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Genetic diversity of *Brucella melitensis* isolates from sheep and goat milk in Iran

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Abstract

The genetic diversity of *Brucella* strains has not been fully understood. To investigate this, the genetic characteristics of 64 isolates of *Brucella melitensis* from sheep and goats' milk were studied using random fragment length polymorphism (RFLP) and multiple locus variable-number tandem repeat analysis (MLVA-16) methods developed in Orsay, France (MLVA-16_{Orsay}). The RFLP analysis revealed that all 64 isolates were of biovar one. The MLVA-typing showed that one sample was simultaneously infected with two strains of *B. melitensis* and the genotype of 65 isolate was analyzed. Four genotypes (47, 42, 43, and 63) were identified using MLVA-8 (panel 1), whereas six genotypes (138, 125, 116, 108, and two unknown genotypes) were identified using MLVA11 (panels 1 and 2A). From the review of MLVA-16 (panels 1, 2A, and 2B), panel 2B showed a very high discriminatory power. Two loci of Bruc04 and Bruc30 from this panel had diversity index values higher than 0.71 and the average diversity index was 0.619. So MLVA-16_{Orsay} 34 showed the genotype indicating a low genetic homogeneity among the isolates. The findings of MLVA genotyping of the isolates suggest that strains of *B. melitensis* isolated from the milk of small ruminants in Iran are most closely related to the isolates from neighboring countries of the Eastern Mediterranean group. To the best of our knowledge, this is the first study to indicate the potential use of MLVA genotyping for simultaneous detection of specimen contamination using two different *B. melitensis* biovars.

Introduction

Brucellosis is an important disease that affects humans and animals with considerable public health importance and economic impact. Gram-negative intracellular bacteria belonging to *Brucella* spp. are the causative agents of brucellosis. Infection by *B. melitensis* in sheep and goats often leads to abortion and reduced milk production.[1,2] Asymptomatic vector animals can infect herds by shedding the pathogen in their body fluids, and humans can also be infected by consuming unpasteurized milk and dairy products. Among *Brucella* species, *B. melitensis* is recognized as the most important pathogen in terms of public health[2-4] causing the highest number of human brucellosis cases worldwide.[5] This species is also the most common *Brucella* species in Iran.[6-10] In areas where the prevalence of this disease is the highest, the only strategy to control brucellosis in small ruminants is to use the *B. melitensis* Rev. 1 vaccine strain.[11,12] This strategy has been implemented in Iran since 1982. Before 2008, only a full dose of 1.00 - 4.00 × 10^9 CFU mL^{-1} vaccine was used to vaccinate lambs and kids in Iran. From 2008 to 2015, the reduced dose of 0.50 - 3.00 × 10^6 CFU mL^{-1} vaccine was used for adult animals. However, since 2015, only the full-dose vaccine has been used for lambs and goats. The annual incidence of human brucellosis has decreased which may be the result of vaccination programs and observing health protocols in the dairy industry. In Iran, this rate reduced from 39 per 100,000 population in 2003 to 16 per 100,000 population in 2016.[10] Currently, *B. melitensis* biovar 1 is the dominant strain in Iran.[8,9,13,14] Although there is a need for further research on the reduction of *B. melitensis* biovar 2 and biovar 3 strains, the predominance of biovar 1 has been reported in livestock herds and human populations.

Most studies have used the random fragment length polymorphism (RFLP) method, presented by Cloeckaert *et al*. to differentiate *B. melitensis* biovars.[15] However, this method cannot differentiate the affected Rev. 1 vaccine strain from biovar 1 of *B. melitensis*. Thus, the multiple

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locus variable-number tandem repeat analysis (MLVA-16) technique has been successfully employed to identify species and to distinguish geographic distribution at the subspecies level in a local outbreak.[16-22] To date, no comprehensive study on the genetic diversity of *B. melitensis* strains in ruminants of Iran has been conducted. Georgi *et al.* only studied two human strains and their genotypes were identified using the MLVA method.[23] Moreover, several studies in Iran have examined *Brucella* isolates from animals using MLVA-16 method, but the genotype of the strains could not be identified.[24,25] Therefore, we examined the strains collected from different parts of Iran, with a high prevalence of brucellosis, using the RFLP and MLVA-16 to determine the biovars among small ruminant brucellosis isolates and investigate the genetic diversity of *B. melitensis* strains.

Materials and Methods

Milk samples. From February 2018 to September 2019, a total of 380 milk samples were collected from 270 sheep and 110 goats (with the age of over 3 years that were between 3 and 8 months old at the time of the vaccination) from 6 provinces with a high prevalence of brucellosis: West Azerbaijan, Hamedan, Lorestan, Kerman, Golestan, and Khuzestan. Then, 50.00 mL of each sample was poured aseptically into tubes and transferred to the laboratory in an ice box.

Bacteriological examination. After centrifugation (5,000 *g*, 10 min at 42.00 ˚C) of the milk samples (10.00 mL), the top fatty layer and sediment mixture were seeded in duplicate plates of *Brucella* agar medium (Quelab, Montreal, Canada) and then incubated at 37.00 ˚C without CO_2 for at least seven days. Finally, suspected colonies were identified by their colonial morphology, Gram staining, urease activity test, inability to produce hydrogen sulfide and oxidase test.[26]

DNA extraction. *Brucella* DNA samples were extracted using the Blood Genomic DNA Extraction Mini Kit (Favorgen, Ping Tung, Taiwan) according to the manufacturer's guidelines. The DNA concentration and quality were also assessed using a Nanodrop spectrophotometer (Thermo Fisher Scientific, Waltham, USA). The extracted DNA was stored at – 20.00 ˚C for further use.

Detection of *B. melitensis* strains. The polymerase chain reaction (PCR) method, described by Baily *et al.*[27] was applied to identify *Brucella* ssp. by detecting the *bcsp31* gene (223 bp). The *B. melitensis* species were identified by detecting the IS711, repetitive genetic element, using specific primers for *B. melitensis* species (731 bp) as previously described by Bricker and Halling.[28]

PCR-RFLP (biovar identification). To identify *B. melitensis* biovars, *omp*2a and *omp2b* genes were amplified by the PCR assay. The primers (Table 1) in this study were similar to those reported by Cloeckaert *et al.*[15] The PCR amplification was carried out using a PCR Master Mix Kit (1.25 mL of Master Mix RED; Ampliqon, Odense, Denmark) was used in 25.00 µL the mixtures containing 2.00 X Master Mix (12.50 µL), primers (0.50 µL each), and the extracted DNA (4.00 µL). The DNA obtained from *B. melitensis* Rev. 1 vaccine strain, supplied by Razi Vaccine and Serum Research Institute (Karaj, Iran), was used as a positive control, while sterile water was used as the negative control. The PCR amplification was performed using a thermo cycler (model QB-96; Quanta Biotech Ltd., London, UK) with the following conditions:

The initial step of 95.00 ˚C for 5 min followed by 30 cycles of 95.00 ˚C for 1 min as denaturation, annealing at 58.00 ˚C for 2 min, extension at 70.00 ˚C for 3 min, and a final extension at 70.00 ˚C for 10 min. The *omp2a and omp2b* genes amplification products were then digested using RFLP with PstI and HinfI restriction enzymes (Thermo Fisher scientific) in 20.00 µL reaction volume containing 6.00 µL PCR product, 2.00 µL of appropriate buffer, 1.00 µL of restriction enzyme, and 11.00 µL of water. The reaction mixture was incubated at 37.00 ˚C for 3 hr and the results of digested DNA were separated using 2.00% agarose gel electrophoresis with gel stain (Smobio, Hsinchu, Taiwan) in 0.50X Tris-Borate-EDTA buffer.[15]

MLVA-16$_{Orsay}$ genotyping. Genotyping of isolates was carried out using MLVA-16$_{Orsay}$ offered by Le Flèche *et al.*[16] and Al Dahouk *et al.*[17] using PCR method. Accordingly, three panels consisting of 16 pairs of primers were determined. Panel 1 contained eight loci (Bruce06, Bruce08, Bruce11, Bruce12, Bruce42, Bruce43, Bruce45, and Bruce55) known as MLVA-8. Panel 2A included three loci (Bruce18, Bruce19, and Bruce21). Panel 1 and Panel 2A are collectively called MLVA-11, which makes it possible to identify *Brucella* species and classify strains based on geographical distribution. The third panel was 2B, which included five loci (Bruce04, Bruce07, Bruce09, Bruce16, and Bruce30). These loci are highly discriminatory and can determine the MLVA-16 combined with Panel 1 and Panel 2B indicating local subspecies and local tracking (tracking local outbreaks). The primers synthesized by Bioneer Co. (Daejeon, South Korea) and provided by Takapouzist (Tehran, Iran), were used according to the instructions by Le Flèche *et al.*[16] The PCR amplification was conducted using a PCR Master Kit (Master Mix RED 1.25 mL; Ampliqon) with 25.00 µL mixtures containing 2.00 X master mix (12.50 µL), the primer (0.50 µL), and the extracted DNA (4.00 µL). The following conditions were met: initial denaturation of 96.00 ˚C for 5 min followed by 30 cycles of denaturation at 96.00 ˚C for 30 sec, annealing at 60.00 ˚C for 30 sec and extension at 70.00 ˚C for 1 min. The final extension was at 70.00 ˚C for 5 min. For gel electrophoresis of panels 1 and 2 loci PCR products, 8.00 µL of the amplification product was loaded into 2.00% and 3.00% agarose gel, respectively. Further, a 100-bp DNA

Table 1. Primers used for MLVA typing of *Brucella melitensis* isolates.

Primer name	PR: imeR: sequence (5'-3')	Amplified size (bp)	References
omp31	F: TGACAGACTTTTTCGCCGAA R: TATGGATTGCAGCACCGC	1,200	
omp2a	F: GGCTATTCAAAATTCTGGCG R: ATCGATTCTCACGCTTTCGT	1,100	34
omp2b	F: CCTTCAGCCAAATCAGAATG R: GGTCAGCATAAAAAGCAAGC	1,200	
BR: uc04	F: CTGACGAAGGGAAGGCAATAAG R: CGATCTGGAGATTATCGGGAAG	152 - 208	
BR: uc06	F: ATGGGATGTGGTAGGGTAATCG R: GCGTGACAATCGACTTTTTGTC	140 - 542	
BR: uc07	F: GCTGACGGGGAAGAACATCTAT R: ACCCTTTTTCAGTCAAGGCAAA	150 - 190	
BR: uc08	ATTATTCGCAGGCTCGTGATTC R: ACAGAAGGTTTTCCAGCTCGTC	312 - 366	
BR: uc09	F: GCGGATTCGTTCTTCAGTTATC R: GGGAGTATGTTTTGGTTGTACATAG	124 - 244	
BR: uc11	F: CTGTTGATCTGACCTTGCAACC R: CCAGACAACAACCTACGTCCTG	257 - 698	
BR: uc12	F: CCAGACAACAACCTACGTCCTG R: GCCCAAGTTCAACAGGAGTTTC	302 - 452	
BR: uc16	F: ACGGGAGTTTTTGTTGCTCAAT R: GGCCATGTTTCCGTTGATTTAT	140 - 240	
BR: uc18	F: TATGTTAGGGCAATAGGGCAGT R: GATGGTTGAGAGCATTGTGAAG	130 - 170	16
BR: uc19	F: GACGACCCGGACCATGTCT R: ACTTCACCGTAACGTCGTGGAT	76 - 190	
BR: uc21	F: CTCATGCGCAACCAAAACA R: GATCTCGTGGTCGATAATCTCATT	148 - 175	
BR: uc30	F: TGACCGCAAAACCATATCCTTC R: TATGTGCAGAGCTTCATGTTCG	119 - 151	
BR: uc42	F: CATCGCCTCAACTATACCGTCA R: ACCGCAAAATTTACGCATCG	164 - 789	
BR: uc43	F: TCTCAAGCCCGATATGGAGAAT R: TATTTTCCGCCTGCCCATAAAC	170 - 194	
BR: uc45	F: ATCCTTGCCTCTCCCTACCAG R: CGGGTAAATATCAATGGCTTGG	133 - 187	
BR: uc55	F: TCAGGCTGTTTCGTCATGTCTT R: AATCTGGCGTTCGAGTTGTTCT	194 - 354	

ladder (Smobio) as well as a 20-bp DNA ladder (Thermo Scientific, Vilnius, Lithuania) were applied as molecular size markers for Panel 1 and Panel 2, respectively.

Statistical analysis. The size of fragments in electrophoresis was evaluated using GeneMapper Software (version 4.1; Applied Biosystems, Foster City, USA) and the band sizes were converted to repeat unit numbers and entered into BioNumerics Software (version 7.6; Applied Maths, Kortrijk, Belgium). Cluster assessment was done using unweighted pair group method with arithmetic averages algorithm and the categorical distance. The genetic variation at each locus was determined by the Hunter Gaston Diversity Index.[29] The MLVA-16 genotypes of *B. melitensis* isolates were compared to the genotypes in the MLVA Bank database (http://mlva.u-psud.fr).

Results

Identification of *B. melitensis* strains and biovars. In this study, a total of 64 *B. melitensis* strains were detected, including 46 and 18 strains isolated from sheep and goat milk, respectively. To determine the biovar type, analysis of *omp2a* and *omp2b* genes by the PCR method revealed 1,100 and 1,200 bp bands in all 64 isolates, respectively (Fig. 1). With enzymatic digestion of the amplicons, the results of this study indicated that in the 64 positive samples, from the digestion of *omp2a* locus by Pst1 and Hinf1 enzymes, P3 and P2 patterns were detected, respectively. From the digestion of the *omp2b* locus by means of Pst1 and Hinf1 enzymes, P1 and P1 patterns were detected. These patterns represented *B. melitensis* biovar 1 (Fig. 2).

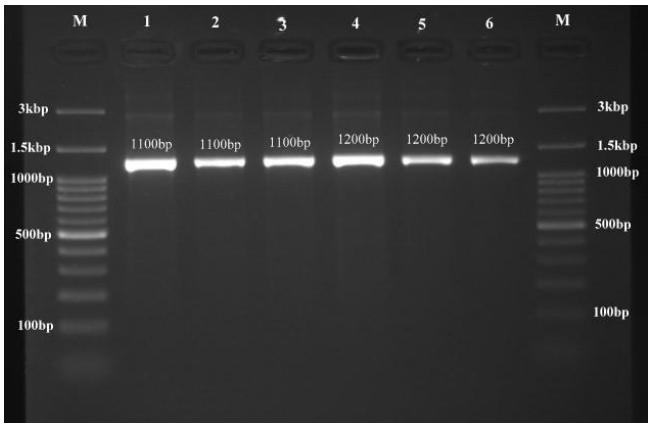


Fig. 1. Agarose gel electrophoresis of touchdown (TD) polymerase chain reaction amplified *omp*2a and *omp2b* genes fragments from *Brucella melitensis* strain isolated. Lane M: Standard DNA marker (100bp DNA ladder); Lane 1: Positive control with *B. melitensis* DNA Rev-1 *omp2a*; Lanes 2 and 3: Amplified loci of *omp2a*; Lane 4: Positive control with *B. melitensis* DNA Rev-1 *omp2b*; Lanes 5 and 6: Amplified loci of *omp2b*.

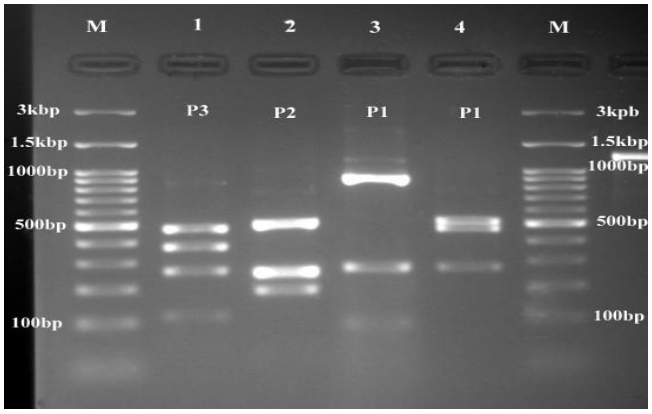


Fig. 2. Agarose gel electrophoresis results of polymerase chain reaction - random fragment length polymorphism patterns obtained from enzymatic digestion of amplified *omp*2a and *omp2b* genes fragments. Lane M: Standard DNA marker (100bp DNA ladder); Lanes 1, and 2 are digestion of *omp2a* fragment by Pst1 (P3) and Hinf1 (P2); Lanes 3, and 4 are digestion of *omp2b* fragment by Pst1 (P1) and Hinf1 (P1).

MLVA genotyping. Since, using the MLVA-16 method, one sheep showed simultaneous infection with two strains with different genotypes, thus, in this study, the genotype of 65 isolate was investigated by this method. Using panel 1 (MLVA-8), four genotypes were detected: Genotype 47 (26 isolates), genotype 42 (14 isolates), genotype 43 (24 isolates) and genotype 63 (1 isolate, ST.m17 strain). Further examination using panel 1 and panel 2A (MLVA-11) indicated six different genotypes, including genotype 138 (3-4-2-13-4-2-3-3-8-36-6; n = 26), genotype 108 (1-5-3-13-2-2-3-2-4-43-8; n = 2), genotype 116 (1-5-3-13-2-2-3-2-4-41-8; n = 12), and genotype 125 (1-5-3-13-3-2-3-2-4-41-8; n = 23). All of these genotypes have been previously described in the MLVA bank. Also, two novel genotypes were identified with a single locus variation in locus 18 which had seven repeats. An isolate (ST.m10 strain) with genotype (1-5-3-13-3-2-3-2-7-41-8) which had a genotype similar to genotype 125 with single locus variants and a ST.m 17 strain, was identified using MLVA-16. This strain compared to other strains registered in the MLVA bank indicated biovar 2. The genotype of this strain (1-5-3-13-2-3-3-2-7-41-8) was similar to that of genotype 111 with single-locus variants. These two strains, together with another biovar 1 strain (ST.m 16 strain) were isolated from sheep, as confirmed by the images obtained by MLVA typing of Bruce06, Bruce55, and Bruce18 strains (Fig. 3). The *B. melitensis* Rev. 1 vaccine strain in Iran showed genotypes 47 and 138, using MLVA-8 and MLVA-11, respectively. Overall, 26 isolates showed this genotype. The MLVA-16 typing assay showed 34 genotypes, with genotype 1 being the predominant genotype among the isolates. Except for genotypes 9, 21, and 33, which were each identified in two isolates from different provinces of Iran, all isolates had different genotypes (Fig. 4). According to the markers, all isolates were homogeneous in two loci of panel1 (Bruce12 with 13 repeats and Bruce45 with three repeats), as they showed only one allele without any variants (Hunter Gaston discriminatory index [HGDI] = 0). Also, all isolates were similar in locus 43, with the number of repeats identical (two repeats), except for one isolate (ST.m 17) with three repeats, which differed from other isolates. The HGDI value was 0.030 at this locus. Besides, in panel 1, loci Bruce06, Bruce08, Bruce11, and Bruce55 had the same HGDI value (0.48). The greatest variation in this panel was related to the Bruce42 locus (0.66). The HGDI values ranged from 0.48 to 0.52 in panel 2A, and from 0.48 to 0.71 in panel 2B. The least variability in panel 2A and panel 2B was related to Bruce21 and Bruce04 (HGDI = 0.48), and the highest variability was related to Bruce16 (0.71) and Bruce04 (0.71), (Table 2).

Table 2. Allelic types and Hunter Gaston diversity index (HGDI) of *Brucella melitensis* strains for 16 loci in this study.

Locus	Allelic types	No. of repeats	HGDI
Panel - 1			
Bruce 06	2	1,3	0.487
Bruce 08	2	4,5	0.487
Bruce 11	2	2,3	0.487
Bruce 12	1	13	0.000
Bruce 42	3	2-4	0.660
Bruce 43	2	2,3	0.030
Bruce 45	1	3	0.000
Bruce 55	2	2,3	0.487
Panel-2A			
Bruce 18	3	4,7,8	0.523
Bruce 19	3	36,41,43	0.523
Bruce 21	2	6,8	0.487
Panel-2B			
Bruce 04	4	2,4-6	0.710
Bruce 07	3	4-6	0.546
Bruce 09	2	3,8	0.487
Bruce 16	4	3-6	0.717
Bruce 30	3	4-6	0.635

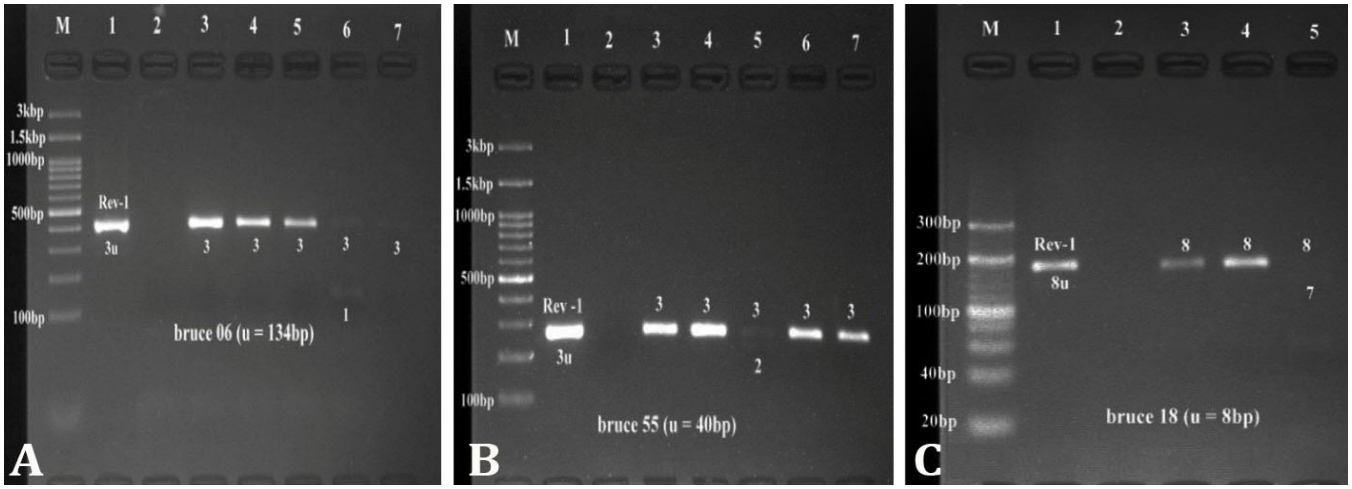


Fig. 3. Amplification patterns of loci Bruce06, Bruce55 and Bruce18 of multiple locus variable-number tandem repeat analysis typing. **A)** Bruce06: Lane M: DNA marker (100bp DNA ladder); Lane 1: Positive control (*Brucella melitensis*-bv1, Rev-1 strain); Lane 2: Negative control (reaction mixture without DNA); Lanes 3-5, 7: Positive samples with three repeats; Lane 6: Positive samples with three repeats and one repeat. **B)** Bruce55: Lane M: DNA marker; Lane 1: Positive control (*B. melitensis*-bv1, Rev-1 strain); Lane 2: Negative control; Lanes 3, 4, 6, and 7: Positive samples with three repeats; Lane 5: Positive samples with three repeats and two repeat. **C)** Bruce18: Lane M: DNA marker (20bp DNA ladder); Lane 1: Positive control (*B. melitensis*-bv1, Rev-1 strain); Lane 2: Negative control; Lanes 3, and 4: Positive samples with eight repeats; and Lane 5: Positive samples with eight repeats and seven repeats.

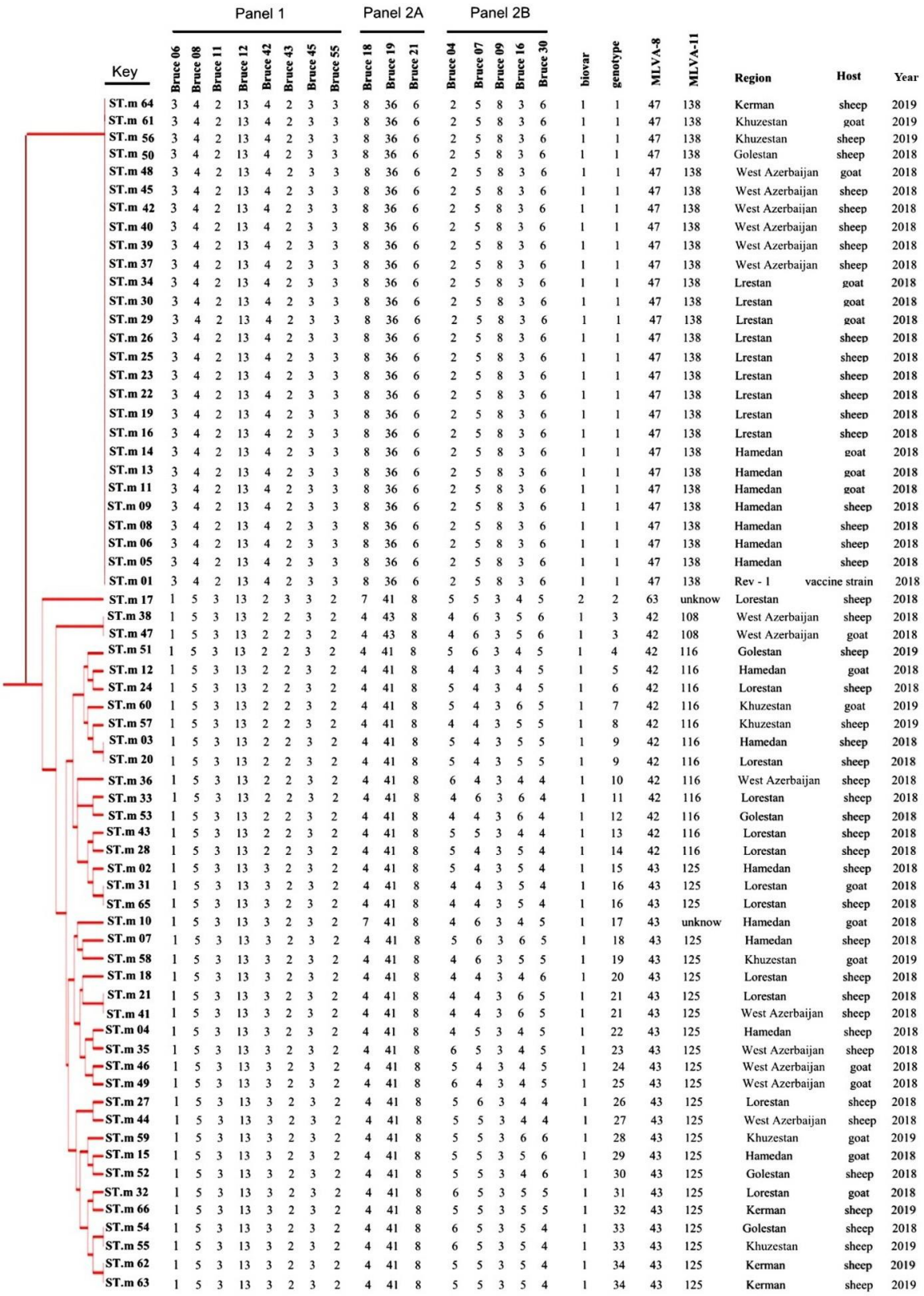
Cluster analysis for *B. melitensis* genotypes. The dendrogram for all 65 genotypes of *B. melitensis* and the vaccine strain Rev-1 (positive control) is presented in Fig. 4. Of these most were genotype 1 (all isolates shared the MLVA-11 genotype 138), which was detected in 26 isolates (Lorestan [42.80%, n = 9], Hamedan [50.00%, n = 7], West Azerbaijan [42.80%, n = 6], Khuzestan [28.50%, n = 2], Kerman [25.00%, n = 1], and Golestan [20.00%, n = 1]). Other common genotypes were genotypes 15 - 34 (all isolates shared the MLVA11 genotype 125), as identified in 23 isolates (Lorestan [28.50%, n = 6], West Azerbaijan [35.70%, n = 5], Khuzestan [42.80%, n = 3], Kerman [75.00%, n = 3], and Golestan [20.00%, n = 1]). The remaining 14 genotypes (genotypes 2 - 14) were identified in 17 other isolates. According to the MLVA-11 analysis, genotype 116 was predominant (identified in 12 isolates; Fig. 4). A total of 39 genotypes belonged to the Eastern Mediterranean group, while 26 genotypes belonged to the American group indicating the Rev. 1 vaccine strain.

Discussion

Brucellosis is an important endemic zoonosis in Iran, but limited information is available regarding the genetic diversity of pathogenic strains.[14] In the present study, during a 19-month period a total of 65 *B. melitensis* isolates were collected from small ruminants in six provinces, namely West Azerbaijan, Hamedan, Lorestan, Kerman, Golestan, and Khuzestan where according to previous studies, the highest prevalence of brucellosis in Iran has been reported.[7-10,14,30] The MLVA-16 which is currently the gold standard method for determining the genotype of *Brucella* strains was used along with the RFLP method.[15,22,31] The results revealed that *B. melitensis* biovar 1 was the predominant strain in Iran (endemic). Overall, 98.46% of the identified isolates were biovar 1.

Since 2012, all studies conducted in Iran have only detected biovar 1.[9,13,14,32,33] Only one study conducted in 2012 reported biovar 2 with a prevalence of 6.80% and biovar 1 with a prevalence of 92.80%.[8] In none of these studies, was biovar 3 identified, while according to previous studies, biovar 3 was prevalent in Iran, although to a lesser extent.[6,7] The present results are in consistent with the findings showing the predominance of biovar 1 in Iran compared to a lower prevalence of biovar 2 and the possible eradication of biovar 3.

The genotype of the Rev. 1 vaccine strain used in Iran as a positive control was determined using the MLVA-16 method.[11] Also, using MLVA-11, it was noticed that this strain had exactly the same genotype as the Rev. 1 vaccine strain used in France, Spain, and South Africa. It also showed the same genotype as the 16M reference strain of the United States and 16M of France with a mutation in the Bruce18 locus.[34] Using the MLVA-16 method, it was found that this strain had the same genotype with a mutation in locus Bruce18 with the reference strain 16M and R1 of the United States and with a mutation in locus Bruce09 with strain R1 and R26 in Spain, which is indicative of possible mutation in Rev-1 vaccine strain used in Iran. Using MLVA-8 and MLVA-11, this strain genotype was determined to be 47 and 138, respectively, while 26 of the isolates identified in the present study with MLVA-8 and MLVA-11 had the same genotype as the Rev. 1 vaccine strain. Thus, not all of the biovars identified in sheep and goat milk are wild-type bacteria, but a high percentage (32.30%, n = 26) of these isolates are Rev. 1 vaccine strain excreted in milk.



Key	Panel 1								Panel 2A			Panel 2B					biovar	genotype	MLVA-8	MLVA-11	Region	Host	Year
	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							
ST.m 64	3	4	2	13	4	2	3	3	8	36	6	2	5	8	3	6	1	1	47	138	Kerman	sheep	2019
ST.m 61	3	4	2	13	4	2	3	3	8	36	6	2	5	8	3	6	1	1	47	138	Khuzestan	goat	2019
ST.m 56	3	4	2	13	4	2	3	3	8	36	6	2	5	8	3	6	1	1	47	138	Khuzestan	sheep	2019
ST.m 50	3	4	2	13	4	2	3	3	8	36	6	2	5	8	3	6	1	1	47	138	Golestan	sheep	2018
ST.m 48	3	4	2	13	4	2	3	3	8	36	6	2	5	8	3	6	1	1	47	138	West Azerbaijan	goat	2018
ST.m 45	3	4	2	13	4	2	3	3	8	36	6	2	5	8	3	6	1	1	47	138	West Azerbaijan	sheep	2018
ST.m 42	3	4	2	13	4	2	3	3	8	36	6	2	5	8	3	6	1	1	47	138	West Azerbaijan	sheep	2018
ST.m 40	3	4	2	13	4	2	3	3	8	36	6	2	5	8	3	6	1	1	47	138	West Azerbaijan	sheep	2018
ST.m 39	3	4	2	13	4	2	3	3	8	36	6	2	5	8	3	6	1	1	47	138	West Azerbaijan	sheep	2018
ST.m 37	3	4	2	13	4	2	3	3	8	36	6	2	5	8	3	6	1	1	47	138	West Azerbaijan	sheep	2018
ST.m 34	3	4	2	13	4	2	3	3	8	36	6	2	5	8	3	6	1	1	47	138	Lrestan	goat	2018
ST.m 30	3	4	2	13	4	2	3	3	8	36	6	2	5	8	3	6	1	1	47	138	Lrestan	goat	2018
ST.m 29	3	4	2	13	4	2	3	3	8	36	6	2	5	8	3	6	1	1	47	138	Lrestan	goat	2018
ST.m 26	3	4	2	13	4	2	3	3	8	36	6	2	5	8	3	6	1	1	47	138	Lrestan	sheep	2018
ST.m 25	3	4	2	13	4	2	3	3	8	36	6	2	5	8	3	6	1	1	47	138	Lrestan	sheep	2018
ST.m 23	3	4	2	13	4	2	3	3	8	36	6	2	5	8	3	6	1	1	47	138	Lrestan	sheep	2018
ST.m 22	3	4	2	13	4	2	3	3	8	36	6	2	5	8	3	6	1	1	47	138	Lrestan	sheep	2018
ST.m 19	3	4	2	13	4	2	3	3	8	36	6	2	5	8	3	6	1	1	47	138	Lrestan	sheep	2018
ST.m 16	3	4	2	13	4	2	3	3	8	36	6	2	5	8	3	6	1	1	47	138	Lrestan	sheep	2018
ST.m 14	3	4	2	13	4	2	3	3	8	36	6	2	5	8	3	6	1	1	47	138	Hamedan	goat	2018
ST.m 13	3	4	2	13	4	2	3	3	8	36	6	2	5	8	3	6	1	1	47	138	Hamedan	goat	2018
ST.m 11	3	4	2	13	4	2	3	3	8	36	6	2	5	8	3	6	1	1	47	138	Hamedan	goat	2018
ST.m 09	3	4	2	13	4	2	3	3	8	36	6	2	5	8	3	6	1	1	47	138	Hamedan	sheep	2018
ST.m 08	3	4	2	13	4	2	3	3	8	36	6	2	5	8	3	6	1	1	47	138	Hamedan	sheep	2018
ST.m 06	3	4	2	13	4	2	3	3	8	36	6	2	5	8	3	6	1	1	47	138	Hamedan	sheep	2018
ST.m 05	3	4	2	13	4	2	3	3	8	36	6	2	5	8	3	6	1	1	47	138	Hamedan	sheep	2018
ST.m 01	3	4	2	13	4	2	3	3	8	36	6	2	5	8	3	6	1	1	47	138	Rev - 1	vaccine strain	2018
ST.m 17	1	5	3	13	2	3	3	2	7	41	8	5	5	3	4	5	2	2	63	unknow	Lorestan	sheep	2018
ST.m 38	1	5	3	13	2	2	3	2	4	43	8	4	6	3	5	6	1	3	42	108	West Azerbaijan	sheep	2018
ST.m 47	1	5	3	13	2	2	3	2	4	43	8	4	6	3	5	6	1	3	42	108	West Azerbaijan	goat	2018
ST.m 51	1	5	3	13	2	2	3	2	4	41	8	5	6	3	4	5	1	4	42	116	Golestan	sheep	2019
ST.m 12	1	5	3	13	2	2	3	2	4	41	8	4	4	3	4	5	1	5	42	116	Hamedan	goat	2018
ST.m 24	1	5	3	13	2	2	3	2	4	41	8	5	4	3	4	5	1	6	42	116	Lorestan	sheep	2018
ST.m 60	1	5	3	13	2	2	3	2	4	41	8	5	4	3	6	5	1	7	42	116	Khuzestan	goat	2019
ST.m 57	1	5	3	13	2	2	3	2	4	41	8	4	4	3	5	5	1	8	42	116	Khuzestan	sheep	2019
ST.m 03	1	5	3	13	2	2	3	2	4	41	8	5	4	3	5	5	1	9	42	116	Hamedan	sheep	2018
ST.m 20	1	5	3	13	2	2	3	2	4	41	8	5	4	3	5	5	1	9	42	116	Lorestan	sheep	2018
ST.m 36	1	5	3	13	2	2	3	2	4	41	8	6	4	3	4	4	1	10	42	116	West Azerbaijan	sheep	2018
ST.m 33	1	5	3	13	2	2	3	2	4	41	8	4	6	3	6	4	1	11	42	116	Lorestan	sheep	2018
ST.m 53	1	5	3	13	2	2	3	2	4	41	8	4	4	3	6	4	1	12	42	116	Golestan	sheep	2018
ST.m 43	1	5	3	13	2	2	3	2	4	41	8	5	5	3	4	4	1	13	42	116	Lorestan	sheep	2018
ST.m 28	1	5	3	13	2	2	3	2	4	41	8	5	4	3	5	4	1	14	42	116	Lorestan	sheep	2018
ST.m 02	1	5	3	13	3	2	3	2	4	41	8	5	4	3	5	4	1	15	43	125	Hamedan	sheep	2018
ST.m 31	1	5	3	13	3	2	3	2	4	41	8	4	4	3	5	4	1	16	43	125	Lorestan	goat	2018
ST.m 65	1	5	3	13	3	2	3	2	4	41	8	4	4	3	5	4	1	16	43	125	Lorestan	sheep	2018
ST.m 10	1	5	3	13	3	2	3	2	7	41	8	4	6	3	4	5	1	17	43	unknow	Hamedan	goat	2018
ST.m 07	1	5	3	13	3	2	3	2	4	41	8	5	6	3	6	5	1	18	43	125	Hamedan	sheep	2018
ST.m 58	1	5	3	13	3	2	3	2	4	41	8	4	6	3	5	5	1	19	43	125	Khuzestan	goat	2019
ST.m 18	1	5	3	13	3	2	3	2	4	41	8	4	4	3	4	6	1	20	43	125	Lorestan	sheep	2018
ST.m 21	1	5	3	13	3	2	3	2	4	41	8	4	4	3	6	5	1	21	43	125	Lorestan	sheep	2018
ST.m 41	1	5	3	13	3	2	3	2	4	41	8	4	4	3	6	5	1	21	43	125	West Azerbaijan	sheep	2018
ST.m 04	1	5	3	13	3	2	3	2	4	41	8	4	5	3	4	5	1	22	43	125	Hamedan	sheep	2018
ST.m 35	1	5	3	13	3	2	3	2	4	41	8	6	5	3	4	5	1	23	43	125	West Azerbaijan	sheep	2018
ST.m 46	1	5	3	13	3	2	3	2	4	41	8	5	4	3	4	5	1	24	43	125	West Azerbaijan	goat	2018
ST.m 49	1	5	3	13	3	2	3	2	4	41	8	6	4	3	4	5	1	25	43	125	West Azerbaijan	goat	2018
ST.m 27	1	5	3	13	3	2	3	2	4	41	8	5	6	3	4	4	1	26	43	125	Lorestan	sheep	2018
ST.m 44	1	5	3	13	3	2	3	2	4	41	8	5	5	3	4	4	1	27	43	125	West Azerbaijan	sheep	2018
ST.m 59	1	5	3	13	3	2	3	2	4	41	8	5	5	3	6	6	1	28	43	125	Khuzestan	goat	2019
ST.m 15	1	5	3	13	3	2	3	2	4	41	8	5	5	3	5	6	1	29	43	125	Hamedan	goat	2018
ST.m 52	1	5	3	13	3	2	3	2	4	41	8	5	5	3	4	6	1	30	43	125	Golestan	sheep	2018
ST.m 32	1	5	3	13	3	2	3	2	4	41	8	6	5	3	5	5	1	31	43	125	Lorestan	goat	2018
ST.m 66	1	5	3	13	3	2	3	2	4	41	8	5	5	3	5	5	1	32	43	125	Kerman	sheep	2019
ST.m 54	1	5	3	13	3	2	3	2	4	41	8	6	5	3	5	4	1	33	43	125	Golestan	sheep	2018
ST.m 55	1	5	3	13	3	2	3	2	4	41	8	6	5	3	5	4	1	33	43	125	Khuzestan	sheep	2019
ST.m 62	1	5	3	13	3	2	3	2	4	41	8	5	5	3	5	4	1	34	43	125	Kerman	sheep	2019
ST.m 63	1	5	3	13	3	2	3	2	4	41	8	5	5	3	5	4	1	34	43	125	Kerman	sheep	2019

Fig. 4. Cluster analysis for 65 small ruminants isolates of *Brucella melitensis* and Rev1 vaccine strain based on the data set of multiple locus variable-number tandem repeat analysis (MLVA)-16$_{Orsay}$. In the columns, the following data are indicated: Biovar, genotype, MLVA-8, MLVA-11, region, host and year of isolation (Year).

type 1 (all isolates shared the MLV

Studies conducted in Spain[35] and Portugal[36] have shown that the vaccine strain could be excreted through milk when adult sheep and goats were vaccinated against brucellosis. Since in Iran, from 2015 onwards, brucellosis vaccination has been performed only in lambs and kids and the milk samples in this study were collected from adult animals (to eliminate the effect of vaccine strains), the vaccine strain could not be identified in milk. It seems that these animals became carriers after receiving the vaccine and maintained the vaccine strain or became carriers due to contact with the vaccinated lambs and kids. Therefore, three years after vaccination, the excretion of vaccine strain in milk could be considered a possible health hazard for milk consumers.

In the present study, the biovar pattern obtained from enzymatic digestion of *omp*2a and *omp2b* genes was in consistent with the results obtained from the MLVA-16. Accordingly, 64 isolates were identified by the enzymatic digestion of the *omp2a* and *omp2b* genes using the Pst1 and Hinf1 enzymes, following the pattern provided by the biovar 1 strains in the MLVA-16 method. Additionally, with MLVA-16 biovar 2, we identified isolate No. ST.m 17 whose biovar pattern was not determined by enzymatic digestion. This study was in line with the study of Vergnaud *et al*.[36] which showed that the MLVA assay could verify *Brucella* species and biovar.

This study also revealed one of the less known capabilities of the MLVA typing method, which is ability to simultaneously detect two or more isolates of *B. melitensis* in contaminated samples. In the present study, simultaneous contamination of two isolates of *B. melitensis* with biovar 1 (strain ST.m 16) and biovar 2 (strain ST.m 17) in a sample obtained from a sheep was recorded (Fig. 3). Since the ST.m 16 and ST.m 17 strains showed the genotypes of the vaccine strain and the wild type of the bacteria, respectively; it can be concluded that this sheep had been first infected with *B. melitensis* biovar 2 and then received the Rev-1 vaccine. In addition, the ST.m 17 strain which showed genotype 63 and genotype 111 (with a mutation in one locus) by MLVA-8 and MLVA-11 methods, respectively, had been observed in neighboring countries such as Afghanistan, Pakistan and Türkiye.[23,37] This isolate seems to have entered Iran from one of these countries and mutated at the Bruce18 locus (Fig. 3C).

The present study showed that, based on the analysis of MLVA-11, *B. melitensis* strains with genotypes 125 and 116, had the highest prevalence rates of 35.30% (23 isolates) and 18.40% (12 isolates), respectively among small ruminants in Iran.

These genotypes had previously been identified separately in a human isolate of *B. melitensis* in the study by Georgi *et al*.[23] and the relevant information has been recorded on the website (http://mlva.u-psud.fr). Also, these genotypes exist in Iran's neighboring countries, such as Türkiye and Iraq. For example, in Türkiye genotype 125 is the dominant genotype of *B. melitensis*.[17, 23,34,38,39] Among the identified isolates, two isolates, namely ST.m 38 and ST.m 47, isolated from sheep and goats, respectively showed genotype 108 based on MLVA-11; this genotype had only been identified in West Azerbaijan province. It has also been reported in neighboring Türkiye which maybe the indicative of livestock exchanges between the two regions. Although these two isolates were isolated from two different herds in West Azerbaijan province, they showed the same genotype (genotype No. 3) based on MLVA-16. It seems that a number of MLVA genotypes is limited to certain geographical areas. There are few comprehensive studies on MLVA typing of *B. melitensis* strains in neighboring countries except for Türkiye. Comparison of the present study with studies conducted in Türkiye showed that in addition to a common genotype among isolates, comparison of genetic diversity showed that in these two countries, the *B. melitensis* strains contained a Bruce42 locus in panel 1 and loci Bruce04, Bruce07, Bruce16, and Bruce30 in panel 2B. The HGDI values were high and similar, indicating the genetic similarity of the studied strains in these countries.

Although the discriminatory power of MLVA- 8 (panel 1) is lower for assessing of the cross-transition among cases, the results of this panel provide useful information regarding the distribution of the genotypes between countries. According to MLVA-8, genotypes 42 and 43, which were more common in the present study, were also observed in other parts of the world.[17, 38,39] While, genotypes 49 and 51, typical of the West Mediterranean family, were not identified in this study, these data show that animal *Brucella* isolates in Iran belong to the Eastern Mediterranean group. Moreover, the results of MLVA-11 analysis (panel 1 and panel 2A) indicated six genotypes, while by adding panel 2B to panel 1 and panel 2A (MLVA–16), the number of genotypes reached 34. Although the HGDI level was high in the Bruce42 locus, panel 1 showed less variability than panels 2A and 2B, and panels 2A and 2B showed high genetic diversities.

In terms of area, the highest genetic diversity was reported in Lorestan Province, with a total of 12, eight, eight, six, five, and three genotypes found in Lorestan, Hamedan, West Azerbaijan, Khuzestan, Golestan, and Kerman provinces, respectively. The isolates obtained from Lorestan, Hamedan, West Azerbaijan, and Khuzestan provinces had almost similar genotypes due to their shared borders and the high rate of livestock movements among them. Genotype 9 was common in Lorestan and Hamedan provinces, while genotype 33 was common in Khuzestan and in Golestan provinces.

The present findings demonstrated that the potential excretion of *B. melitensis* Rev. 1 vaccine strain in milk poses a health risk to humans. Therefore, some potential dangers of Rev. 1 vaccination should be considered in

national control programs. This study showed that genetic diversity was very high among *B. melitensis* isolates in small ruminants in Iran. The MLVA typing was not only suitable to differentiate vaccine strains from other strains of *B. melitensis,* but it could also distinguish two different *B. melitensis* biovars simultaneously in any given infected animal or human. Additionally, MLVA typing could be significantly effective in the epidemiological trace back investigation of *Brucella* infections, assisting the control and management programs of brucellosis in Iran. The present study presented epidemiological data of *B. melitensis* isolates in Iran using MLVA genotypes. However, despite limitations such as the length of the sampling period and the low number of isolated bacteria, further research is needed to obtain more information about the epidemiology of brucellosis in Iran.

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Conflicts of interest

The authors declare that they have no conflicts of interest.

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