Original Article

Multiple Locus Variable-Number Tandem Repeat Analysis Genotyping of Human *Brucella melitensis* Isolates from West of Iran

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Abstract

Background & Objective: Brucellosis is an endemic disease with a high prevalence in Iran whose highest frequency is in the western region of the country. Genetic diversity investigation is an important method to determine the epidemiological relationship of *Brucella* isolates in different geographical areas. Therefore, the present study aimed to investigate the genetic diversity of human *Brucella melitensis (B. melitensis)* strains using the Multiple Locus Variable-Number Tandem Repeat Analysis (MLVA) Typing method in the west of the country.
Materials & Methods: In this study, 20 strains of isolated *B. melitensis* were collected from the human serum samples of suspected Brucellosis in the west of the country and were analyzed by MLVA-16 method.
Results: The results showed that 3 genotype numbers 42, 43 and 47 were identified using MLVA-8 method and using MLVA-11 method genotypes 125, 138 and 111 were recognized. Also, 16 different genotypes were detected from the analysis of the isolates by MLVA-16 method which shows a high degree of polymorphism among the isolates due to the high genetic diversity of the isolates in Panel 2B loci.
Conclusion: The results showed the high genetic diversity of *B. melitensis* isolates in the west of the country and their genetic relationship with the known strains in the neighboring countries of the Eastern Mediterranean area, as well as the importance of the MLVA method in identifying the source of infection.

Keywords: Brucellosis, *Brucella melitensis*, Genotype, MLVA, Human, Iran

Introduction

Brucellosis is a common infection between human and animals that affects the general health of society (1, 2). The genus *Brucella* until now contains 12 species. Most species are specific to the host except for three species of *B. melitensis*, *B. abortus*, and *B. suis*, which cause a common disease between humans and livestock. *B. melitensis* is the main cause of human brucellosis, so that one of the common causes of brucellosis in Iran is *B. melitensis* strain (3–5). The main way that humans get infected with Brucellosis is to consume milk and animal products contaminated with *Brucella* bacteria (1, 6). Most regions of Iran are endemic to brucellosis, and the annual incidence of Brucellosis is still high. The annual number of human Brucellosis is 29.83 cases per 100,000 individuals from 2001 to 2009 in Iran (7). Compared to other regions, the west of the country has a high level of brucellosis infection (8, 9).

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Therefore, investigating the epidemiology of brucellosis in order to identify the source of infection and to identify the genotype of the causative strains is of great importance. MLVA-16 is a suitable method for identifying species and investigating geographic distribution at the subspecies level and identifies the source of infection (10-14). The information about the strains identified by this method is available on the site http://mlva.u-psud.fr and the genotype of the investigated isolates can be compared through the information on the site and identify the epidemiological information about each strain. Since the genetic diversity of *B. melitensis* strains in the west of the country has not been determined, the present study was conducted to determine the genetic diversity of *B. melitensis* strains isolated from patients with Brucellosis in the west of the country by the MLVA-16 method and investigate the epidemiological relationships among the isolates

Materials and Methods

Sampling

From March 2020 to September 2021, a total of 100 blood samples, proved to be positive by Wright tests (Titer higher than 1/80), were collected by health centers from six western provinces of the country (Lorestan (19 samples), Kermanshah (20 samples), Hamedan (35 samples), Kurdistan (15 samples), Ilam (11 samples) and referred to the veterinary laboratory of Lorestan University. Serum samples were kept at -20°C for molecular tests until processing.

DNA extraction

200 µL of each serum sample were transferred to a microtube for DNA extraction using DNA Purification Mini Kit (GeneAll, Korea) just as the manufacturer instructions. The quality and quantity of extracted DNA was evaluated by 1% agarose gel and Nanodrop spectrophotometer, respectively (Thermo Scientific, Waltham, USA). Finally, the extracted DNA was stored at -20°C for use in PCR reaction.

Diagnosis of B. melitensis strains

First, the isolates *(Brucella spp.)* were identified by detecting the bcsp31 gene with a length of 223 bp by PCR method as previously described by Baily et al. (15). Then, *B. millitensis* species were diagnosed by detecting IS711 repetitive genetic element with specific primers of *B. millitensis* species with a size of 731 bp as previously described by Bricker and Halling, (16).

MLVA-16Orsay genotyping

Using MLVA-16 assays, Genotyping of each isolate was identified as described by Le Fleche (11) and Al Dahouk et al. (10) using the PCR method. Accordingly, three panels containing panel 1 for species identification (Bruce06, Bruce08, Bruce11, Bruce12, Bruce42, Bruce43, Bruce45, and Bruce55) and panels 2A (Bruce18, Bruce19, and Bruce21) and 2B (Bruce04, Bruce07, Bruce09, Bruce16, and Bruce30) for more subspecies differentiation. The PCR conditions were performed using Primer pairs as described by Le Flèche et al. (11). These steps were as follows: initial denaturation of 96 °C for 5 min, followed by 30 cycles of denaturation at 96 °C for 30 seconds, annealing at 60°C for 30 s and extension at 70°C for 1 min. The final extension was at 70°C for 5 min. The PCR amplification products were separated into 3% and 2% agarose gel (Merck, Germany) containing 2.5 µg/mL DNA safe stain (Cinnagen, Iran) for panel 1 and panel 2 loci, respectively. Electrophoresis was carried out in 0.5x Tris/Borate/EDTA (TBE) buffer. The PCR products were visualized under a UV transilluminator (E-Box, Iran) and the 100-bp DNA ladder and a 20-bp DNA ladder (thermo scientific, Lithuania) were used as molecular size markers for panel 1 and panel 2, respectively.

Data analysis

Analysis of electrophoresis patterns was performed by the BioCalculator software version 3.0.05 (Qiagen, Germany), and fragment sizes converted to repeat unit numbers were imported into BioNumerics 7.6 (Applied Maths, Belgium) as a character data set. The cluster analysis was performed using the UPGMA (unweighted pair group method with

arithmetic mean) algorithm and the categorical distance. Genetic diversity at each locus was evaluated using the Hunter Gaston diversity index (HGDI) (17). The MLVA-16Orsay genotypes of *B. melitensis* and isolates were compared to genotypes from isolates available in the MLVA bank database (http://mlva.u-psud.fr).

Results

Among 100 serum samples suspected of *Brucellosis* (identified by Wright's test, with a titer higher than 1/80) in this work, 52 isolates were detected with the bcsp31 gene of 223bp *Brucella* spp. Furthermore, the examination of 52 Brucella isolates led to the detection of 20 *B. melitensis* isolates by detecting the IS711 repetitive genetic element with specific primers of *B. melitensis* species with a size of 731bp (Figure 1).

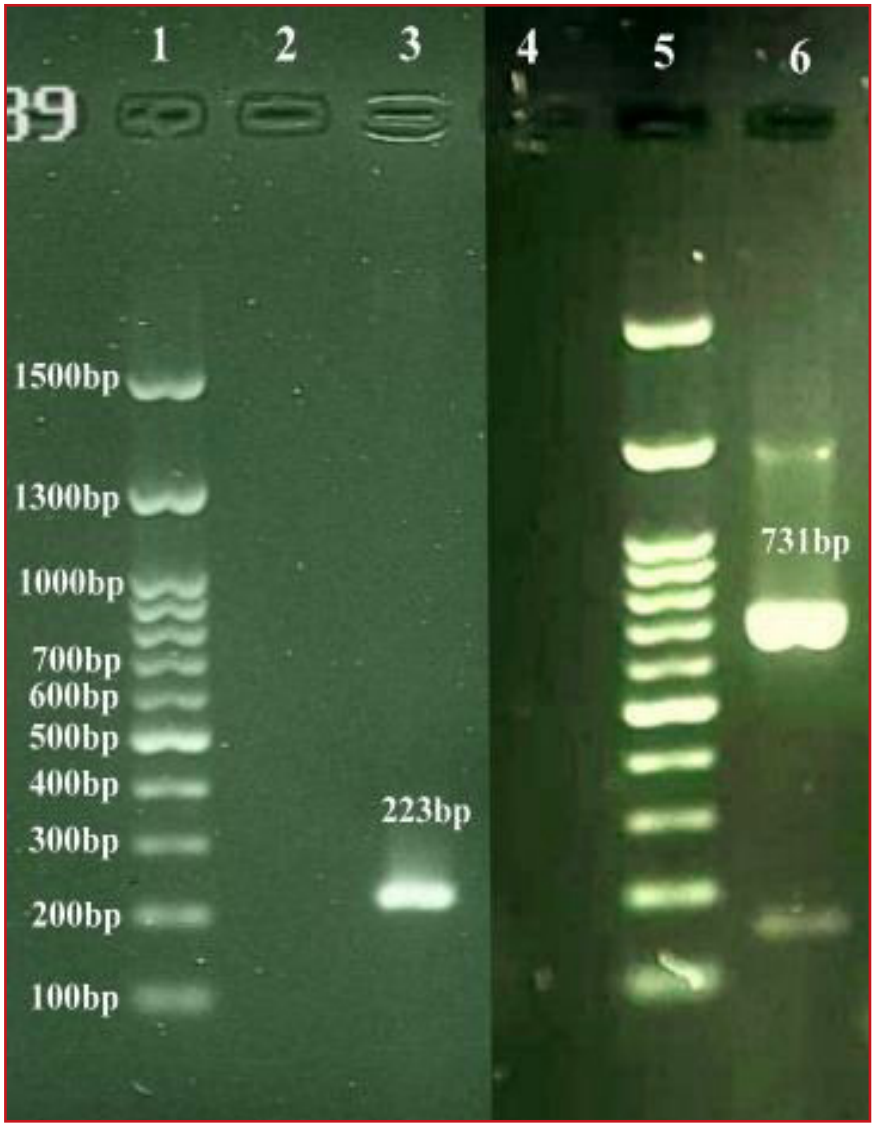
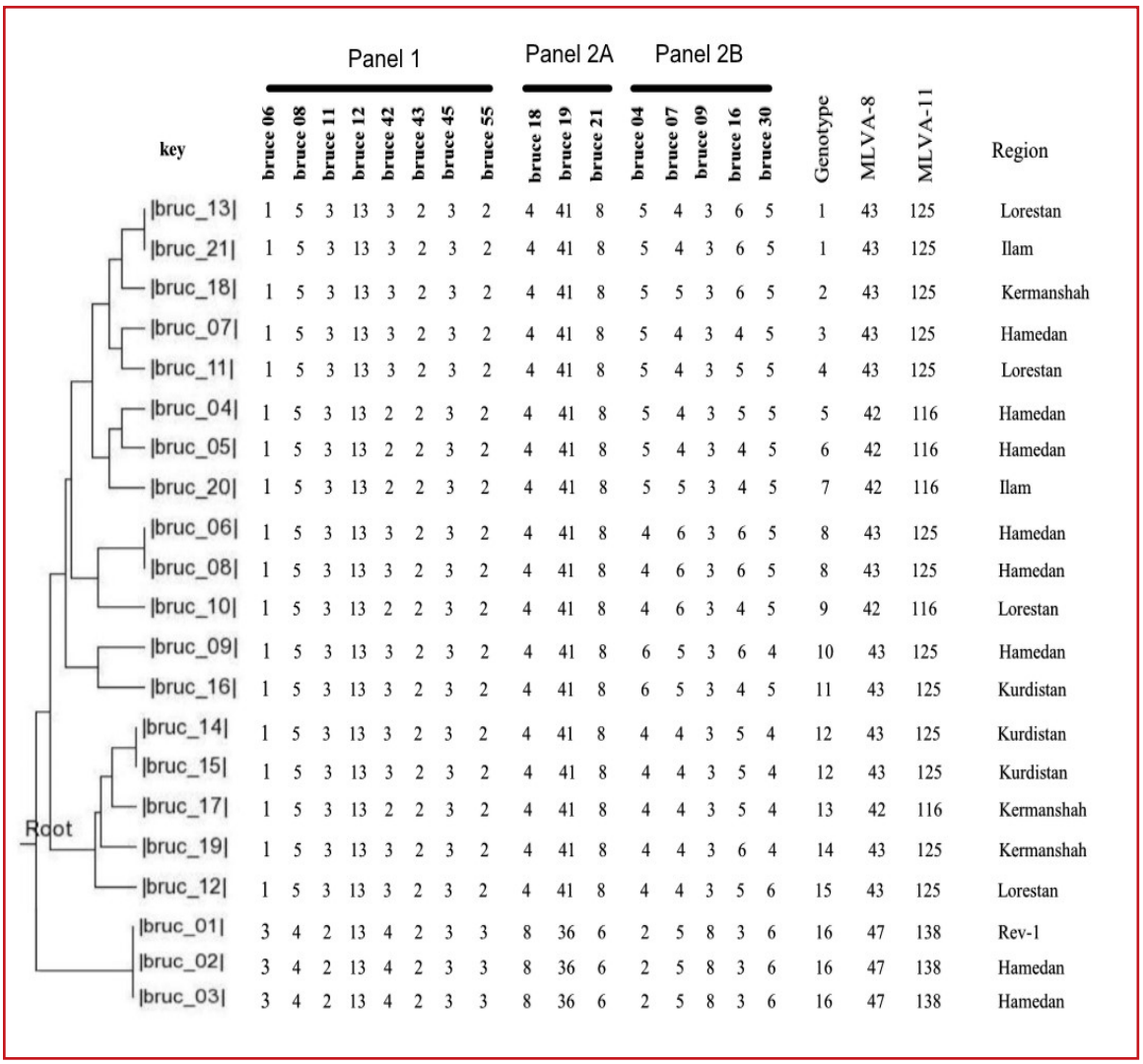


Figure 1. Agarose gel electrophoresis of PCR amplified *bcsp31* and *IS711* genes fragments from *Brucella spp.* and *B. melitensis* strain was isolated from the serum of patients with a titer higher than 1/80 (using Wright's test). Lanes 1 and 5: Standard DNA marker (100bp DNA ladder); Lane 2: Negitive control; for *Brucella spp.*; Lane 3: Positive control with *Brucella spp.* DNA (223bp); Lane 4: Negitive control for B. melitensis; Lane 6 Positive control with *B. melitensis* DNA (731bp)

After detecting the isolates of *B. melitensis*, the genotype of each isolate was then detected by the MLVA typing method so that the genotypes 42 (5 isolates), 43 (13 isolates), and 47 (2 isolates) were detected using the MLVA-8 method (Panel 1). Furthermore, genotypes 116 (1-5-3-13-2-2-3-2-4-41-8; n=5), 125 (1-5-3-13-3-2-3-2-4-41-8, n= 13), and 138 (3-4-2-13-4-2-3-3-8-36-6; n= 2) were detected using the MLVA-11 method (Panel 2A).

Moreover, 16 different genotypes were obtained using the full MLVA-16 method (Panels 1, 2A, and 2B) (Figure 2). Except for genotypes 1, 8, 12, and 16, which were detected in more than one isolate, other isolates indicated different genotypes. Genotype 1 was obtained from two different provinces of Lorestan and Ilam (Iran). Except for genotype 16, which belonged to the American group, all the genotypes obtained in this study belonged to the East Mediterranean group.



key	bruce 06	bruce 08	bruce 11	bruce 12	bruce 42	bruce 43	bruce 45	bruce 55	bruce 18	bruce 19	bruce 21	bruce 04	bruce 07	bruce 09	bruce 16	bruce 30	Genotype	MLVA-8	MLVA-11	Region
|bruc_13|	1	5	3	13	3	2	3	2	4	41	8	5	4	3	6	5	1	43	125	Lorestan
|bruc_21|	1	5	3	13	3	2	3	2	4	41	8	5	4	3	6	5	1	43	125	Ilam
|bruc_18|	1	5	3	13	3	2	3	2	4	41	8	5	5	3	6	5	2	43	125	Kermanshah
|bruc_07|	1	5	3	13	3	2	3	2	4	41	8	5	4	3	4	5	3	43	125	Hamedan
|bruc_11|	1	5	3	13	3	2	3	2	4	41	8	5	4	3	5	5	4	43	125	Lorestan
|bruc_04|	1	5	3	13	2	2	3	2	4	41	8	5	4	3	5	5	5	42	116	Hamedan
|bruc_05|	1	5	3	13	2	2	3	2	4	41	8	5	4	3	4	5	6	42	116	Hamedan
|bruc_20|	1	5	3	13	2	2	3	2	4	41	8	5	5	3	4	5	7	42	116	Ilam
|bruc_06|	1	5	3	13	3	2	3	2	4	41	8	4	6	3	6	5	8	43	125	Hamedan
|bruc_08|	1	5	3	13	3	2	3	2	4	41	8	4	6	3	6	5	8	43	125	Hamedan
|bruc_10|	1	5	3	13	2	2	3	2	4	41	8	4	6	3	4	5	9	42	116	Lorestan
|bruc_09|	1	5	3	13	3	2	3	2	4	41	8	6	5	3	6	4	10	43	125	Hamedan
|bruc_16|	1	5	3	13	3	2	3	2	4	41	8	6	5	3	4	5	11	43	125	Kurdistan
|bruc_14|	1	5	3	13	3	2	3	2	4	41	8	4	4	3	5	4	12	43	125	Kurdistan
|bruc_15|	1	5	3	13	3	2	3	2	4	41	8	4	4	3	5	4	12	43	125	Kurdistan
|bruc_17|	1	5	3	13	2	2	3	2	4	41	8	4	4	3	5	4	13	42	116	Kermanshah
|bruc_19|	1	5	3	13	3	2	3	2	4	41	8	4	4	3	6	4	14	43	125	Kermanshah
|bruc_12|	1	5	3	13	3	2	3	2	4	41	8	4	4	3	5	6	15	43	125	Lorestan
|bruc_01|	3	4	2	13	4	2	3	3	8	36	6	2	5	8	3	6	16	47	138	Rev-1
|bruc_02|	3	4	2	13	4	2	3	3	8	36	6	2	5	8	3	6	16	47	138	Hamedan
|bruc_03|	3	4	2	13	4	2	3	3	8	36	6	2	5	8	3	6	16	47	138	Hamedan

Figure 2. Cluster analysis for 20 human isolates of *B. melitensis* and Rev1 vaccine strain based on the data set of MLVA-16Orsay. In the columns, the following data are indicated: Panels, genotype, MLVA-8, MLVA-11, region

The levels of polymorphism in the isolates using the Hunter Gaston diversity index (HGDI) method indicated that the level of polymorphism was low in Panels 1 and 2A, and the highest level of polymorphism was found in Panel 2B so that all isolates in bruce12, bruce43, and bruce45 loci of Panel 1 did not show any polymorphism (HGDI= 0.000) and only one allele was detected. Bruce06, bruce08, bruce11, and bruce55 loci from Panel 1 along with the loci of Panel 2B indicated a low level of polymorphism (HGDI=0.198). Furthermore, the bruce42 locus of Panel 1 indicated a high level of polymorphism (HGDI=0.531) along with the loci of Panel 2B. The highest variation in Panel 2B was related to the Bruce16 locus (HGDI=0.752) (Table 1).

Table 1. Allelic types and Hunter and Gaston diversity index (HGDI) of *B. melitensis* isolated for 16 loci in this study

Locus	Allelic types	No. of repeats	HGDI
Panel - 1			
Bruce06	2	(1, 3)	0.189
Bruce08	2	(4, 5)	0.189
Bruce11	2	(2, 3)	0.189
Bruce12	1	(13)	0.000
Bruce42	3	(2-4)	0.531
Bruce43	1	(2)	0.000
Bruce45	1	(3)	0.000
Bruce55	2	(2, 3)	0.189
Panel–2A			
Bruce18	2	(4,8)	0.189
Bruce19	2	(36, 41)	0.189
Bruce21	2	(6, 8)	0.189
Panel-2B			
Bruce04	4	(2, 4-6)	0.694
Bruce07	3	(4-6)	0.584
Bruce09	2	(3,8)	0.189
Bruce16	4	(3-6)	0.752
Bruce30	3	(4-6)	0.584

Discussion

Brucellosis is an important infectious disease with a high prevalence in Iran (4, 8, 9, 18, 19). A bacterium from *Brucella genus* is the main cause of brucellosis. Among the members of this genus, B. melitensis has great importance owing to the severity of the disease (1, 20, 21). However, there is little information on the genetic characteristics of pathogenic strains of this species in Iran. In the present study, 20 *B. melitensis* isolates were detected from the examination of 52 *Brucella spp*. isolates from the serum of patients in the west of Iran. The genetic diversity and epidemiological relations of the isolates were then investigated using the MLVA typing method. The prevalence rate of *B. melitensis* species was 38.4% among 52 *Brucella* isolates from the serum of people with brucellosis, indicating the importance of other *Brucella* species *(such as Brucella abortus)* causing brucellosis in humans.

The examination of Panel 1 proved among genotypes 42, 43, and 47, genotype 43 was the dominant genotype with the highest prevalence rates in Lorestan, Hamedan, and Kurdistan. This genotype was also dominant in China, United Arab Emirates and Turkey (10, 14, 22-25). Genotypes 125 and 116 with prevalence rates of 65% and 25%, respectively, (which belonged to the East Mediterranean group) were detected from Panels 1 and 2A (MLVA-11). The differentiation power of MLVA-11 was higher than MLVA-8 for assessing cross-transmission among samples. The results of these panels provided more useful information about the distribution of genotypes among the countries.

Genotypes 125 and 116 were also reported in some Eastern Mediterranean countries such as Turkey, Iraq, Pakistan, China, and Kazakhstan (10, 23, 26, 27). Genotype 125 was dominant in the west of Iran and in the neighboring country, Turkey (14, 22). It might be due to the transfer of livestock between these two regions. Georgi's (23) study on numerous samples of *B. melitensis* collected from different countries indicated that the two isolates of human *B. melitensis* isolated from Iran had genotypes 125 and 116. Their information is presented on http://mlva.u-psud.fr confirming the dominance of these two genotypes in Iran and the result was consistent with the present study. Among the studied isolates, two strains (bruc-02, bruc-03) with genotype 138 were detected, indicating their belonging to the American group.

The positive control indicated genotype 138 was related to the vaccine strain, Rev-1 (bruc-01); hence, the genotype of the two isolates showed that two people were infected with *B. melitensis* vaccine strain and were from Hamadan province. These people were probably in contact with the Rev-1 vaccine or were in contact with animals infected by the vaccine strain. The presence of the vaccine strain in human infection requires a wider study in this area.

The examination of isolates at a higher level with MLVA-16 (MLVA-11+ Panel 2B) detected 16 genotypes of isolates. It was due to the very high differentiation power of Panel 2B. Only 3 genotypes were detected in more than one isolate and the rest of them had different genotypes.

Except for the bruce09 locus, the other four loci of panel 2B had HGDI>0.500, indicating high genetic diversity among *B. melitensis* subspecies. The highest genetic diversity in Panel 2B belonged to the bruce 16 loci (HGDI=0.752). This locus had higher diversity than the rest of theloci in Panel 2B in Turkey and China (14, 22, 28). The highest genetic diversity was found in Hamadan province. A total of 6, 4, 2, 3, and 2 genotypes were detected in Hamadan, Lorestan, Kurdistan, Kermanshah, and Ilam provinces, respectively. The isolates obtained from these provinces almost had similar genotypes owing to the same borders of the provinces and the high transfer of livestock between the provinces. Therefore, the present study shows that in order to prevent the further Prevalence of brucellosis in the western regions of the country, serious preventive measures should be taken, such as educating people more about the brucellosis disease, identifying infected animals and eliminating them. Also, more serious management of animal transport in border areas should be put on the agenda.

Conclusion

The present study indicated that the MLVA typing method could be significantly effective in the epidemiological tracking of Brucella infections and in promoting the control and management of *brucellosis* in Iran. It was due to the highest genetic diversity among *B.melitensis* isolates from human sources in western Iran. The epidemiological data of *B. melitensis* isolates in the west of Iran and their genetic similarity with isolates from neighboring countries using the MLVA method was reported. However, broader studies are needed to obtain more information about the epidemiology of brucellosis in other regions of Iran.

Author Contributions

All authors have made contributions to designing the study, obtaining the data, analyzing the data, and preparing the article. All authors have agreed the manuscript to submit.

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Conflicts of Interest

The authors declare no conflict of interest.

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