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Investigation of Mutations in the Rifampin-Resistance-Determining Region of the *rpoB* Gene of *Brucella melitensis* by Gene Analysis

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Abstract

Background: RNA polymerase beta subunit (*rpoB*) gene analysis in bacterial communities is known as a method for determining rifampin sensitivity and genetic diversity among *Brucella* spp. Detection of antibiotic resistance among *Brucella* isolates can be a critical approach to control brucellosis. However, *rpoB* gene analysis of *Brucella melitensis* for assessing rifampicin resistance has not yet been performed in Iran, which is considered an endemic area for brucellosis.

Objectives: The aim of this study was to analyze the whole sequence of *rpoB* genes of different *B. melitensis* isolates from humans to identify the single-nucleotide polymorphisms (SNPs) and mutations related to rifampin resistance and to analyze the genetic diversity of these bacteria in Iran.

Methods: Between 2017 and 2019, a total of 156 blood samples along with 12 synovial fluid specimens were collected from brucellosis patients in different Iranian provinces and subjected to bacterial culture in *Brucella* selective media. *Brucella* identification was carried out using classical biotyping and molecular examinations. Polymerase chain reaction (PCR)-based amplification of the *rpoB* gene was performed by specific *rpoB* primers for whole gene sequencing. The antimicrobial susceptibility of *Brucella* isolates was assessed using disk diffusion susceptibility tests and minimal inhibitory concentration (MIC) methods. The presence of rifampin-binding sites and SNPs were investigated through *rpoB* whole gene sequencing.

Results: Clinical *B. melitensis* isolates were obtained from blood (13) and synovial fluid (1) samples of patients from different regions of Iran. The results of MIC and disk diffusion susceptibility tests showed that all the isolates were sensitive to rifampin except for one isolate showing intermediate rifampin resistance based on the standards defined for slow-growing bacteria by the Clinical and Laboratory Standards Institute (CLSI). Gene analysis for identifying the mutations related to rifampin resistance and investigating genetic diversity showed that none of the *B. melitensis* isolates had missense mutations, confirming the susceptibility of all the studied isolates to rifampin.

Conclusions: The present study revealed that *rpoB* gene analysis could be used for the efficient and precise identifying of the mutations related to rifampin resistance, investigating rifampin binding sites, and genotyping *Brucella* species. Furthermore, the identification of *B. melitensis* isolates with intermediate resistance to rifampicin highlighted the importance of periodically carrying out antibiotic susceptibility testing. The molecular detection of *rpoB* mutations in different *Brucella* isolates may help to prevent the spread of rifampin-resistant *Brucella* spp. among humans and livestock.

Keywords: *rpoB*, Rifampin Resistance, *Brucella melitensis*

1. Background

Brucellosis is known as a zoonotic infection caused by *Brucella* spp. belonging to the class of alpha-proteobacteria and the family of *Brucellaceae*. This disease is responsible for high morbidity among animals and humans, leading to considerable health and economic costs in many countries (1). The main species responsible for brucellosis in Iran are *Brucella melitensis* and *B. abortus* (1). According to different Iranian reports, *B. melitensis* has been identified as the most prevalent bacteria responsible for human brucellosis; however, *B. abortus* has also been reported to a much lesser extent (1). On the other hand, antibiotic therapy is the main approach used for the efficient treatment of human brucellosis. Nonetheless, the intracellular properties of this pathogen cause the relapse of the disease in 5 - 14% of the patients subjected to antibiotic treatment (2, 3).

A six-week course of combination therapy with rifampin and doxycycline is recommended as the treatment

of choice by the World Health Organization (WHO) (4). Rifampin combined with quinolones appeared to be significantly less efficient compared to the combination of doxycycline with either streptomycin or rifampin. Rifampin is an important and efficient antibiotic for treating brucellosis and has been repeatedly recommended as the best first-line therapy (4, 5). This antibiotic has a good bacteriostatic or bactericidal effect with ideal intracellular penetration and clear synergism with other antibiotics proposed by the WHO for brucellosis treatment (6). However, there have been several reports on increasing rifampin resistance rates among *Brucella* isolates from Iran (7-9), Egypt (10), Turkey (11), Saudi Arabia (12), and Qatar (13). Moreover, combined rifampin and doxycycline administration poses problems in Middle East countries because of its potential to stimulate rifampin resistance in other infections, especially tuberculosis (2).

Accordingly, antimicrobial susceptibility testing by E-tests as well as SNP analysis have been recommended as efficient approaches to monitor the effectiveness of antibiotics in the treatment of human brucellosis. This is particularly the case when studying *B. melitensis* clinical populations in endemic regions (14). On the other hand, the use of the DNA-directed RNA polymerase subunit beta (*rpoB*) gene, expressing the b-subunit of RNA polymerase, appeared to be highly effective for detecting the *rpoB* mutations causing rifampin resistance (15). The evaluation of the rifampin susceptibility profiles of *B. melitensis* and B. abortus is critical as this antibiotic is the most widely recommended and applied therapeutic agent for the treatment of human brucellosis (16, 17).

Resistance to rifampin is a growing problem in both developed and developing countries (18-20). This issue is even more difficult to handle in patients with immunodeficiency disorders (14, 21). Therefore, the rapid detection of antibiotic resistance is of overwhelming importance for the efficient management of brucellosis. Some investigations put into light the genetic basis of resistance against anti-brucellosis antibiotics among *Brucella* isolates (15, 22). Resistance against rifampin is most possibly due to point mutations as well as small insertions and deletions in the *rpoB* gene expressing the RNA polymerase beta subunit (23, 24). Also, some mutation sites in the *rpoB* gene have been identified as biomarkers for rifampin resistance in *Brucella* spp. (25).

Moreover, *rpoB* genotyping allows the identification of new bacterial species and facilitates the analysis of bacterial communities (26, 27). The frequency and nature of *rpoB* gene mutations vary significantly among different bacterial species (23, 24). However, to our knowledge, there is no data available on specific mutational patterns of rifampin resistance in the *Brucella* strains isolated from Iranian patients. In this study, *rpoB* mutations of the *Brucella* strains isolated from different parts of Iran were evaluated by whole *rpoB* gene sequencing of a 4134-bp segment. We also analyzed specific interactions between the ligands and proteins expressed by *rpoB* in the isolated *Brucella* species.

2. Objectives

The aim of the current study was to determine SNP(s) in the *rpoB* gene by sequencing the whole gene from different clinical isolates of *B. melitensis* to investigate any association between SNPs and rifampin resistance.

3. Methods

3.1. Bacteria Isolation

A total of 156 blood and 12 synovial fluid specimens were collected from brucellosis patients from different provinces of Iran (Alborz, Qom, Kerman, Tehran, Hamadan, Khorasan Razavi, Kermanshah, and Yazd) and cultured in *Brucella* selective media. All the samples were collected from 2017 to 2019. Inclusion criteria for patients were positive serological tests and presenting brucellosis symptoms. Patients with positive serological results for the Rose Bengal test (RBT), serum agglutination test (SAT), and 2-Mercaptoethanol (2-ME) test were selected for bacterial culture. The most common symptoms observed among patients were fever and fatigue accompanied with one or more of the following symptoms: night sweats, chills, weight loss, headache, arthralgia, myalgia, weakness, arthritis, and malaise.

All the samples were inoculated into *Brucella* selective agar supplemented with vancomycin (10.0 mg), cycloheximide (50.0 mg), nystatin (50,000 IU), polymyxin B (2,500 IU), nalidixic acid (2.5 mg), and bacitracin (12,500 IU) (Oxoid, UK) along with 5% inactivated horse serum. Then the culture plates were incubated at 37°C under 10% CO_2 for 10 days. The characterization of *Brucella* isolates was done by classical biotyping according to previous works (1). Genomic bacterial DNA was extracted using fresh cultures by the Exgene Cell SV kit (Gene All, South Korea) based on the manufacturer's protocol. The DNA integrity of the isolated bacteria was evaluated by 1.5 % agarose gel electrophoresis, and DNA concentration was evaluated by reading ODs at 260/280 nm by a Nanodrop Spectrophotometer. Bacterial DNA was stored at -20°C until further analysis.

3.2. Molecular Typing

The extracted genomic DNA was subjected to species-level identification according to IS711-based PCR (AMOS-PCR) and Bruce-ladder PCR to detect *Brucella* spp. The AMOS-PCR amplification with 5-primer multiplex was done at the thermal program of one cycle of denaturation at 95°C (5 min) and 40 cycles of denaturation at 95°C (30

s), annealing at 55°C (60 s), and extension at 72°C (3 min), followed by a final extension at 72°C (10 min) (28). Molecular typing by multiplex PCR (Bruce-ladder) with 16-primer multiplex PCR was done applying an initial denaturation at 95°C (5 min) and 35 cycles of denaturation at 95°C (30 s), annealing at 56°C (90 s), and extension at 72°C (3 min), followed by a final extension at 72°C (10 min) (29). *Brucella* abortus 544 and *B. melitensis* 16M were used as appropriate positive controls for PCR experiments. A sample without DNA was used as the negative control. The amplicons were separated by 1.5 % agarose gel electrophoresis. All the PCR primers used have been shown in Table 1.

3.3. RpoB Gene Amplification

The PCR amplification of the *rpoB* gene was performed using specific *rpoB* primers for whole gene amplification (15). The reaction mixture (25 μL) consisted of 0.5 mM of each primer, 0.05 IU of Taq polymerase, 0.2 mM deoxynucleotide triphosphate, 10 mM Tris–HCl (pH 8), 50 mM KCl, 1.5 mM $MgCl_2$, and 100 ng genomic DNA (evaluated by reading O.D. at 260 nm) (27, 30).

3.4. Sequence Analysis of rpoB Amplicons

After purification by a PCR purification kit, an ABI Prism 377 sequencer Genetic Analyzer (Applied Biosystems Foster City, CA) was utilized for sequencing PCR products based on the Big-Dye Terminator protocol (Applied Biosystems) (Gene All, Seoul, South Korea). The assembling of *rpoB* sequences was performed by Mega 6 software, and the amino acids were deduced according to the CLUSTAL W (31). For nucleotide diversity evaluation, all *rpoB* sequences were generated by specific primers and compared to those available in the NCBI database. Mutations were analyzed twice to confirm our findings.

3.5. Antimicrobial Susceptibility Testing

The minimum inhibitory concentration (MIC) of rifampin against clinical *Brucella* isolates was determined using the disk-diffusion susceptibility test (5 μg rifampin per disk) and rifampin E-test (0.016 - 256 μg/mL) (E-test®, Liofilchem, Italy) following CLSI guidelines. Bacterial suspensions were prepared for each isolate from fresh and pure colonies with 0.5 McFarland turbidity standards and spread onto Muller-Hinton agar (Sigma- Aldrich) supplemented with 5% sheep's blood. All the isolates were incubated at 37°C with 10% CO2, and the results were read after 48 hours. The breakpoints of *Brucella* isolates against rifampin were presented as MIC50 and MIC90. The CLSI guideline for slow-growing bacteria (*Haemophilus* spp.) was used for interpreting the results (32). All antibiotics were assessed in duplicate against all the isolates. Furthermore, *Escherichia coli* (ATCC 25922) and *B. melitensis* 16M strain (ATCC 23456) were applied as reference strains in antimicrobial susceptibility tests.

3.6. Rifampin-Binding Site and SNP Analyses for the rpoB Gene

Nine crystal structures (*Thermus acquaticus* (1YNN), *T. thermophilus* (4OIR), *Mycobacterium smegmatis* (6CCV), *E. coli* (5UAL), and *M. tuberculosis* (5UHC, 5UHB, 5UHD, 5UHG, and 5UH6)) bound to rifampin, and related molecules were identified by searching the PDB database. An in-house python script was written, performing multiple sequence alignments to compare the binding sites of the provided crystal structures with 11 clinical isolates. The generated alignment for the binding site was color-coded, allowing for discerning identical and mutated positions in the sequence. Furthermore, the mutations related to rifampin resistance were investigated at Cd 154 (GTT/TTT), Cd 526 (GAC/TAC, GAC/AAC, GAC/GGC), Cd 536 (CAC/CTC, CAC/TAC), Cd 539 (CGC/AGC), Cd 541 (TCG/TTG), and Cd 574 (CCG/CTG) of the *rpoB* gene by aligning with the reference sequence using Mega 6 software (22).

4. Results

4.1. Bacteria Isolation and Identification

Typical phenotypic features of *Brucella* spp., such as small smooth-surface honey-colored and translucent, shiny colonies, were observed in 14 isolated bacteria. A collection of clinical *B. melitensis* isolates (blood (13), synovial fluid (1)) was obtained from different regions of Iran. All the isolated bacteria grew after five days of incubation at 37°C under 10% carbon dioxide (CO_2). The isolated bacteria were Gram-negative with biovar levels as *B. melitensis* (i.e., biovar one or three).

4.2. Molecular Identification

The identity of *Brucella* species was confirmed at the molecular level using AMOS-PCR and Bruce-ladder PCR. A total of 11 *B. melitensis* strains were isolated from 168 human samples. These strains belonged to two different biovars, and the biovar one (10 cases from Qom, Kerman, Tehran, Hamadan, Khorasan Razavi, Kermanshah, and Yazd provinces) was more common than the biovar three (4 cases from Alborz province). All the isolated bacteria were recognized as wild type *B. melitensis* by both AMOS-PCR (a PCR product of 731 bp, Figure 1) and Bruce-ladder PCR (products of 1682, 794, 587, 450, 152, and 1,071 bp, Figure 2).

4.3. Analysis of Genetic Distances

The *rpoB* gene of the 14 clinical *Brucella* isolates was characterized through full-length sequencing. The nucleotide and deduced amino acid sequences of *Brucella* isolates were edited by Mega 6 software and deposited in the NCBI database with the accession numbers of MK790247, MK598748, MK790249, MK790248, MK790251, MK790252, MK629658, MK629659, MK629660, MK790250, MK629661, MW589198, MW168443, and MW589199. All nucleotides and

Table 1. Primer Sets and Expected Amplicon Sizes Related to Different *Brucella* Species

Strain Amplicon & Primer Set	Primer Sequence (5'-3')	DNA Target	Size (bp)	References
AMOS PCR		IS711	498	(28)
IS711f	TGCCGATCACTTTCAAGGGCCTTCAT			
AB r	GACGAACGGAATTTTTCCAATCCC			
AMOS PCR		IS711	731	(28)
IS711 f	TGCCGATCACTTTCAAGGGCCTTCAT			
BM r	AAATCGCGTCCTTGCTGGTCTGA			
AMOS PCR		IS711	976	(28)
IS711	TGCCGATCACTTTCAAGGGCCTTCAT			
B. ovis	CGGGTTCTGGCACCATCGTCG			
AMOS PCR		IS711	285	(28)
IS711	TGCCGATCACTTTCAAGGGCCTTCAT			
B. suis	GCGCGGTTTTCTGAAGGTTCAGG			
Bruce-ladder PCR		Glycosyl transferase, gene *wboA*	1,682	(29)
BMEI0998f	ATC CTA TTG CCC CGATAA GG			
BMEI0997r	GCT TCG CAT TTT CACTGT AGC			
Bruce-ladder PCR		Immunodominant antigen, gene *bp26*	450	(29)
BMEI0535f	GCG CAT TCT TCG GTTATG AA			
BMEI0536r	CGC AGG CGA AAA CAGCTA TAA			
Bruce-ladder PCR		Outer membrane Protein, gene *omp31*	1071	(29)
BMEII0843f	TTT ACA CAG GCA ATCCAG CA			
BMEII0844r	GCG TCC AGT TGT TGTTGA TG			
Bruce-ladder PCR		Polysaccharide deacetylase	794	(29)
BMEI1436f	ACG CAG ACG ACC TTCGGTAT			
BMEI1435r	TTT ATC CAT CGC CCTGTCAC			
Bruce-ladder PCR		Erythritol catabolism, gene *eryC* (Derythrulose-1-phosphate dehydrogenase)	587	(29)
BMEII0428f	GCC GCT ATT ATG TGGACT GG			
BMEII0428r	AAT GAC TTC ACG GTCGTT CG			
Bruce-ladder PCR		ABC transporter binding protein	272	(29)
BR0953f	GGA ACA CTA CGC CACCTT GT			
BR0953r	GAT GGA GCA AAC GCTGAA G			
Bruce-ladder PCR		Ribosomal protein S12, gene *rpsL*	218	(29)
BMEI0752f	CAG GCA AAC CCT CAG AAG C			
BMEI0752r	GAT GTG GTA ACG CAC ACC AA			
Bruce-ladder PCR		Transcriptionalregulator, CRP family	152	(29)
BMEII0987f	CGC AGA CAG TGA CCATCA AA			
BMEII0987r	GTA TTC AGC CCC CGTTAC CT			
rpoB		*rpoB* gene	4134	(15, 25)
1rB	ATGGCTCAGACCCATTCTTTC			
4134rB	TTATTCTGCCGCGTCCGGAA			

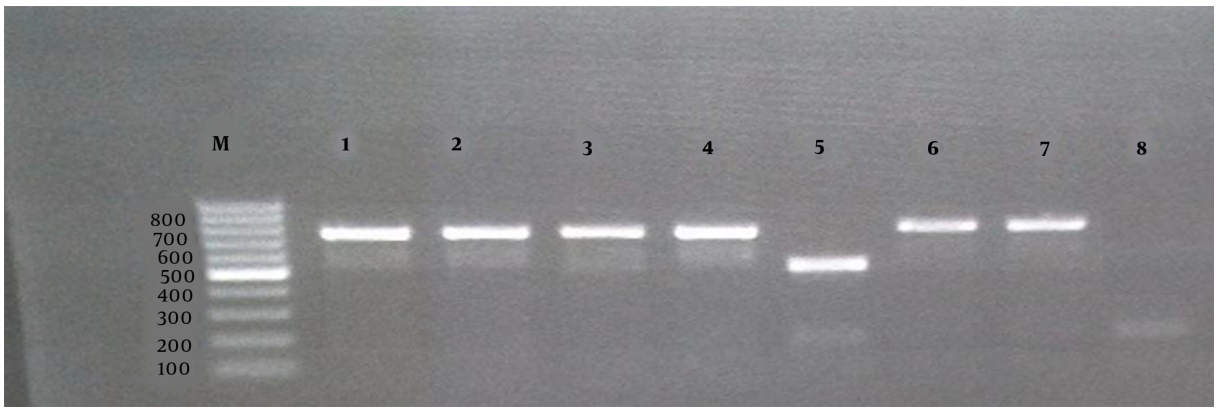

Figure 1. Agarose gel electrophoresis (1%) of the PCR products amplified by the AMOS-PCR of bacterial DNA samples. The "M" shows the DNA marker (100 bp DNA ladder). Lanes 1, 2, 3, 4, and 7 show *Brucella melitensis* field strains; Lane 5, *B. abortus* 544 as the reference; Lane 6, *B. melitensis* 16 M as the reference; Lane 8, negative control.

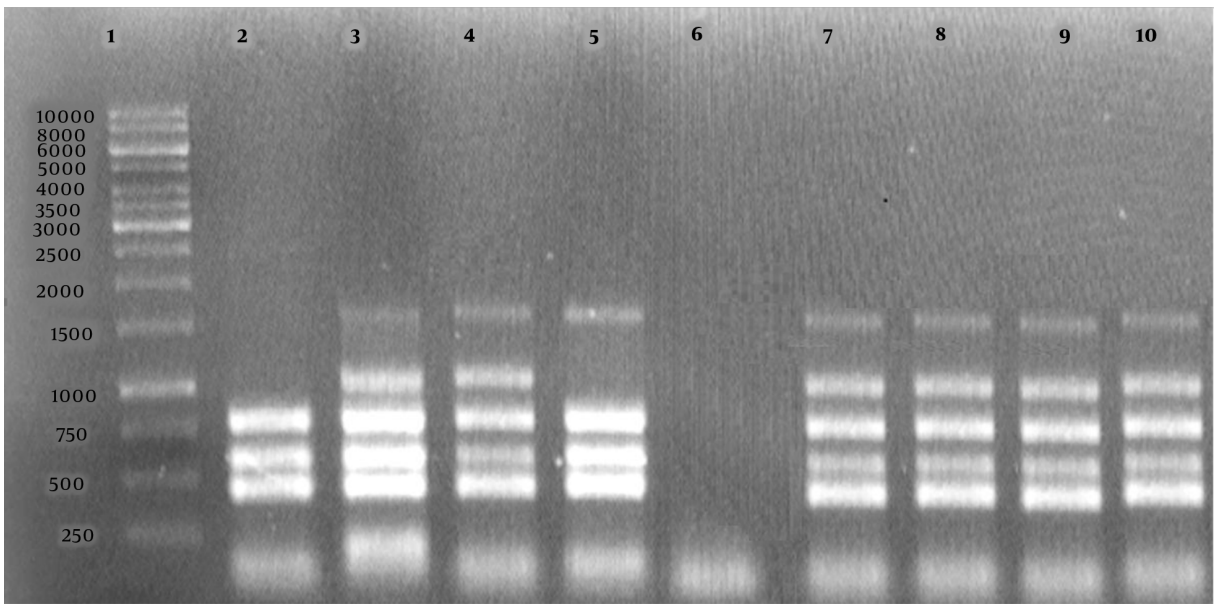

Figure 2. Agarose gel electrophoresis (1%) of the PCR products amplified by the Bruce-ladder PCR of bacterial DNA samples. Lane 1 shows the DNA marker (1000 bp DNA ladder). Lane 2 shows *Brucella abortus* RB51; Lane 3, *B. melitensis* Rev1; Lane 4, *B. melitensis* 16 M; Lane 5, *B. abortus* 544; Lane 6, negative control, Lanes 7 - 10, *B. melitensis* field strains.

deduced amino acids were scrutinized to detect missense mutations. Nucleotide sequences of the *rpoB* gene among the *Brucella* isolates showed 99% identity. The results obtained for reference strains, including *B. melitensis* B115, Rev 1, 16M, and *B. melitensis*, along with other isolates identified from other regions, were compared with those resulting from this study. Gene analysis for identifying the mutations related to rifampin resistance and evaluating genetic diversity showed that none of the *B. melitensis* isolates had missense mutations, confirming the fact that all the clinical isolates from different provinces were susceptible to rifampin. Also, according to our results, *rpoB* typing grouped the majority of the isolates from provinces of Alborz, Qom, Kerman, Hamadan, Khorasan Razavi, Kermanshah, and Yazd in the *rpoB* type 2 (629-Val (GTG), 985-Val (GTC), 1249-Met (ATG), and 1309-Leu (CTA)) while only one of them (from Tehran) belonged to the *rpoB* type 1 (629-Ala (GCG), 985-Ala (GCC), 1249-Met (ATG) and 1309-Leu (CTG)) (Table 2).

4.4. Antimicrobial Susceptibility Testing

According to CLSI breakpoints for slow-growing bacteria (*Haemophilus* spp.), we evaluated the antibiotic sensitiv-

Table 2. The Results of the Disk Diffusion Test and E-test Compared with the Sequencing Results for Mutation-Specific Codons Related to Rifampicin Resistance in *Brucella melitensis*

Brucella melitensis Isolates [a]	Isolates' Properties						Mutation Specific Codons Associated with Rifampicin-Resistant Phenotype [b]					
	Accession numbers	Province	Isolates	Genotyp	MIC results [c] (μg/ml)	Disk diffusion [d] (mm)	Cde 154GTT	Cd 526 GAC	Cd 536 CAC	Cd 539 CGC	Cd 541 TCG	Cd 574 CCG
16 M	Reference strain	-	Reference strain	2	0.75	38	GTT	GAC	CAC	CGC	TCG	CCG
***B. melitensis* bv3**	MK629658	Alborz	Blood	2	1	22	GTT	GAC	CAC	CGC	TCG	CCG
***B. melitensis* bv3**	MK629659	Alborz	Blood	2	0.38	29	GTT	GAC	CAC	CGC	TCG	CCG
***B. melitensis* bv1**	MK629660	Alborz	Blood	2	2	17	GTT	GAC	CAC	CGC	TCG	CCG
***B. melitensis* bv1**	MK629661	Qom	Blood	2	1	22	GTT	GAC	CAC	CGC	TCG	CCG
***B. melitensis* bv1**	MK790247	Kerman	Blood	2	0.38	30	GTT	GAC	CAC	CGC	TCG	CCG
***B. melitensis* bv1**	MK790248	Tehran	Synovial fluid	1	0.38	28	GTT	GAC	CAC	CGC	TCG	CCG
***B. melitensis* bv1**	MK598748	Alborz	Blood	2	0.38	29	GTT	GAC	CAC	CGC	TCG	CCG
***B. melitensis* bv1**	MK790249	Khorasan	Blood	2	0.38	27	GTT	GAC	CAC	CGC	TCG	CCG
***B. melitensis* bv1**	MK790250	Hamadan	Blood	2	0.38	30	GTT	GAC	CAC	CGC	TCG	CCG
***B. melitensis* bv1**	MK790251	Kermanshah	Blood	2	1	22	GTT	GAC	CAC	CGC	TCG	CCG
***B. melitensis* bv1**	MK790252	Yazd	Blood	2	0.38	33	GTT	GAC	CAC	CGC	TCG	CCG
***B. melitensis* bv3**	MW589198	Alborz	Blood	2	0.38	35	GTT	GAC	CAC	CGC	TCG	CCG
***B. melitensis* bv1**	MW168443	Alborz	Blood	2	0.125	38	GTT	GAC	CAC	CGC	TCG	CCG
***B. melitensis* bv3**	MW589199	Alborz	Blood	2	1	25	GTT	GAC	CAC	CGC	TCG	CCG

Abbreviations: Cd, codon; bv, biovar.
[a] Reference strains: *B. melitensis* 16M (ATCC 23456)
[b] The numbering of *rpoB* codons is based on the published *B. melitensis* 16 M nucleotide sequence (accession number AE 009516).
[c] Range: 0.125-2 μg/mL, MIC50: 0.38 μg/ml, MIC90: 1 μg/mL. MIC value of rifampin: $\leq$ 1 μg/mL sensitive, 2 μg/mL intermediate-sensitive, $\geq$ 4 μg/mL resistant.
[d] Disk diffusion value of rifampin: $\geq$ 20 mm sensitive, 17 - 19 mm intermediate-sensitive, $\leq$ 16 mm resistant.

ity of the collected isolates. Considering the antibiotic sensitivity of the isolated strains based on the disk diffusion test, inhibition zone diameters ranged from 17 to 38 mm. The range of MIC values for rifampin was from 0.125 to 2 μg/mL. All the clinical isolates were sensitive to rifampin except for one of them, which revealed intermediate rifampin resistance. The MIC50 and MIC90 values were reported as 0.38 and 1 μg/ml, respectively. The intermediate rifampin resistance observed in one of the isolates could probably lead to resistant phenotype (Table 2).

4.5. RpoB Analysis for Detecting SNPs

The rifampin-resistant strain showed no mutations at the codons 154, 526, 536, 539, 541, and 574 of the *rpoB* gene (Figure 3). Eleven *rpoB* sequences were aligned with nine *rpoB* crystal structures from *T.s acquaticus* (1YNN), *T. thermophilus* (4OIR), *M. smegmatis* (6CCV), *E. coli* (5UAL), and *M. tuberculosis* (5UHC, 5UHB, 5UHD, 5UHG, and 5UH6). In the present study, the *rpoB* sequence identity with the sequences of their crystal structures ranged from 53% to 57%. Furthermore, binding residues were mapped to analyze conservation in the *rpoB* rifampin-binding site among the isolates through the comparison of the identified binding residues with structure-ligand complexes (Figure 3).

5. Discussion

The worldwide distribution of *Brucella* spp. infections in different hosts has highlighted the crucial need for various local laboratories to apply the same typing methods to facilitate data exchange and comparison. According to our results, *rpoB* typing showed that most of the assessed Iranian *Brucella* isolates belonged to the *rpoB* type 2. These findings are in agreement with those of a study showing similar missense mutations at the same location (i.e., the codon 985) of the *rpoB* gene in three *B. melitensis* type 2 strains (25, 27). Other *rpoB* missense mutations of the genotype 2 of *B. melitensis* have also been observed in other studies (25-27, 30). The genotype 1 of *B. melitensis*, harboring the *rpoB* gene type 1, was identified in a single isolate from Iranian capital, Tehran (accession number: MK790248). On the other side, *rpoB* type 2 was found in all the other isolates collected from other Iranian provinces.

In this study, we combined microbiological and genomic analyses to evaluate specific ligand-protein interactions with rifampin according to the *rpoB* gene of *Brucella* spp. For this purpose, 14 *Brucella* isolates were recovered from 168 human specimens. We sequenced the full-length *rpoB* gene and investigated the discriminative power of rifampin interactive properties. The *rpoB* is a highly conserved housekeeping gene that is present in all bacteria due to its critical function in cellular metabolism (25, 26). Indeed, amino acid substitutions secondary to *rpoB* point mutations have been demonstrated to have important implications in the development of rifampin resistance. In previous studies, two *B. melitensis* strains showed the following mutations: Val154Phe, Asp526Tyr/Asn/Gly,

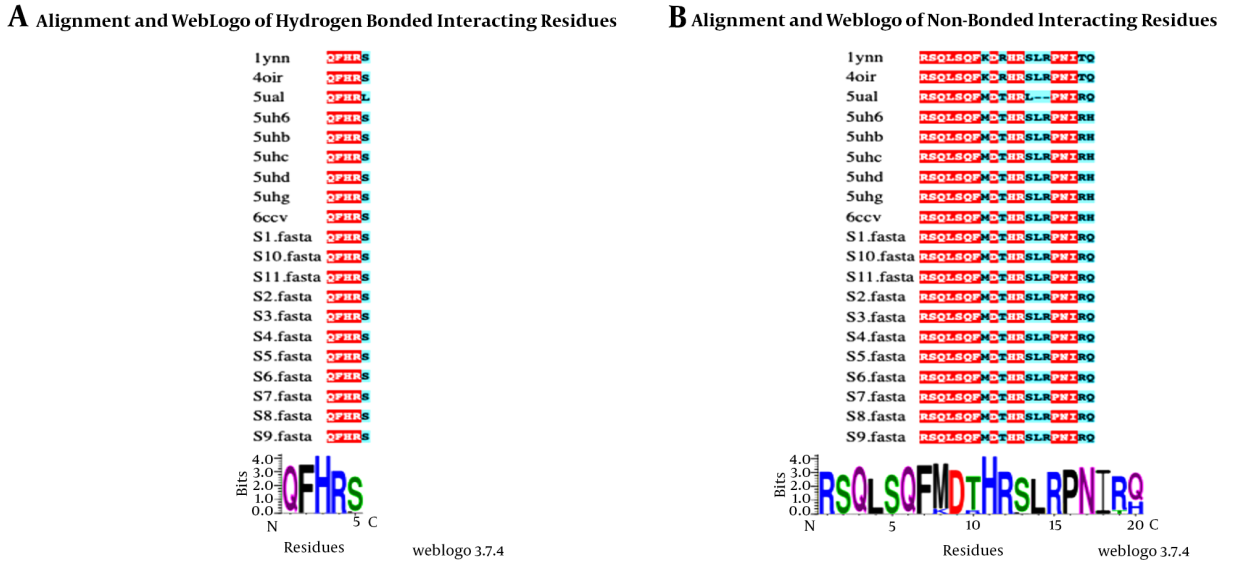

Figure 3. A, The amino acids forming hydrogen bonds with rifampin in PDB structures (identified from the PDB sum database). B, The amino acids that formed non-bonded interactions with rifampin. Identical amino acid positions have been shaded in red whereas cyan-shaded positions indicate amino acid substitutions. A web logo has been presented for both bonded and non-bonded interactions.

His536Leu/Tyr, Arg539Ser, Ser541Leu, and Pro574Leu, leading to rifampin resistance (15).

Rifampin is one of the broad-spectrum and most effective antibiotics used in multidrug regimens to treat brucellosis. Resistance against rifampin in *Brucella* spp. can easily occur in human infections (15). It has been reported that *rpoB* gene mutations can be involved in the development of rifampin resistance in different bacterial strains such as *Brucella* species (25, 26). In our analysis, all the *B. melitensis* isolated were recognized as rifampin-susceptible based on molecular testing. These findings were further confirmed by the results of the E-test, as well as disk-diffusion susceptibility tests. We found no reports investigating the molecular mechanisms of rifampin-resistance among *B. melitensis* bacteria in Iranian patients with brucellosis. In a recent investigation in our institution, in vitro antimicrobial susceptibility tests showed a MIC90 value of 1.5 mg/L and up to 1.7% intermediate susceptibility to rifampin in *B. melitensis* isolates (7). However, our results showed that all the isolates tested in this study were susceptible to rifampin. Moreover, none of them showed the most frequently observed change (i.e., His 536) in *B. melitensis* isolates. All the isolates displayed a common sequence of *rpoB* at the position of 125 to 720 (a fragment with 595 amino acids), which is specific for *B. melitensis* 16M. Our results confirmed the findings of previous studies showing neither mutations nor rifampin resistance in isolates with intermediate susceptibility to rifampin (22, 30).

The present *rpoB* data, as well as the characterization of *rpoB* mutations, showed that the *B. melitensis* isolates originating from the patients first diagnosed with brucellosis were all susceptible to rifampin. Various investigations on Iranian samples have also reported similar susceptibility findings based on the MIC method (MIC50 and 90 as 0.38 and 1 μg/mL, MIC range of 0.125 - 1.5 mg/L) (7, 33). Therefore, the selection of rifampin as the first choice for the treatment of brucellosis is supported by these findings. The results of this study also supported the applicability of molecular testing for investigating rifampin susceptibility. On the other hand, in vitro antimicrobial susceptibility testing of *Brucella* can be performed by several other techniques, including agar dilution, broth microdilution, and the E-test (34). However, antimicrobial susceptibility testing for highly zoonotic bacteria such as *Brucella* needs a laboratory with level-3 biosafety, which may not be accessible in many regions (35).

Moreover, traditional antibiotic susceptibility tests require several passaging of *Brucella*, which is a high-risk and time-consuming activity (22). Furthermore, in vitro antimicrobial susceptibility methods for *Brucella* spp. need proper standardization (36). Therefore, molecular approaches could be safer and more convenient for studying antimicrobial resistance in *Brucella* spp. Different studies have reported many missense mutations in the *rpoB* gene of *M. tuberculosis*, *Legionella pneumophila*, *Helicobacter pylori*, and *E. coli*, developing resistance to rifampicin (37, 38). To date, the susceptibility of *B. melitensis* clinical isolates to rifampin had not been assessed in Iran using molec-

ular methods based on *rpoB* gene mutation analysis. In this study; however, no rifampin resistance-associated missense mutations were detected in the *rpoB* gene of the *B. melitensis* strains isolated from Iranian patients. Nevertheless, phenotypically intermediate resistance to rifampin is, per se, a matter of concern. Therefore, SNP analysis can be proposed as a useful technique for regular screening of rifampin resistance in *B. melitensis* isolates.

5.1. Conclusions

According to our results, *rpoB* gene SNP analysis was performed to characterize rifampin resistance-associated missense mutations in the *B. melitensis* strains isolated from the Iranian patients first-diagnosed with brucellosis. All the isolates showed a common sequence at the analyzed positions (i.e., Cd 154, Cd 526, Cd 536, Cd 539, Cd 541, and Cd 574) of the *rpoB* gene, and none of them showed the *rpoB* gene missense mutations previously reported in rifampicin-resistant *B. melitensis*. All the studied isolates were found to be rifampicin-susceptible according to molecular analysis. These findings were further confirmed by the results of the E-test and disk-diffusion susceptibility tests. Furthermore, the *rpoB* gene displayed potential polymorphisms that could be used for the differentiation of all species of *Brucella* and their biovars. These observations highlight the need for regular monitoring of rifampin susceptibility in *Brucella* spp. by molecular methods in vitro.

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Footnotes

Authors' Contribution: Study concept and design: M. D.; Acquisition of data: N.B.; Analysis and interpretation of data: M. D. and S.H.; Drafting of the manuscript: M. D.; Critical revision of the manuscript for important intellectual content: M. D. and S.H.; Statistical analysis: M. D. and S.H.; Administrative, technical, and material support: GA.G.

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