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Molecular Typing of *Brucella melitensis* Isolated from Patients and Animals by Pulsed Field Gel Electrophoresis from Iran

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Authors' contributions

This work was carried out in collaboration between all authors. Authors MYA, MRA and RM designed the study, managed the literature search and wrote up the work. Author NB wrote the first draft of the manuscript. Authors AF, PM and MK managed the data. All authors read and approved the final manuscript.

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ABSTRACT

Background: The Brucellosis is recognized as a significant public health problem, with major economic and financial burdens in countries where the disease remains endemic. The disease is one of the most important worldwide zoonosis affecting livestock and humans. The aim of the present study was to genotypically characterize *Brucella* strains isolated from human and animal samples in two provinces of Iran.
Methods: Twenty seven *Brucella* strains isolated from patients and animals during April 2015 -

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December 2016. Thirteen human and fourteen animal strains were identified by biochemical tests and confirmed by amplification of fragment 1100bp *omp2a* gene using Polymerase Chain Reaction (PCR) method. Isolates were genotypically characterized by *XbaI* digestion and pulsed-field gel electrophoresis (PFGE) technique.

Results: In PFGE analysis totally, 7 common clones and 3 single clones were obtained. In genotyping, in two clones/clusters, human isolates were grouped with genotypes from animal isolates. Also PFGE results indicated 21 *Brucella* strains an overall similarity higher than 90%, and three clusters based on 100% similarity were revealed.

Conclusion: According to dendrogram clinical strains had a high degree of homology compared to animal strains. The information of this study indicating a close genetic relatedness or common origin of the isolates in the two geographical region of Iran and it implies that *B. melitensis* cross-infected between human and livestock.

Keywords: Brucella melitensis; molecular typing; PFGE.

1. INTRODUCTION

Brucellosis is a major public health and economic problem in many parts of the world and remains endemic in developing and some developed countries such as: Southeast Asia, Latin America, Southern Europe, Africa, and the Middle East including Iran [1,2]. Annually, more than 500000 cases of human brucellosis are reported globally to the World Health Organization [3]. Trends of human brucellosis in Iran was studied between 1991 till 2008 and reported prevalence rates vary in different parts of Iran. The results of previous studies indicated that the prevalence rate of the brucellosis in Iran since 2001 to 2009 was 24.56% and in different provinces has been reported from 0.5% to 10.9% [2]. According to the report of the Ministry of Health and Medical Education in 2009, the incidence of human brucellosis was about 31-41 per 100 000 populations in the province of Hamadan, west of Iran [4]. The prevalence of brucellosis among goats /sheep was 10.2% and among cows was 17.5% in the 1990s [5].

In the most of studies in Iran reported *Brucella melitensis* is the predominant species of human and animal brucellosis [6,7,8]. Information about the existence of *Brucella* in a region is essential for diagnosis and epidemiological of diseases and helps to plan the preventive programs and treatment strategies [9]. Although the species of *Brucella* can be discriminated by conventional phenotypic tests based on lipopolysaccharide antigens, phage typing, dye sensitivities, CO2 requirements, and H2S production, but their sensitivity is low and they consume a lot of time (requiring 1-4 days) [10]. Several genes have been used for DNA replication, however, the *omp2* locus (*omp2a and omp2b*) coding for porin proteins and closely linked in the *Brucella* genome as potential targets for molecular typing, and identification of *Brucella* at the species, biovar or strain level [11]. Recently for molecular epidemiological studies, various molecular methods developed based on genetic diversity and specific identification of DNA sequences of *Brucella* associated with the genus, species and biovars [12,13].

Among molecular methods, Pulse-field gel electrophoresis (PFGE) is a powerful technique which has been introduced for molecular typing of microorganisms [14]. PFGE is considered as a gold standard in molecular typing for most of bacteria and the possibility genetic diversity, genetic distance between strains, finding the sources of contamination and distribution of organisms and epidemiological studies has clearly shown among *Brucella* species [15]. This study evaluated the molecular typing of *Brucella* strains and investigation of relationship among *Brucella* genotypes isolated from human and livestock by PFGE technique in our region, Hamadan and Tehran cities of Iran.

2. MATERIALS AND METHODS

2.1 Bacterial Isolates

Twenty seven *Brucella* strains isolated from humans and animals in two different regions of Iran were used in this study for PFGE typing. Thirteen human isolates were isolated from blood cultures of brucellosis patients who referred to Sina hospital of Hamadan, west of Iran in April 2015 to December 2016. Patients had symptoms such as chills, fever, night sweats, anorexia, and positive serologic Wright test with ≥1/160 titer in their serums .The blood samples were cultured in the BACTEC blood culture system (9050 BD

Company, U.S.A.) and incubated at 37°C for 7 days [16,17,18].

A total of 14 animals isolates were obtained from the Department of Microbiology, Iran University of Medical Sciences, Tehran, Iran. These strains isolated from blood, retropharyngeal and lymph nodes of different animals (sheep and goat) with seropositive tests for brucellosis. Identification of total *Brucella* isolates performed by microbial standard methods including Gram stain, the production of H2S, the CO2-dependence, urea hydrolysis , oxidase, catalase, methyl red , indole tests and sensitivity to thionin, basic fuchsin dyes, additionally the agglutination with mono-specific A and M antisera was performed [19] .

All isolates were kept frozen at −80°C in *Brucella* broth (Becton Dickinson, Sparks, MD, USA) with 20% glycerol in Department of Microbiology, Hamadan University of Medical Science, Hamadan, Iran.

2.2 PCR Assay

For confirmation of *Brucella* isolates, *omp2a* gene amplification was performed. For extraction of DNA, several bacterial colonies were mixed with 0.5 ml of distilled water and centrifuged at 12000×g for 5 min. The supernatant was discarded and 100 µl of NaOH 50mM were added to the tubes and were placed at 95°C for 30 min. Finally, tubes centrifuged at 12000 ×g for 5 min. The supernatant used for PCR as template DNA [20]. The PCR was performed using specific primers as forward: F: 5'-GGCTATTCAAAATTCTGGCG-3' and reverse R: 5'-ATCGATTCTCACGCTTTCGT-3' for *omp2a* gene in PCR Thermal Cycler (Bio-Rad, USA) [21].

The PCR was carried out in 25 µL reactions contained 12.5 µL Master Mix (Pars Tous Co, IRAN), 8.5 µL distilled water, 1 µL (10 pmol) each primer (Bioneer Co, Korea), and 2 µL of template DNA. The cycling conditions were set as follows: (1) 95°C for 5 min; (2) 94°C for 45 sec; (3) 52°C for 45 sec; (4) 72°C for 1 min; and (5) 72°C for 7 min. Steps 2 through 4 were repeated for 35 cycles. Ten micro liters of PCR product was subjected to horizontal electrophoresis in a 1% agarose gel in 1×Tris-boric-EDTA (TBE) buffer. A 1 kb plus ladder (Bioneer Co., Korea) was used as the molecular marker. Gels were viewed and recorded by ultraviolet transillumination, using a UV imager (UVP). Sterile distilled water was used as the negative control and *B. melitensis* biovar1 was used as positive control.

2.3 Pulsed-Field Gel Electrophoresis Typing

2.3.1 Genomic DNA extraction

Genomic DNA of *Brucella* and vaccine strains were prepared in agarose plugs and digested by restriction enzyme *XbaI* as described previously with some modifications [22]. Briefly,*the Brucella* isolates and *B.melitensis* vaccine Rev1 as positive control, were grown on *Brucella* broth media(Merck, Germany) at 37ºC for 48 h in 5-10% CO2. Cells centrifuged at 13000×g for 5 min and cells sediment were washed by buffer (100 Mm Tris–HCl [pH 8.0], and 100 mM EDTA [pH8.0]) then tubes centrifuged and resuspended in above buffer and turbidity was measured to 1.4 at 610 nm. The plugs prepared using 100 µl of cells suspension and were mixed with 100 µl low-melting-point agarose 2% (LMP; Merck, Germany) then dispensed into plug molds. The agarose plugs were transferred to 4 ºC for 1 h to solidify, then plugs were transferred into lysis buffer included 50 mM Tris–HCl [pH 8.0], 50 mM EDTA [pH 8.0], 1% Sodium lauryl sarcosine, 1% SDS and 0.1 mg/ml of proteinase K (Sigma, Germany) and incubated overnight at 55ºC in a shaking water bath. The supernatant was discarded and washing was performed in six stages, included twice with sterile distilled water and four times with 10 ml of TE buffer (10 mM Tris–HCl [pH 8.0], 1 mM EDTA) for 30 min each time.

2.3.2 Restriction endonuclease digestion of DNA

A slice 2×2 mm of each plug was cut in100 µl of 10X restriction buffer (Thermo Fisher Scientific, Waltham, MA) for 45 min on ice. The restriction buffer was removed and replaced with 100 µl of 1X restriction buffer containing 30 units of *XbaI enzyme* (Thermo Fisher Scientific, Waltham, MA). The plug slices were transferred to 4ºC for 45 min and were incubated an overnight at 37ºC.

2.3.3 Pulsed-field gel electrophoresis

The digested plugs were run in 1% agarose gel (Merck, Germany) with 0.5X Tris-borate-EDTA buffer (PH 8) to separate the bands on Pulsed-Field Gel Electrophoresis system (Rottaphor Biometra, Version 6, Electric Fields, Germany) by running for 22 h at 14°C, switch angle 120°,

switching directions every 5 sec and ending with 35 sec. Lambda Ladder PFGE Marker (NEB: N0340) was used as the molecular reference marker. Gels were stained with ethidium bromide (0.5 mg /ml) and viewed and recorded under UV transillumination (UV, VilberLourmat).

2.3.4 Analysis of PFGE

Gel images were analyzed using BioNumerics software version 7.5 (Applied Maths, StMartens-Latem, and Belgium). Dendrograms were obtained for all of the strains. Clusters were identified at a cut-off value of 80% and classify of isolates performed with more than 80% similarity in genotypes [23].

3. RESULTS

Twenty-seven *Brucella* strains included 13 human isolates (*B. melitensis*) and 14 animal isolates (*B. melitensis)* were obtained from different sources. The Gram negative coccobacilli with transparent and convex colonies, negative for H2S production, CO2 requirement, methyl red, indole and positive for urease, oxidase and catalase tests, grow on media plates containing thionin and basic fuchsin and agglutination with monospecific anti-M sera identified as *B. melitensis* biovar 1.

3.1 PCR Results

After biochemical tests, identification of *Brucella* strains, confirmed by PCR technique using *omp2a* gene primers and obtained specific DNA fragment with 1100 bp for all of the 27 *Brucella* isolates and *B.melitensis* biovar1 with no DNA band in negative control (distilled water).

3.2 PFGE Results

The PFGE method by *XbaI* was used for access typing and genetic relationship between the *Brucella* strains isolated from human and animal samples (Fig 1). From 27 animal and human *B. melitensis* isolates, 7 common clones /clusters (I, II, III, IV, V, VI, and VII) and 3 single clones were obtained (Fig 2). The similar strains in two different geographic locations were seen. Cluster II had the predominant clones with 8 members. In this cluster, 5 members obtained from patients and 3 members obtained from sheep. In cluster II, four isolates had full similarity in genotype that one of them belongs to animal strain and the others were belonging to human strains. In a case of isolates, in cluster VII likewise, similar isolates include one animal (sheep) and the others were patients with complete similarity and different geographical regions. In cluster 1, there were two animal strains, originated from sheep. In cluster III, there were 3 animal strains, two strains originated from sheep and another originated from gout (9A strain). In clusters of IV and V there were animal strains originated from sheep and in cluster of VI; there were four human strains with complete similarities in each two strains together. Among the 27 isolates, 21(80%) isolates had full or close genetic similarity > 80%. Characterization and distribution of animal and human *B.melitensis* strains were shown in Table 1.

4. DISCUSSION

Brucellosis is one of the most serious problems in public health and zoonosis transmittable to humans. The diseases have long been spread throughout the world particularly in Iran as an endemic area and in some of provinces, sporadic cases are found every seasons [7]. Twenty-seven *B.melitensis* were isolated from patient's blood culture (13 isolates) and animal samples (14 isolates). After identify *Brucella* isolates by microbiology standard tests, PCR method confirmed the presence of *omp2a* gene, which indicated 1100 bp amplification in all isolates. Previous studies indicated PCR technique has increasingly been used as a contributing method in the determination and diagnosis of *Brucella* species [12,24] and *omp2a* gene is specific for *Brucella* genus in studies such as those conducted by Huber and Kazemi [25,26]. They showed that PCR was a sensitive and suitable method for detecting *Brucella* species. According to our results all of human and animal *Brucella* isolates obtained were *B.melitensis* biovar 1 (100%). The Previous studies indicated *B. melitensis* is predominant strain and human major pathogen in different regions of European, the Mediterranean countries [27] and *B. melitensis* biovar 1 was the predominant cause of human brucellosis associated with sporadic cases and outbreak in humans and widely spreading in some areas of Iran [6,28]. In the present study typing of isolates in pathogens bacteria was performed for studies of outbreak and analyses of population survey of bacteria based on their genotypes, analysis of predominant genotypes, distribution and determine the probability of transmission of isolates between hosts. PFGE is a gold standard for evaluation of epidemiological status and genetic relationship between pathogens [29].

In this study by the PFGE technique, 27 *B. melitensis* and vaccine strains (*B. melitensis* Rev1) genomic DNA digested with restrictive endonuclease *XbaI.* The results showed two isolates with complete genotypes similarities were found among animal and patient isolates in cluster II and VII. This indicated that these isolates were transmitted and could be spread from animal to human and is circulating between animals and human in two different geographical areas close to each other. Therefore, animal was considered as a source of these isolates.

Some of studies reported the incidence of brucellosis in human is directly associated with the brucellosis status of animals in a particular geographic area [30,31]. Like other studies, there was a high conformity between *Brucella* strains isolated from animal and human [6,7]. The study of Thakur and Thapliyal [32], revealed a prevalence rate of 4.97% in samples obtained from persons exposed to animals. In the current study, the results of PFGE analysis showed there was a high homology in more genotypes of *Brucella* strains. Full or close genetic similarity among strains may be due to common sources of infection. It seems that *B. melitensis* might originate from bacterial common clone and was circulating in population of animals and human in our region. There are major factors that play

Table 1. Characterization and distribution of *B. melitensis* in PFGE analysis

Key	Cluster	Members No (%)	Animal isolates No	Human isolates No
1	I	2(7.4)	2	0
2	II	8(29.7)	3	5
3	III	3(11. 1)	3	0
4	IV	2(7.4)	2	0
5	V	2(7.4)	2	0
6	VI	4(14.8)	0	4
7	VII	3(11.1)	1	2
8	single clone (VIII, IX, X)	3(11.1)	1	2
Total	**10**	**27(100)**	**14 (51.9)**	**13 (48.1)**

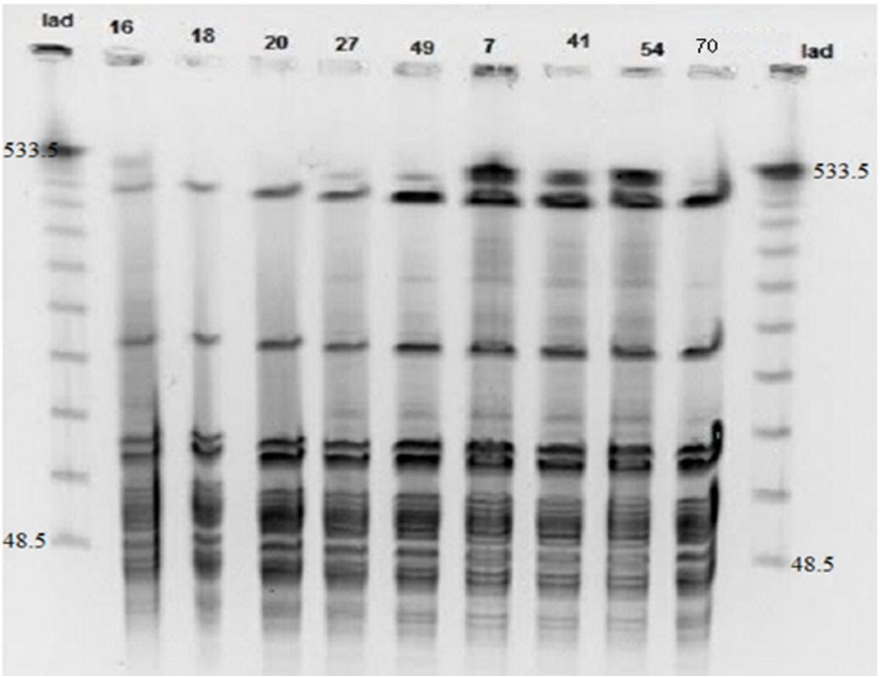

Fig 1. The Result of PFGE. Lanes 16-18-20-27 animal strains; lanes 49-7-41-54-70 human strains; Lane Ladder. Lambda Ladder PFGE Marker (NEB: N0340)

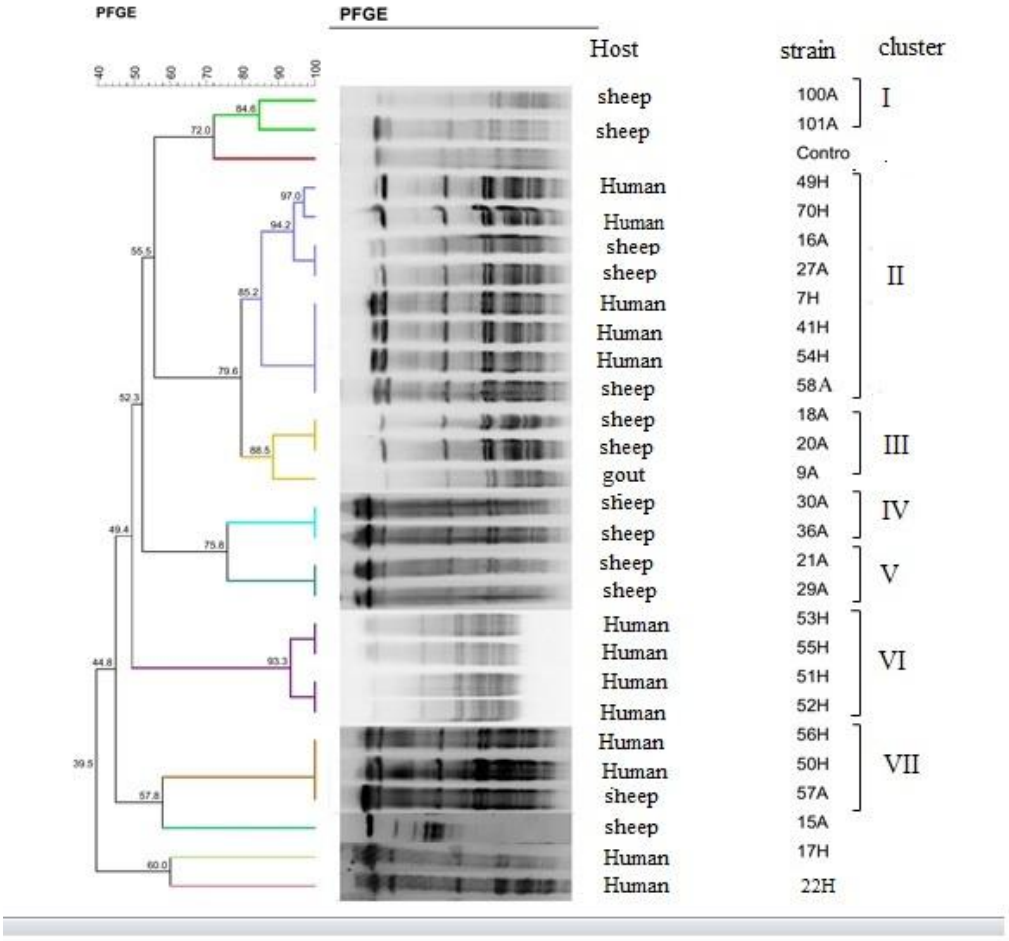

Fig. 2. Dendrogram for 27 *B. melitensis* and Rev. 1 vaccine isolates based on a PFGE analysis. A: animal; H: human

important role in transmission of *Brucella spp*. from animals to human. These include illegal movements and animal trafficking, inadequate attention to livestock health infrastructure in animal husbandry developments and Rancher's Habits. Rancher's habits have an important role in disease transmission between animals and humans [33]. Also our results indicated three isolates with close genotype similarities among animal strains (Sheep and Goat) in cluster III. These results showed *Brucella* strains were circulating between sheep and goat population. Based on PFGE dendrogram, there was little genetic similarity among *B. melitensis* vaccine strain (Rev1) with others animal and human isolates. These results indicated origin of these isolates were different from vaccine strain and showed animal or human brucellosis in our region were not caused by this type of vaccine. It is difficult to distinguish between infections from vaccine or non-vaccine strains of *Brucella spp.* by the application of serological and conventional bacteriological methods. It has been demonstrated that PFGE genomic DNA analysis is more discriminative in exhibiting phylogenetic diversity than analyses based on phenotype characterization [34]. According to comparative analysis obtained genetic supports to the possibility typing methods for separating bacterial strains with the same phenotype. The PFGE analysis showed is a powerful tool for epidemiological and molecular typing of bacteria and was used in *Brucella* successfully [22,35]. Unlike our study there are some reports which have shown the real possibility that strains used in the animal's vaccination can represent a source of human and animal brucellosis [36] .In South Africa Human infection with the vaccine strain Rev.1 has been reported, following horizontal infection among sheep [37]. These

results are consistent with the finding of the pishva etal. They reported after *Brucella* vaccination cattle and sheep, with close contact with each other, Rev-1 vaccine strain was isolated from 2 aborted fetuses [6].

Another important results in the current study indicated that complete similarities were not found among the animal or human isolates in single clones. Furthermore, these isolates were not transmitted from animal to human.

Compare the results of this study with the results of other studies from some of countries such as, China, New Zealand and Iraq revealed that the *Brucella* isolates from Iran by PFGE method is distinctive from those isolates and these isolates are unique in our country [15,22,38]. Iran's geographical position has always been an important risk factor in the spread of infectious diseases, mainly from the eastern and western neighbors such as Afghanistan, Pakistan and Iraq, and it is possible new *Brucella* strains enter the country.

These results and events indicated epidemic genotypes of disease. This study also indicated that analysis by PFGE will be helpful to manage infections caused by *B. melitensis* based on their genotypes PFGE analysis is a powerful tool for epidemiological and molecular typing of bacteria which was used in *Brucella* successfully [22,36].

5. CONCLUSION

In this study PFGE analysis of *XbaI*-digested genomic DNA indicates effectively discriminated animal and human *Brucella* strains. PFGE revealed genetic relationship and presence of different clusters in *Brucella* strains. Various profiles will be used as a tool for continuous surveillance of the *B.melitensis* strains and to trace the source of future infections in Iran.

CONSENT

All authors declare that 'written informed consent was obtained from the patient for publication of this paper and accompanying images.

ETHICAL APPROVAL

The ethics committee of the Hamadan University of medical science, Hamadan, Iran approved this study.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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