Molecular characterization of mutations in \textit{gyrA}, \textit{parC}, \textit{rpoB}, and \textit{aadA1} genes of human Brucellosis

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Summary

Brucellosis or Malta fever is a life-threatening systemic human infection and one of the most common zoonotic diseases worldwide, causing considerable public health and economic problems. The Determination of antibiotic susceptibility profile of \textit{Brucella} spp. in each region or country is clinically important and has essential role in epidemiological studies and infection control policy planning. The present study aimed to determine the occurrence of the most common antimicrobial resistance genes among \textit{Brucella} spp. isolated from Iranian patients.
This cross-sectional multicenter study was conducted on non-repetitive blood specimen from patients suspected to have brucellosis for a period of two years from January 2015 to January 2017 referred to hospitals in Tehran, Ahvaz, Hamadan, Qazvin and Mianeh in Iran. The presence of mutations in gyrA, parC, rpoB, and aadA1 genes within Brucella isolates were investigated by PCR method. Ninety-one blood samples were included in this study. Presence of the Brucella spp. was confirmed in 52 (55%) samples molecularly. PCR screening for the presence of antibiotic resistance mechanisms showed that 26 (50%), 4 (7.7%) and 2 (3.8%) of isolates were positive for aadA1, parC and gyrA genes, respectively. None of the isolates contained rpoB genes. Concurrent presence of parC/aadA1 genes was found in 3 (5.8%) of isolates. In conclusion, this is the first study to investigate the frequency of antibiotic resistance mutations in Brucella isolates in Iran, detecting antibiotic resistance-inducing mutations to commonly used antibiotics.

Key words
Brucellosis, Brucella, Antibiotic resistance, Mutation

Introduction
Brucellosis or Malta fever is a life-threatening systemic infection and one of the most common zoonotic diseases worldwide resulting in considerable public health issues and economic losses.1,2 Annually, more than 500,000 new cases are suspected to have brucellosis, with substantial residual disability but minimal reported mortality, worldwide. The disease is still endemic in Middle East countries, Africa, Latin America and Central Asia and is considered an important cause of travel-associated morbidity.3,4 Brucella is a Gram-negative facultative intracellular pathogen and consists of numerous different species with zoonotic potential, the main being Brucella melitensis, Brucella abortus, Brucella suis, and Brucella canis mainly affecting sheep and goats, cattle, swine, and dogs, respectively.5

Generally, the transmission routes to human occurs mainly by direct contact with infected animals, consumption of contaminated animal products, or through the inhaling airborne agents.6 According to literature review, the brucellosis appears in the forms of the acute, sub-acute or chronic and presents itself usually with flu-like symptoms; including fever, weakness, malaise and weight loss.7 Cardiac and neurological complications can also occur at the chronic stage of brucellosis.8

The optimal treatment of brucellosis requires a combined regimen of antibiotics that may include doxycycline, rifampicin, streptomycin, gentamicin, and trimethoprim-sulfamethoxazole.9 Since the Brucella spp. are intracellular pathogens, antibiotic choice and duration of treatment are important for maximal effectiveness.10 Furthermore, the site of
infection and the associated clinical symptoms can necessitate prolongation of treatment duration.\(^{11}\) Although, a variety of recommended antimicrobial agents have activity in vitro against *Brucella* spp., sporadic cases of antibiotic resistance and disease relapse have been reported.\(^{12}\)

Fluoroquinolone and rifampicin resistance is commonly observed due to chromosomal mutations and alternation in the antibiotic target sites.\(^{13,14}\) These mutations include mutations in the target genes encoding DNA gyrase (*gyrA*), or topoisomerase IV (*parC*) inducing resistance to fluoroquinolones.\(^{13}\) One of the mutations in *gyrA* and *parC* that can cause resistance to this group of antibiotics was detected by rapid polymerase chain reaction (PCR) mismatch amplification mutation assay (MAMA PCR) of *E. coli*.\(^{13}\) DNA-dependent RNA polymerase (*rpoB*) is considered as a cause for resistance to rifampicin.\(^{14}\) Multiplex allele-specific PCR (MAS-PCR) assay based on standard PCR has been used to detect the most important rifampicin resistance-inducing mutations in *rpoB* gene.\(^{15}\) Moreover of significance to brucellosis, aminoglycoside adenyltransferase (*aadA1*), a member of integrin gene cassettes, can cause resistance to streptomycin.\(^{15}\) The determination of the antibiotic susceptibility profile of *Brucella* spp. is clinically important and has essential role in management of the infection, in control policies and epidemiological concerns. Therefore, the aim of this study was to determine the rate of occurrence of the most common antimicrobial resistance genes among *Brucella* spp. isolated from Iranian patients.

**Materials and methods**

**Study design and subjects**

This cross-sectional study was conducted on non-repetitive blood specimen from patients suspected to have brucellosis according to clinical signs and symptoms and positive standard agglutination test (SAT) for a period of two years from January 2015 to January 2017. They had been referred to hospitals in Tehran, Ahvaz, Hamadan, Qazvin and Mianeh in Iran. During the study, demographic and clinical information of patients suspected to have brucellosis according to general physician diagnosis were collected in standardized data collection forms. SAT tests were performed for all suspected patients and the blood specimen from those with positive STA (more or equal to 1/80) were examined for detection of resistance mutations. This study was approved by the Ethics Committee of the National Institute for Medical Research Development (NIMAD), Iran and was in accordance with the declaration of Helsinki.

**DNA extraction and detection of antimicrobial resistance genes:**

DNA was extracted from 2 mL blood specimen with a commercial kit (Qiagen, Germany) using the standard protocol. The extracted DNA suspension was stored at –20°C for further analysis. The integrity of DNA was confirmed by amplification of a β-globin (BG) as housekeeping gene, using the BG primers.\(^{16}\) *Brucella* isolates were genotypically identified by amplification of BCSP31 gene by PCR method as previously described.\(^{17}\) PCR amplification was performed to detect *aadA1* gene for streptomycin resistance according to the primers and conditions described by Abbasoglu et al.\(^{18}\)

**MAMA PCR protocol for mutation detection of *gyrA* and *parC***

The MAMA primers for mutation detection are shown in Table 1. In each PCR, a forward primer and a MAMA primer were used for mutation detection in *gyrA* and *parC* genes. These primers generate a short PCR product from the wild-type gene, but fail to produce this product from any gene with a mutation at the location covered by the mismatch positions on the MAMA primer. The third control primer that is expected to anneal efficiently to all gene alleles, was used in conjunction with the forward primer to generate a longer PCR product as an internal control.\(^{15}\)

Four PCR reactions were carried out on each template and 1 μL of template DNA was added to a final volume of 50 μL containing 0.35 μM forward primer, 0.25 μM MAMA primer, 0.10 μM control primer, 25 μl of Amplicon PCR Master Mix (2X) and nuclease-free water to 50 μl. The reaction was performed on a thermal cycler (Applied Biosystems, USA) by an initial denaturation at 95°C for 3 min and 35 cycles of denaturation at 95°C for 30 s, annealing at 54°C for 30 s and extension at 72°C for 40 s, with a final step of 72°C for 5 min. PCR products were visualized on horizontal 1.0% agarose gels in 1X TAE buffer, loaded with 9 μl of reaction mix and stained with DNA safe stain. O’GeneRuler 100bp DNA ladder (Thermo Scientific) was used during electrophoresis to estimate the sizes of the bands obtained.

**Multiplex allele-specific PCR (MAS-PCR):**

MAS-PCR was performed using three allele-specific primers targeting mutated codons 516 of the *rpoB* gene as described by Woods et al.\(^{15}\) The complete list of primers used in this study is listed in Table 1. The MAS-PCR primers bind specifically to wild-type sequences of the *rpoB* gene (codons 516). In the presence of a mutated allele, no amplification is noted, resulting in the absence of the corresponding band on agarose gel.\(^{15}\)
Results

Ninety-one blood samples were included in the present study. Blood samples were obtained from 58 (63.7%) males and 33 (36.3%) females with a mean age of 26.6 ± 20.6 year, ranging from 1 year to 86 years old. The majority of isolates were obtained from Mianeh city [63 (69.2%)] followed by Qazvin city [11 (12.1%)]. Demographic and clinical characteristics of patients are summarized in Table 2. Presence of *Brucella* spp. was molecularly confirmed in 52 (55%) samples. From them, PCR screening for the presence of antibiotic resistance genes showed that 26 (50%) isolates were positive for *aadA1* gene, 4 (7.7%) isolates were positive for *parC* gene, and 2 (3.8%) isolates were positive for *gyrB* gene. None of the isolates contained *gyrA* and *rpoB* genes. The co-occurrence of *parC/aadA1* genes was found in 3 (5.8%) of isolates (Table 3).

Discussion

Infection by *Brucella* species is a major zoonotic disease and brucellosis is an important international public health problem. We evaluated certain antibiotic resistance genes of 91 human *Brucella* isolates from different geographical regions of Iran between obtained between 2015 and 2017. In the present study, mutation in *rpoB* gene (responsible for rifampicin resistance) was not detected in any of our *Brucella* isolates. On the other hand, 50% and 11.5% of isolates were resistant to streptomycin and fluoroquinolones by the presence of mutation in *aadA1* gene and *parC* or *gyrB* genes, respectively. The highest rate of resistant-isolates were seen in western regions of Iran (Mianeh and Ahvaz).

According to World Health Organization (WHO) recommendations, a standard regimen for the successful treatment of brucellosis in adults includes doxycycline plus streptomycin or rifampicin. In addition, second-line agents such as quinolones or trimethoprim-sulfamethoxazole can be administered for children under the age of 8 or patients with treatment failure or repeated relapses. The only evidence of streptomycin and ciprofloxacin resistance in human *Brucella* isolates was reported by Farazi et al. in Markazi Province, central part of Iran. Other reports from Iran (Hamedan, Kashan and Tehran) have demonstrated predominantly susceptible *Brucella* isolates to

<table>
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<tr>
<th>Primers</th>
<th>Primer Sequences</th>
<th>References</th>
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<tbody>
<tr>
<td>BG-F</td>
<td>5′-CAATTCATCCACGTTGCACC-3′</td>
<td>15</td>
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<tr>
<td>BG-R</td>
<td>5′-ACACACCTGTCACATGC-3′</td>
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<tr>
<td>BCSP31-F</td>
<td>5′-AAGGCAAGTGGAAGATTT-3′</td>
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<tr>
<td>BCSP31-R</td>
<td>5′-CCTGTTCCAAGAACTTCG-3′</td>
<td></td>
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<tr>
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<td>5′-GTGGATGCGGCTGAAAGCC-3′</td>
<td>17</td>
</tr>
<tr>
<td>aadA1-R</td>
<td>5′-AATGGCCAGTCGCGAGCG-3′</td>
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<tr>
<td>gyrA-F</td>
<td>5′-GACTGCGAGGAGAATTACAC-3′</td>
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<td>rpoB-R</td>
<td>5′-GTCGCCCGATCAAGGAACGTA-3′</td>
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drugs of choice, with the exception of rifampicin,\textsuperscript{18,20,21} These differences in antibiotic susceptibility may reflect intrinsic properties of different \textit{Brucella} species according to geographical distribution or different patterns of antibiotic use in different parts of Iran. In our neighboring country Turkey, a total of 56 \textit{B. meli-
isolates were evaluated for doxycycline, streptomycin, rifampicin, trimethoprim-sulfamethoxazole and tigecycline susceptibility, which all of them were susceptible with a MIC90 lower than 2 mg/L. In an in vitro study performed in Malaysia, all *B. melitensis* isolates were noted to be sensitive to all the antimicrobial agents tested except for rifampicin where elevated MIC >1μg/mL was noted in 30 out of 41 isolates. In another Asian study performed in Kazakhstan, all *B. melitensis* isolates were susceptible to streptomycin, tetracycline and doxycycline and 97.3% of the isolates were susceptible to gentamycin; while, only 37.4% of isolates were susceptible to rifampicin. In China, of 85 *B. melitesis* human isolates, all were susceptible to minocycline, sparfloxacin, doxycycline, tetracycline, ciprofloxacin, gentamicin and levofloxacin, while 1.0% and 7.0% of the isolates were resistant to rifampicin and co-trimoxazole, respectively. Finally, in a peruvian study, all of 48 *B. melitensis* isolates were generally susceptible to the evaluated antibiotics, with the exception of single isolates with reduced susceptibility to rifampicin and to trimethoprim-sulfamethoxazole.

Determination of antibiotic resistant strains by in vitro susceptibility tests are insufficient, since the presence of cryptic resistance genes and interaction of these genes with broad-spectrum antibiotics can alter antibiotic resistance percentages. The prevalence of *aadA1* gene mutation, related to streptomycin resistance and mostly associated with integrons that may transfer among bacteria, may pose a significant risk for the emergence of resistant-strains and treatment failure. The presence of *gyrB* and *parC* mutations however, may not isolatedly lead to fluoroquinolones resistance due to interplay of different mechanisms.

In conclusion, as recognition of *Brucella* species and their resistance patterns is difficult by conventional cultures, ours was the first study in Iran that demonstrating that the investigation of antibiotic resistance genes in these isolates can be performed by molecular methods. These results suggest that the empiric use of antibiotics for the treatment of brucellosis can be based on logical and easy to perform molecular assays.

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**Conflict of Interest:**
None to be declared
Περίληψη

Μοριακός χαρακτηρισμός μεταλλάξεων στα γονίδια gyrA, parA, parC, rpoB και aadA1 που σχετίζονται με αντιμικροβιακή αντοχή κλινικών στελεχών Brucella spp.

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Η βρουκέλλωση ή πυρετός της Μάλτας είναι μία απειλητική για τη ζωή, συστηματική λοίμωξη και μια από τις συχνότερες ζωονόσους παγκοσμίως, που προκαλεί σημαντικές προβλήματα στη δημόσια υγεία και την οικονομία. Ο καθορισμός της ευαισθησίας στα αντιβιοτικά της Brucella spp. είναι σημαντικός στην κλινική πράξη και παίζει ουσιαστικό ρόλο τόσο στις επιδημιολογικές μελέτες, όσο και στο σχεδιασμό της στρατηγικής για τον έλεγχο της λοίμωξης. Σκοπός της παρούσας μελέτης ήταν ο καθορισμός της επίπτωσης των συχνότερων μεταλλάξεων σχετιζόμενων με αντιμικροβιακή αντοχή σε συγκεκριμένα γονίδια Brucella spp. σε ασθενείς στο Ιράν. Ωστόσο, δεν έχουν ακόμη υπογεγραμμιστεί στα γονίδια Brucella spp. στην περιοχή Ιράν. Ουσιαστικά, η παρούσα μελέτη αποτελεί την πρώτη μελέτη διερεύνησης της συχνότητας των μεταλλάξεων αντιμικροβιακής αντοχής σε γονίδια Brucella spp. σε ασθενείς στο Ιράν.
References


