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Genetic Diversity and Phylogenetic Relationship of Clinical Isolates of *Brucella melitensis* Based on Gene Polymorphism of β Subunit of RNA Polymerase (*rpoB*) Gene in Iran

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ABSTRACT

Background: The prevalence of *Brucella* infections in animals and humans has indicated the important need for different regional/local reference laboratories to use valid species-determining approaches to facilitate and compare data exchange. The purpose of current study was to evaluate the RNA Polymerase Beta Subunit (*rpoB*) as a molecular marker in *Brucella* species differentiation and to determine the genotype of *Brucella melitensis* species using single-nucleotide polymorphism (SNP) analysis.

Materials & Methods: In this study, blood and cerebrospinal fluid (CSF) samples were taken from 108 patients with brucellosis. After culturing the samples insupplemented *Brucella* agar, eleven isolates of *Brucella* bacteria were isolated and identified by classical and molecular biotyping methods. Then the complete sequence of their *rpoB* gene was multiplied and sequenced. Sequencing results were analyzed by Mega6 program.

Results: According to the results, the *rpoB* gene was able to differentiate between *Brucella* species and other bacteria.Moreover, the *rpoB* typing grouped the majority of Iranian isolates in the rpoB type 2, while only one strain belonged to the rpoB type 1. Among the 10 isolates of *rpoB* type 2, there are six different isolates with only one unique type-2 SNPs in codon 985, which gives rise to new genotype 2 variants.

Conclusion: Our results shown a high discriminative power of *rpoB* gene among *B. melitensis* strains from some regions of Iran, which leads to accurate genotype and identification of these bacteria.

Keywords: RpoB, *Brucella melitensis,* SNP analysis, Phylogenetic

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Introduction

Brucellosis is the most common zoonotic disease that infects animals and humans through contaminated animals and their products. The disease is caused by an intracellular small gram- coccobacillus of the genus *Brucella,* which has no capsules, flagella and spores cause destructive disease that leads to great economic losses to the livestock industry by reducing milk production Humans are usually infected through consumption of contaminated dairy products or close contact with infected animals **(1)**. Human infection can lead to a chronic debilitating disease whose nonspecific symptoms affect various organs **(2, 3)**. The genus *Brucella* has six classic species, including: . *Brucella abortus*, *Brucella*

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melitensis, *Brucella ovis*, *Brucella suis*, *Brucella neotomae*, *Brucella canis*. *B. melitensis*, *B. abortus*, *B. canis*, and *B. suis* can cause brucellosis in human **(4)**. In addition, six non-classical species are known as *Brucella* species. Reports published in the last decade in Iran have introduced *B. melitensis* as the main pathogen responsible of brucellosis in human. Although. *B. abortus* was also involved to much lesser extent **(6, 5)**. Rapid and accurate identification of *Brucella* species can indicate geographical and host origin. Currently, differentiation between species and biovars of *Brucella* are done using various analyze according to phage typing, phenotypic characteristics of lipopolysaccharide antigen, color sensitivity, need for carbon dioxide, the production of sulfide-hydrogen gas and metabolic properties **(7)**. Diagnosis and detection of brucellosis based on biochemical tests is inefficient to differentiate *Brucella* species due to the presence of unknown behavioral strains in these experiments **(8)**. Also, these experiments are potentially dangerous, complex and time consuming for laboratory technicians. Furthermore, *Brucella* identification complication can occurr due to similar differences between some species and inconstancy in reporting several phenotypic traits **(9, 10)**. The use of genetic characteristics has been investigated using molecular DNA technology to address these shortcomings. Many PCR-based methods have been developed for rapid identification of *Brucella* species. For this purposes, molecular tests of the genome using the BCSP31 gene or 16SrRNA-23S operon are sufficient **(11, 12)**, but for the other aims such as epidemiological tracking, more accurate methods are required. Due to the high DNA homology (above 90%) among *Brucella* species **(14, 13)**, the significant genetic differences are mononucleotide polymorphisms, and regions with high genomic diversity between species are very rare **(15)**. Several PCR-based methods have been used to determine the exact molecular biomarkers to determine *Brucella* molecular type **(12)**. Various studies have reported that the use of the RNA polymerase (*rpoB*) β subunit gene, which is very suitable for phylogenetic analysis and identification *Brucella* strains, especially in highly homologous isolates (8, 15, 16 RpoB-based genotyping also allows the identification of new bacterial species and analysis of the bacterial community **(16)**. It can also describe *rpoB* gene mutations that play a very important role in rifampicin resistance **(17)**. the present study evaluates the effectsof single nucleotide polymorphism (SNP) assays on the *rpoB* gene to show intra-species diversity occurring in different *B. melitensis* strains collected from various regions of Iran. (this part of manuscript is too long).

Materials and Methods

Sampling and bacteria isolation

In this study, a collection of 106 blood and 2 cerebrospinal fluid samples from patients with brucellosis symptoms from2017-2019 were cultured by the Brucellosis Department of Razi Vaccine and Serum Research Institute (Karaj, Iran). All samples were cultured in selective *Brucella* agar medium with Cycloheximide (50.0mg), Vancomycin (10.0mg), polymyxin B (2,500 IU), Bacitracin (12,500 IU), Nystatin (50,000 IU), and Nalidixic acid (2.5mg) (Oxoid, Basingstoke, UK) and 5% of inactivated horse serum. Bacterial cultures were incubated for 10 days at 37 ° C and 10% carbon dioxide. After this steps , the isolated bacteria were identified by classical typing methods **(5, 18)**. Brucella isolated from patients and standard bacterium of *B.melitensis* 16M (ATCC 23456) were then used in this study to analyze *rpoB* gene by single nucleotide polymorphism. Genomic DNA extraction was performed based on the manufacturing protocol by a high purity PCR template preparation kit (Roche, Germany) on isolated bacteria. The integrity of DNA was evaluated by 1% agarose gel and the concentration of DNA was assessed at 260/280 nm by a Nanodrop Spectrophotometer (Wilmington, DE, USA). The DNA of bacterial samples were then preserved at − 20 °C for analysis.

Molecular confirmation of isolated bacteria

Molecular identification of isolated bacteria in *Brucella* agar-specific medium was performed based on the IS711-based polymerase chain reaction (AMOS-PCR) and Bruce-ladder PCR were carried out on all extracted DNA to analyze the *Brucella* presence in samples. Amos-PCR amplification using 5 primers on the IS711 gene (Table 1) was done in a thermal program of 1 cycle of 95 ° C for 5 minutes, followed by 40 cycles of denaturation at 95 ° C for 30 seconds, annealing temperature at 55 ° C for 60 seconds, extension at 72 ° C for 3 minutes and final extension at 72 ° C for 10 minutes **(19).** Molecular typing also was performed by multiplex PCR (Bruce-ladder) with 16 primers on 8 different *Brucella* genes (Table 1) as follows: 95 ° C for 5 minutes, followed by 30 cycles at 95 ° C for 30 minutes,56 ° C for 90 seconds, 72 ° C for 3 minutes and at 72 ° C for 10 minutes **(4)**. All reactions of PCR were conducted in a total volume of 25 µL including 0.2 mM deoxynucleotide triphosphate, 0.5 mM of each primer, 10 mM Tris–HCl (pH 8), 50 mM KCl, 1.5 mM $MgCl_2$, and 0.05 IU of Taq polymerase. The PCR products were run by electrophoresis on the 1.5 % agarose gel. All applied primers in this study are mentioned in the table 1.

PCR assay on the rpoB gene and its genotyping assignment

The PCR amplifications of the *rpoB* gene was done with specific primers using the *B. melitensis* 16M (accession number AE009516) as reference. Upstream primer, 1rB (5-ATGGCTCAGACCCATTCTTTC-3), and a downstream primer, 4134rB (5-TTATTCTGCCGCGTCCGGAA-3) were used to amplify the whole length of rpoB gene with 4,134-bp **(17)**. PCR amplifications were performed with

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25 µL of PCR mixture comprised of 10 mM Tris–HCl (pH 8), 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM deoxynucleotide triphosphate, 0.05 IU of Taq polymerase, 0.5 mM of each primer and 100 ng of genomic DNA (evaluated by O.D. at 260 nm). To determine the *rpoB* types of different *B. melitensis* phenotypic biovars, the *rpoB* molecular targeting of specific residues on the codons 629, 985, 1249, and 1309 were performed using Mega 6 software **(20)**.

Sequence analysis

The purification of PCR products were performed by PCR product purification kit (GeneAll Company, South Korea) and sent to Fazapjooh Company for sequencing. The *rpoB* sequences were then aligned and assembled by the Mega 6 software program **(21)**. In addition, the amino acids were deduced by the CLUSTAL W method of the Mega 6 program. After that all generated sequences with specific primers and length of 4134 bp, were compared by *rpoB* gene of *B. melitensis* 16M (accession number AE009516) for evaluation of nucleotide diversity. Sequencing was performed twice to confirm the results of reported mutations in this study.

Phylogenetic analysis

The consensuses data of all sequences were evaluated using the NCBI (Blast) alignment search tool to confirm *rpoB* genes matching. A phylogenetic tree was then drawn with Bootstrap 1000 **(22)**. Bootstrap values from 1000 Bootstrap datasets were displayed at the end of the nodes.

Table 1. Primers used to identify isolated bacteria and PCR product sizes expected for different types of *Brucella*

Bacterial strains	Primer set	Primer sequence (5-3')	DNA target	size (bp)	References
B. abortus	IS711 AB	TGCCGATCACTTTCAAGGGCCTTCAT GACGAACGGAATTTTTCCAATCCC	IS711	498	(19)
B. melitensis	IS711 BM	TGCCGATCACTTTCAAGGGCCTTCAT AAATCGCGTCCTTGCTGGTCTGA	IS711	731	(19)
B.ovis	IS711 *B.ovis*	TGCCGATCACTTTCAAGGGCCTTCAT CGGGTTCTGGCACCATCGTCG	IS711	976	(19)
B.suis	IS711 *B.suis*	TGCCGATCACTTTCAAGGGCCTTCAT GCGCGGTTTTCTGAAGGTTCAGG	IS711	285	(19)
B. abortus *B. melitensis* *B. melitensis Rev.1*	BMEI0998f BMEI0997r	ATC CTA TTG CCC CGATAA GG GCT TCG CAT TTT CACTGT AGC	Glycosyltransferase, gene wboA	1,682	(4)
B. abortus *B. melitensis* *B. melitensis Rev.1*	BMEI0535f BMEI0536r	GCG CAT TCT TCG GTTATG AA CGC AGG CGA AAA CAGCTA TAA	Immunodominant antigen, gene bp26	450	(4)
Deletion of 25,061 bp in BMEII826–BMEII0850 in *B. abortus*	BMEII0843f BMEII0844r	TTT ACA CAG GCA ATCCAG CA GCG TCC AGT TGT TGTTGA TG	Outer membrane protein, gene omp31	1071	(4)
B. abortus *B. melitensis* *B. melitensis Rev.1*	BMEI1436f BMEI1435r	ACG CAG ACG ACC TTCGGTAT TTT ATC CAT CGC CCTGTCAC	Polysaccharide deacetylase	794	(4)
B. abortus *B. melitensis* *B. melitensis Rev.1*	BMEII0428f BMEII0428r	GCC GCT ATT ATG TGGACT GG AAT GAC TTC ACG GTCGTT CG	Erythritol catabolism, gene eryC (Derythrulose-1-phosphate dehydrogenase)	587	(4)
Deletion of 2,653 bp in BR0951–BR0955 in *B. melitensis* and *B. abortus*	BR0953f BR0953r	GGA ACA CTA CGC CACCTT GT GAT GGA GCA AAC GCTGAA G	ABC transporter binding protein	272	(4)
Point mutation in BMEI0752 in *B. melitensis* Rev.1	BMEI0752f BMEI0752r	CAG GCA AAC CCT CAG AAG C GAT GTG GTA ACG CAC ACC AA	Ribosomal protein S12, gene *rpsL*	218	(4)
B. abortus *B. melitensis* *B. melitensis Rev.1*	BMEII0987f BMEII0987r	CGC AGA CAG TGA CCATCA AA GTA TTC AGC CCC CGTTAC CT	Transcriptional regulator, CRP family	152	(4)

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Results

Isolation and identification of bacteria

In this study, *Brucella* isolates (n=11) were detected from 108 blood samples from cases of brucellosis, 10 bacterial isolates from blood and 1 bacterial isolate from cerebrospinal fluid. Common phenotypic characteristics of *Brucella* species were observed from isolated bacteria including small glossy t shiny, and honey colonies with smooth surface. Eleven bacterial isolates were grown after 5 days of incubation at 37°C with 10% carbon dioxide. Isolated bacteria were gram-negative in gram staining, did not produce hydrogen sulfide and did not lyse with standard phages in the classic *Brucella* detection method, and also grew on Fuchsin and tionine dyes, so according to OIE standard tables for the identification Brucellae at the biovar level, it was identified as belonging to *B. melitensis* bivar 1 and 3 (Table 2).

Table 2. *B. melitensis* isolates and identification using different molecular typing methods.

Isolate	Accession number	Source	Biotype	Bruce-ladder	Amos PCR	year	Place
S1	MK629658	Human blood	*B. melitensis* bv3	*B. melitensis*	*B. melitensis*	2018	Karaj
S2	MK629659	Human blood	*B. melitensis* bv3	*B. melitensis*	*B. melitensis*	2016	Karaj
S3	MK629660	Human blood	*B. melitensis* bv1	*B. melitensis*	*B. melitensis*	2016	Karaj
S4	MK629661	Human blood	*B. melitensis bv1*	*B. melitensis*	*B. melitensis*	2015	Qom
S5	MK790247	Human blood	*B. melitensis* bv1	*B. melitensis*	*B. melitensis*	2019	Kerman
S6	MK790248	Human synovial fluid	*B. melitensis* bv1	*B. melitensis*	*B. melitensis*	2016	Tehran
S7	MK598748	Human blood	*B. melitensis* bv1	*B. melitensis*	*B. melitensis*	2017	Karaj
S8	MK790249	Human blood	*B. melitensis* bv1	*B. melitensis*	*B. melitensis*	2018	Mashhad
S9	MK790250	Human blood	*B. melitensis* bv1	*B. melitensis*	*B. melitensis*	2015	Hamadan
S10	MK790251	Human blood	*B. melitensis* bv1	*B. melitensis*	*B. melitensis*	2019	Kermanshah
S11	MK790252	Human blood	*B. melitensis* bv1	*B. melitensis*	*B. melitensis*	1017	Yazd

Molecular confirmation of isolated bacteria

Isolated bacteria were identified and confirmed at the molecular level using AMOC PCR and Bruce-ladder multiplex methods at the species level. In terms of biotype results, it was found that the clinical isolates belonged to *B. melitensis* bivar 1 or 3. For the AMOS-PCR assay, a product of 731 bp for all isolates, indicating the genus *B. melitensis,* was identified. All isolates in Bruce-ladder PCR reaction was also confirmed the amplification of PCR products with 152, 450, 587, 794, 1,071 and 1682 bp as filed type of *B. melitensis*.

Determination of *Brucella* genotype based on *rpoB* gene analysis

The differentiating power of *rpoB* gene for *Brucella* detection was first confirmed for Iranian *Brucella* isolates using single nucleotide polymorphism analysis of *rpoB* gene, moreover, the results were compared with the sequences recorded in the NCBI database. According to our results, most Iranian isolates in this study were classified as the *rpoB* with 629-Val (GTG), 985-Val (GTC), 1249-Met (ATG) and 1309-Leu (CTA) genomic maps While only one of them belonged to *rpoB* type 1 with genomic map (629-Ala (GCG), 985-Ala (GCC), 1249-Met (ATG) and 1309-Leu (CTG). The gene and amino acid sequences were stored in the NCBI database under access numbers MK629658, MK629659, MK629660, MK629661, MK790247, MK790248, MK598748, MK790249, MK790250, MK790251 and MK790252. Also, the ability of *rpoB* gene differentiation to identify *Brucella* Iranian isolates was confirmed for the first time in this study. Most of the Iranian isolates in this study belonged to *rpoB* type 2 and only one of them belonged to *rpoB* type 1. Also, no spatial grouping of *rpoB* type 3 was identified in this study. Of the 10 *rpoB* type 2 strains, six strains with only one single nucleotide polymorphism at codon985, showed different variants, indicating the missense 985 -Ala (GCC) mutation instead of Val (GTC).

Therefore, these strains should be considered as a new variants of genotype 2 (Accession number: MK629658, MK629660, MK629661, MK790247, MK790249 and MK790252) (Fig 1). In the other four isolates, no mutation pattern was observed in codon 985 of type 2 genotype.

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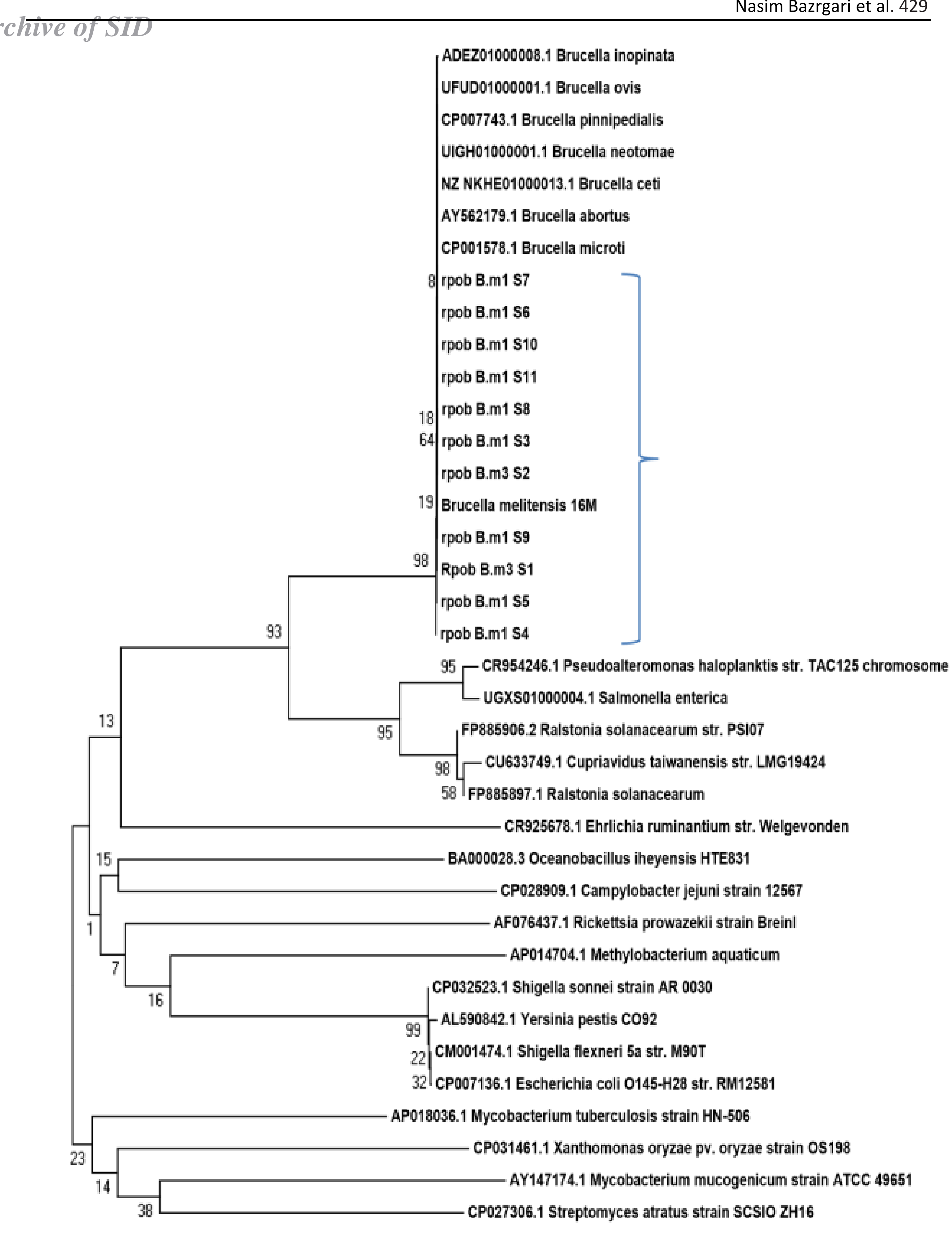

Figure 1. Phylogenetic relationship using the Neighbor-Joining method shows the power of gene differentiation between *Brucella* species and other bacteria in the figure. The percentages of replications in which related species are grouped in the Bootstrap test (1000 replicates) are shown next to the branches. The Maximum Composite Likelihood method showed evolutionary distances. Evolutionary analysis was performed on MEGA6.

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Discussion

Identification of *Brucella* species is one of the most important programs in brucellosis eradication and control issues as well as epidemiological trace analysis in human and animal. the worldwide distribution of *Brucella* infections in animals and humans highlights the vital need of different regional/local reference laboratories to use the same typing methods of *Brucella* bacteria in order to facilitate comparison and data exchange. At present, the identification Brucella species and subspecies according to the different characterization's analysis consisting of biochemical phenotype, growth needs and serology. These tests are time consuming and increase the risk of infection for laboratory staff. In addition, the limited diversity of some *Brucella* species and biovares in biochemical properties may lead to conflicting data and complex interpretations **(23)**. In some studies, it has been showed that clinical strains of *B. melitensis* isolated from human specimens show unusual phenotypic patterns in fuchsin and tionine dye sensitivity tests **(15, 16)**. More recently, the *B. melitensis* biovar types 1, 2, and 3 have been isolated from numerous studies in African, Asian, South American, and European countries that differ in color sensitivity **(24)**. Moreover, the biotypical characteristics of *Brucella* spp. isolates in Israel were serologically known as *B. melitensis* biovar 1, but showed unusual sensitivity to penicillin, and the dyes of fuchsin and tionine **(9)**. Recent observations, however, indicate that differences between the species of *B. melitensis* are not limited to the agglutination pattern. Color sensitivity, while indicating a phenotypic feature, is also a factor for the *B. abortus* biovars identification **(25)**. Hence, various molecular methods have been designed to identify *Brucella* species, for example, it has been shown that PCR-RFLP method is a fast and practical technique, particularly for differentiation and identification between different *Brucella* species and biovars of *B. abortus* and *B. melitensis* in human blood samples **(26)**. In a further study, pulse-field gel electrophoresis (PFGE) was found to be a more reliable and useful method for the molecular typing of *Brucella* strains and the determination of genetic similarity between *Brucella* isolates in humans and animals than PCR RFLP **(27)**. Also, optimized molecular hybrid methods are able to simultaneously identify and differentiate *B. abortus* and *B. melitensis* species with high specificity and sensitivity in clinical specimens **(28)**. Furthermore, detection and differentiate of *B. melitensis* and *B. abortus* species by real-time PCR and high resolution melting analysis (HRM) curves in human blood has been expressed as a useful method compared to PCR-RFLP **(29)**. In this study, comparison of *B. melitensis rpoB* sequences led to molecular and phylogenetic classification of 11 clinical isolates from different provinces of Iran (Figure 1). *RpoB*-based molecular typing enables us to determine and differentiate *Brucella* intraspecific genotypes and to analyze *Brucella* species from each other simultaneously based on a single nucleotide polymorphic analysis on the *rpoB* gene, which is not possible simultaneously in other molecular differentiation methods. Various studies have reported that the analysis of phylogenetic relationships based on *rpoB* gene was approximately three times more accurate than that obtained with 16S rRNA analyses in identifying highly homologous species of *Brucella* **(8, 15, 30)**. Also, the 16S rRNA locus lacks sufficient sequence diversity to differentiate *Brucella* species **(30)**.

Furthermore, New identification methods have recently been reported by targeting the rpoB gene fragment located between positions 625, 985,1249, and 1390 to identify Brucella species.

Using this approach makes it possible to identify and differentiation closely identical bacteria with high homology in the genome. This method also revealed high sensitivity in differentiating *Brucella* spp. genotypes by *rpoB* gene sequencing **(8, 31)**. Different genotypes of *B. melitensis* have been identified based on *rpoB* types due to the combination of mononucleotide polymorphisms (SNPs) in codons 629, 985, 1249 and 1309 **(16)**. Up to now, 3 important genotypes according to the B*. melitensis rpoB type* have been demonstrated in different counties, including the *rpoB* type 1, 629-Ala (GCG), 985-Ala (GCC), 1249-Met (ATG) and 1309-Leu (CTG); the *rpoB* type 2, 629-Val (GTG), 985-Val (GTC), 1249-Met (ATG) and 1309-Leu (CTA); and the *rpoB* type 3, 629-Ala (GCG), 985-Ala (GCC),1249-Ile (ATA) and 1309-Leu (CTG) (16, 20). The finding of the current study are consistent with the findings of a Spanish study that showed similar missense mutations at the same location (codon 985) for three of *B. melitensis rpoB* type 2. Other missense mutations have been reported in Turkey in genotype 2 of *B. mensensis*, including only two of the three missense mutations that were identified by Marianli et al. **(16)**. According to Tan et al., most of the strains collected from American and European countries belong to type 1 *rpoB*, while type 2 *rpoB* is mainly reported among strains collected from Asia, Africa and Europe **(8)**. However, our results showed that both type 1 and type 2 of *rpoB* were present in the Iranian isolates of *B. melitensis*. Phylogenetic analyzes performed in other studies also confirmed the use of neighbor-joining method in this study (15, 32). Finally, it can be confirmed that despite the high DNA homology in the genus *Brucella*, the *rpoB* gene can act as a highly specific and stable molecular marker in this gene **(15, 32)**.

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This method also allows for rapid differentiation and identification of *Brucella*.

Conclusion

In the present study, the genotyping results of *B. melitensis* isolates using single nucleotide polymorphism analysis on the rpoB gene led to the successful identification and classification of Iranian clinical isolates and provided a better understanding of the distribution and transmission of *Brucella* spp. infecting human at a regional level. It was also found that biovar level detection could be performed without the need for special laboratory facilities.

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Conflict of Interest

Authors declared no conflict of interests.

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