thus the presence of new emerging strains and their source should be continually considered. Our study showed other important result in the PFGE analysis that single clones (8a, 9a, 10a) were not similar to each other and with other human and animal isolates and had specific patterns.

In this study, PCR-RFLP and PFGE showed genetic relatedness and diversity among isolates, but they were not equivalent. We found the PFGE analysis had a more powerful and stronger distinction among *Brucella* strains than PCR-RFLP (Fig. 4). Many studies reported PFGE is a tool with high powerful separation and has been demonstrated capable to discriminate within and between species of microorganisms (Rivoal et al., 2005). The genetic patterns produced in this study showed that *B. melitensis* 20236 strain is circulating between animals and humans in two provinces. Therefore the use of molecular typing methods for identification of animal and human *Brucella* isolates will be helpful in managing of different infections caused by *Brucella* strains based upon their genotypes.

5. Conclusion

The results of this study showed the PFGE method is the more reliable and useful assay for molecular typing of *Brucella* strains and is more preferred to PCR-RFLP in determination of genetic similarity among human and animals Brucella isolates. The presented data showed PCR-RFLP analysis was not able to differentiate between *B. melitensis* biovars and vaccine strain, and it is better to use as a supplementary molecular typing method along with PFGE.

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Ethical approval:

This study was approved by the ethics committee of the Hamadan University of Medical Science, Hamadan, Iran.

Conflict of interest

This study no Conflict Interest.

References

Al Dahouk, S., Tomasom, H., Prenger-Berninghoff, E., 2005.Identification of Brucella species and biotypes using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP). APMIS.112, 908–929.

Alamian, S., Esmaelizad, M., Zahraei, T., Etemadi, A., Mohammadi, M., Afshar, D., Ghaderi, S., 2017. A Novel PCR Assay for Detecting *Brucella abortus* and *Brucella melitensis*. Osong. Public. Health. Res. Perspect. 8 (1), 65–70.

Allardet-Servent, A., Bourg, G., Ramuz, M., Pages, M., Bellis, M., Roizes, G., 1988. DNA polymorphism in strains of the genus *Brucella*. J. Bacteriol. 170, 4603–4607.

Alton, G.G., Jones, L.M., Angus, R.D., Verger, J.M., 1988. Techniques for the Brucellosis Laboratory. Institut National de la Recherche Agronomique, Paris.

Bahmani, N., Naseri, Z., Alikhani, M.Y., Karami, M., Kalvandi, R., 2016. Evaluation of antibacterial effects of Withania coagulans and Cynara cardunculus extracts on clinical isolates of *Brucella*strains. Int. J. Med. Res. Health. Sci. 5 (9), 90–95.

Bardenstein, S., Mandelboim, M., Ficht, T.A., Miriam baum, M., Banai, M., 2002. Identification of the *Brucella melitensis* vaccine strain Rev. 1 in animals and humans in Israel by PCR analysis of the PstI site polymorphism of its *omp*2 gene. J. Clin. Microbiol. 40, 1475–1480.

Benkirane, A., 2006. Ovine and caprine brucellosis: world distribution and control/eradication strategies in West Asia/North Africa region. Small. Rumin. Res. 62, 19–25.

Bikas, C., Jelastopulu, E., Leotsinidis, M., Kondakis, X., 2003. Epidemiology of human brucellosis in a rural area of north-western Peloponnese in Greece. Eur. J. Epidemiol. 18, 267–274.

Bras, R., Vet, Ci, 2012. Laboratorial diagnosis of animal brucellosis. Bioterrorism. Biodefense. 19 (3), 117.

Esmaeili, H., 2014. Brucellosis in Islamic republic of Iran. J. Med. Bacteriol. 3(3, 4), 47-57.

Georgi, E., Walter, M.C., Pfalzgraf, M.-T., Northoff, B.H., Holdt, L.M., Scholz, H.C., 2017. Whole genome sequencing of *Brucella melitensis* isolated from 57 patients in Germany reveals high diversity in strains from Middle East. PLoS ONE. 12 (4), e0175425.

Hanot Mambres, D., Boarbi, S., Michel, P., Bouker, N., Escobar-Calle, L., Desqueper, D., 2017. Imported human brucellosis in Belgium: bio and molecular typing of bacterial isolates, 1996–2015. PLoS ONE 12 (4), e0174756.

Hasanjani, M.R., Mohrez, M., Smailnejad, S.M., Soleimani, M.J., Hajiahmadi, M., 2004. Epidemiological features and clinical manifestations in 469 adult patients with brucellosis in Babol, northern Iran. Epidemiol. Infect. 132, 1109–1114.

Hossain, M.T., Kim, Y.O., Kong, I.S., 2013. Multiplex PCR for the detection and differentiation of *Vibrio parahaemolyticus* strains using the *groEL*, *tdh* and *trh* genes. Mol. Cell. Probes. 27 (5–6), 171–175.

Hossain, M.T., Kim, Y.R., Kong, I.S., 2014. PCR-restriction fragment length polymorphism analysis using groEL gene to differentiate pathogenic Vibrio species. Diagn. Microbiol. Infect. Dis. 78, 9–11.

Jensen, A.E., Cheville, N.F., Thoen, C.O., MacMillan, A.P., Miller, W.G., 1999. Genomic fingerprinting and development of a dendrogram for *Brucella* spp. isolated from seals, porpoises and dolphins. J. Vet. Diagn. Invest. 11, 152–157.

Mirnejad, R., Jazi, F.M., Mostafaei, S., Sedighi, M., 2017. Epidemiology of brucellosis in Iran: a comprehensive systematic review and meta-analysis study. Microb. Pathog. 109, 239–247.

Mirnejad, R., Majdi, A., Taghizoghi, N., Piranfar, V., 2013. Molecular Typing of Brucella melitensis and B. abortus From Human Blood samples Using PCR-RFLP Method. Jundishapur. J. Microbiol. 6 (6), 71–77.

Motaharinia, Y., Rezaee, M.A., Rashidi. A., Jalil, A., Rezaie, M.J., Shapouri, R., Hossieni, W., Rahmani, M.R., 2013. Induction of protective immunity against brucellosis in mice by vaccination witha combination of naloxone, alum, and heat-killed Brucella melitensis 16 M. J. Microbiol. Immunol. Infect.46, 253e258.

Nagalingam, M., Shome, R., Balamurugan, V., Shome, B.R., NarayanaRao, K., Isloor, S., 2012. Molecular typing of Brucella species isolates from livestock and human. Trop. animal. Health. produc. 44 (1), 5–9.

Aloufi, A.D., Memish, Z.A., Assiri, A.M., McNabb, S.J., 2016. Trends of reported human cases of brucellosis, Kingdom of Saudi Arabia, 2004–2012. J. Epidemiol. Glob. Health. 6 (1), 11–18.

Pieterson, P M., Pefanis, S.,1988. The characteristics of a variant strain of Brucella melitensis. Rev 1.j. vete. res. 55(1),15-17.

Pishva, E., Salehi, M., Gharavi, M., 2008. Relationship between *Brucella* immunocomplex and glomerulopathies. J. Clin. Infect. Dis. 5, 127–132.

Queipo-ortuno, M.I., Morata, P., Ocon, P., Manchado, P., Colmenero, J.D., 1997. Rapid diagnosis of human brucellosis by peripheral-blood PCR. J. Clin. Microbiol. 35 (11), 2927–2930.

Refai, M., 2002. Incidence and control of brucellosis in the Near East region. Vet. Microbiol. 90 (1), 81–110.

Ridler, A.L., Leyland, M.J., Fenwick, S.G., West, D.M., 2005. Demonstration of polymorphism among *Brucella ovis* field isolates by pulsed-field gel electrophoresis. Vet. Microbiol. 108, 69–74.

Rivoal, K., Ragimbeau, C., Salvat, G., Colin, P., Ermel, G., 2005. Genomic diversity of *Campylobacter coli* and *Campylobacter jejuni* isolates recovered from free-range broiler farms and comparison with isolates of various origins. Appl. Environ. Microbiol. 71, 6216–6227.

Rostami, H., Tavana, A.M., Tavakoli, H.R., Tutunchian, M., 2015. Prevalence study of brucellosis in Iranian military forces during 2001–2009. J. Health. Polic. Sust. Health. 2, 1–5.

Salehi, M., Salehi, R., Rahmani, M., 2006. Isolation of *Brucella abortus* Using PCR-RFLP Analysis. Iranian. J. Publ. Health. 35, 22–27.

Sofian, M., Aghakhan, i.A., Velayati, A.A., Banifazl, M., Eslamifa, r A., Ramezani, A., 2008. Risk factors for human brucellosis in Iran: a case-control study. Int. J. Infect. Dis. 12, 157–161.

Tenover, F.C., Arbeit, R.D., Goering, R.V., Mickelsen, P.A., Murray, B.E., Persing, D.H., Swaminathan, B., 1995. Interpreting chromosomal DNA restriction patterns produced by pulsed-field gel electrophoresis: criteria for bacterial strain typing. J. Clin. Microbiol. 33, 2233.

Whatmore, A.M., 2009. Current understanding of the genetic diversity of *Brucella*, an expanding genus of zoonotic pathogens. Infect. Genet. Evol. 9 (6), 1168–1184.