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Original Article

Molecular Survey of *Brucella melitensis* Field Isolates using Sequence-Based PCR of Outer Membrane Protein 31

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Abstract

Sequence-based Polymerase Chain Reaction (PCR) has been introduced as an effective and reliable method for bacterial strain typing, which could provide a reliable typing approach for clinical laboratories. This study aimed to describe the reproducibility and performance of the Outer Membrane Protein 31 (Omp31)-based PCR, as a molecular genotyping tool for *Brucella melitensis* (*B. melitensis*) typing. The 31 KD outer-membrane protein of *Brucella*, which encodes the *Omp31* gene, can be applied as an antigen to diagnose brucellosis. For this purpose, 146 samples were taken from human blood samples, bovine and camel lymph nodes, as well as sheep and goat aborted fetuses, including fetal kidney, abomasum, liver, lung, spleen, and heart for bacteriological investigation. The molecular detection of the *Omp31* and *IS711* genes was performed using the isolated *B. melitensis* (n=14). The sequencing of the *Omp31* gene of *B. melitensis* in the Iranian field isolates was also performed for the whole gene sequencing. The homology of all sequences was then checked with the reported National Center for Biotechnology Information sequences using a basic local alignment search tool for the nucleotide diversity evaluation. The findings revealed that *B. melitensis* isolates were recovered from 14 examined cases and confirmed by the IS711-based PCR with a PCR product of 731 bp. Moreover, 14 Iranian *B. melitensis* sequences clustered together as a monophyletic grouping with bootstrap support of 63, and they were closely related to the *B. melitensis* reference isolates. This Omp31-based phylogenetic placement strongly indicates the monophyletic origin of the Iranian *B. melitensis* in different animals and human hosts.

Keywords: Brucella melitensis; Omp31; Phylogenetic analysis; Sequence-based PCR

1. Introduction

Brucella melitensis (*B. melitensis*) is a Gram-negative facultative intracellular bacteria that is known as the main species contributing to ovine, caprine, and human brucellosis (1, 2). A layer of peptidoglycan could be found in the cell wall of *Brucella*, which is closely related to the outer membrane. The outer membrane is composed of phospholipids lipopolysaccharide (LPS) and proteins (3). The outer membrane proteins (Omps) are categorized as the proteins of 94 or 88 kDa (also named as group 1), the proteins of 36-38 kDa (also named as group 2), and the proteins of 25-27, 31-34 kDa (also named as group 3) (4-6). The *Omp31* gene encodes the major 31-kDa *Brucella* Omps located in chromosome I of *B. melitensis* and plays an essential role in interacting with the host cells (4). It is a suitable gene candidate for the species-based identification of *Brucella* species (spp.) (7). It is important to highlight that the diversity of genes encoding the major Omps in species, biovars, and strain levels is of taxonomical and epidemiological importance (8). It has been proved that the *Omp31* gene could be a useful antigen candidate in

differentiating *Brucella* spp., except for *B. neotomae*, using southern blot hybridization and Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP) tests (9, 10). Recently, many investigations have evaluated the outer membrane proteins of brucellosis, including *Omp31*, as better candidates for the PCR-based *Brucella* diagnosis (5, 9, 11, 12). The *Omp31* gene could remove the limitations of brucellosis diagnostic antigens, which primarily use LPS or the whole bacteria and easily show cross-reaction with Gram-negative bacteria, especially *Yersinia enterocolitica* O:9 (13, 14). The high conservation of the *Omp31* gene was identified to have only nine nucleotide differences in *Brucella ovis* (*B. ovis*). This difference induces different antigenic properties of the *Omp31* gene of *B. ovis* (10). However, there is no genomic analysis of these proteins in different biovars of *B. melitensis* and different hosts, such as humans, goats, sheep cattle, and camels, in Iran. Therefore, it was attempted to investigate the genomic characterization of *Omp31* from the Iranian isolates of *B. melitensis* and compare them with the registered *Omp31* gens in the GenBank using a sequence-based PCR for *B. melitensis* genotyping. This approach reveals amino acid and nucleotide variations which can save this genome as an applicable gene for developing specific diagnostic tests. The present study analyzed the *Omp31* gene of 14 Iranian human and animal field isolates of *B. melitensis* for the presence of nucleotide variations by PCR amplification and sequence analysis. The results were confirmed with AMOS PCR assay, with the AMOS being an abbreviation for four *Brucella* spp. identified as *B. abortus, B. melitensis, B. ovis, and B. suis*.

2. Materials and Methods

2.1. Sample Collection

The tested samples (n=146) included human blood samples (n=34), lymph nodes (cows: n=49, camels: n=35), and samples from aborted fetuses (sheep: n=26, goats: n=2), including fetal kidney, abomasum, liver, lung, spleen, and heart. From 2018 to 2020, all suspected samples were transferred to the Brucellosis Department of the Razi Vaccine and Serum Research Institute in Karaj, Iran. Cows, goats, and sheep samples had a history of abortion on the farm. All visceral organ samples were collected and submitted in a sterile plastic bag and preserved at -20˚C until further investigations. Human cases of brucellosis were patients who were referred to the Razi Vaccine and Serum Research Institute with clinical brucellosis complaints and symptoms compatible with positive Rose Bengal test, Wright, and 2ME tests.

2.2. *Brucella melitensis* Isolation and Identification

A bacteriological investigation was performed on all individual lymph node samples, aborted fetal organs, and human blood under appropriate protection in biosafety cabinets. All samples were inoculated on a *Brucella* selective supplement for the isolation of pathogenic *Brucella*. The selective supplement comprised of Bacitracin (12,500 IU), Polymyxin B (2,500 IU), Cycloheximide (50 mg), Nystatin (50,000 IU), Vancomycin (10.0 mg), and Nalidixic acid (2.5 mg) (Oxoid, UK), inactivated 5% horse serum in *Brucella* agar (Himedia, India), and incubated with 10% CO_2 for 21 days at 37˚C. The morphology and purity of the isolated bacteria were characterized using Gram stain and the regular panel of biotyping tests based on Alton, Jones (15). The procedure included agglutination with specific *Brucella* antisera, monospecific antisera M and A, CO_2 dependence, and H_2S production, as well as agglutination by acriflavine, growth in colored basic fuchsine and thionine media, and lysis by specific phages (15). Reference phages of *Brucella* and monospecific *Brucella* antisera of A and M antigens were routinely prepared and applied for *Brucella* diagnosis in our center.

2.3. Genomic DNA Extraction

The extraction of the total genomic DNA of bacteria was performed using the Exgene Cell SV kit (Gene All, South Korea), based on the manufacture's protocol. The concentration of DNA was tested at 260/280 nm by ND-1000 Nanodrop (Wilmington, USA). Furthermore, the integrity of DNA was tested by 1% agarose gel and stored at -20˚C, and finally, the PCR analysis was performed.

2.4. Molecular Identification of *Brucella melitensis* Isolates

The molecular identification of *B. melitensis* isolates was performed on the extracted DNA according to IS711-based PCR by AMOS (i.e., *abortus*, *melitensis*, *ovis*, and *suis*) PCR, as described previously (16). The AMOS PCR conditions were as follows: initial denaturation at 95˚C for 5 min, 35 cycles of denaturation at 95˚C for 30 sec, annealing for 60 sec at 55˚C, extension at 72˚C for 3 min, and a final extension at 72˚C for 10 min (16). The PCR mixture reaction containing the five-primer cocktail (0.5µl of IS711, and 0.5µl *B. melitensis*, *B. abortus*, *B. ovis*, *B. suis*-specific primer), 2µl of template DNA, 5µl ddH_2O, and 12.5µl RED Master Mix PCR (Amplicon, Denmark) was employed in this study. Visualization of the amplified PCR products was conducted using electrophoresis on a 1% agarose gel (Table 1).

2.5. PCR Amplification of the *Omp31* Gene

The specific primers for the *Omp31* gene of *B. melitensis* were designed based on the available *Omp31* nucleotide sequences on the National Center for Biotechnology Information (NCBI) database (Table 1). PCR amplification of the *Omp31* gene of the 14 *B. melitensis* Iranian field isolates was also performed. The reaction mixture (25µl) consisted of 2µl of genomic DNA, 8.5µl of ddH_2O, 12.5µl of RED Master Mix PCR (Amplicon, Denmark), and 1µl of each primer (10 pmol/µl). PCR amplification was carried out through the following stages: the initial denaturation at 94˚C for 5 min, 35 cycles of denaturation at 94˚C for 60 sec, annealing at 56˚C for 45 sec, and extension at 72˚C for 60 sec, followed by a final extension at 72˚C for 5 min. Subsequently, the PCR products were visualized for the presence of a single band of expected molecular weight on 1% agarose gel.

Table 1. Primer sets and the expected amplicon sizes specific to different *Brucella* species

Strain amplicon	Primer set	Primer sequence (5-3')	DNA target	Size (bp)	References
AMOS PCR	IS711 AB	TGCCGATCACTTTCAAGGGCCTTCAT GACGAACGGAATTTTTCCAATCCC	IS711	498	(16)
AMOS PCR	IS711 BM	TGCCGATCACTTTCAAGGGCCTTCAT AAATCGCGTCCTTGCTGGTCTGA	IS711	731	(16)
AMOS PCR	IS711 *B.ovis*	TGCCGATCACTTTCAAGGGCCTTCAT CGGGTTCTGGCACCATCGTCG	IS711	976	(16)
AMOS PCR	IS711 *B.suis*	TGCCGATCACTTTCAAGGGCCTTCAT GCGCGGTTTTCTGAAGGTTCAGG	IS711	285	(16)
OMP31	OMP31 F OMP31 R	5' ATGAAATCCGTAATTTTGGCGTC-3' 5'-TTAGAACTTGTAGTTCAGAC 3'	OMP31	723	Designed in this study

2.6. Genomic and Bioinformatic Analysis of *Omp31* Gene

The PCR products of *Omp31* were purified by the purification kit (GeneAll, South Korea) and sent for sequencing by ABI Prism 377 sequencer genetic analyzer equipment (Applied Biosystems Foster City, CA). The assembling of the *Omp31* sequences was performed by the Mega 6 software program. Furthermore, the amino acids of the *Omp31* were deduced by the CLUSTAL W (17). The homology of sequences was checked with the reported NCBI sequences using a basic local alignment search tool for the nucleotide diversity evaluation. Gene polymorphisms of the *Omp31* were also evaluated twice to confirm the findings of the present study. The analysis of the *Omp31* amino acid sequence was performed as well, using Basic Local Alignment Search Tool (BLAST, NCBI) and Mega 6 software. The *Omp31* nucleotide sequence of *B. melitensis* of Iranian isolates was deposited in the NCBI.

2.7. Phylogenetic Analysis

The sequence data were analyzed using BLAST

(NCBI), Mega 6, and Vector NTI (Invitrogen) software. Finally, the results of nucleotides and proteins were compared with other existing sequences in the NCBI data bank, and then, phylogenetic trees were depicted using the maximum likelihood method (18, 19). A phylogenetic analysis was performed using 14 reported full-length *Omp31* gene sequences from *Brucella* isolates representing all already-known *Brucella spp*.

3. Results

B. melitensis isolates (n=14) were recovered from 146 examined cases (Table 2). The *B. melitensis* isolates were from human blood (n=6), ovine aborted fetuses (n=2), goat aborted fetuses (n=2), bovine lymph nodes (n=2,) and camel lymph nodes (n=2). The isolated bacteria showed common phenotypic features of *B. melitensis*, including Gram-negative, and formed small honey-colored, shiny, and translucent colonies with a smooth surface. All isolates grew in 10% carbon dioxide (CO_2) after incubation at 37°C for 5 to 10 days. Bacteria isolates were characterized to the biovar level, and identity was confirmed to species/vaccine level for all isolates using the AMOS PCR (Table 2). All isolates were identified as wild types of *B. melitensis* through the AMOS PCR with a PCR product of 731 bp.

Table 2. Details of Iranian animal and human field isolates of *B. melitensis* used in this study and their *Omp31* gene accession numbers in the NCBI

Accession number	*Brucella spp.*	Biovar	Isolate	Source	Host	Province
MK611562.1	*B. melitensis*	1	B.m1	Blood	Human	Esfahan
MK611565.1	*B. melitensis*	1	Razi-K-B.m1	Blood	Human	Alborz
MK611566.1	*B. melitensis*	2	Razi-K2-B.m2	Lymph node	Cow	Kerman
MK611567.1	*B. melitensis*	2	Razi-K-B.m2	Lymph node	Cow	Kerman
MK611570.1	*B. melitensis*	1	Razi-Z-B.m1	Aborted fetus	Goat	Zanjan
MK611571.1	*B. melitensis*	1	Razi-K3-B.m1	Blood	Human	Esfahan
MK611572.1	*B. melitensis*	1	Razi-M-B.m1	Aborted fetus	Sheep	Mazandaran
MK611563.1	*B. melitensis*	3	Razi B.m3	Blood	Human	Alborz
MK611564.1	*B. melitensis*	3	Razi-Kh-B.m3	Aborted fetus	Sheep	Khorasan Razavi
MK611568.1	*B. melitensis*	1	Razi-K2-B.m1	Blood	Human	Kermanshah
MK611569.1	*B. melitensis*	1	Razi-T-B.m1	Blood	Human	Tehran
MW792535	*B. melitensis*	1	Razi-H-B.m1	Lymph node	Camel	Hormozgan
MW792536	*B. melitensis*	1	Razi-H2-B.m1	Lymph node	Camel	Hormozgan
MW792537	*B. melitensis*	1	Razi-F-B.m1	Aborted fetus	Goat	Fars

The occurrence and distribution of *B. melitensis* infection in Iranian human and animal hosts were confirmed in different provinces of Iran, including Esfahan, Alborz, Zanjan, Kerman, Mazandaran, Khorasan Razavi, Kermanshah, Hormozgan, Tehran, and Fars. The *Omp31* sequences of 14 *B. melitensis* isolates were identical in nucleotides. Additionally, these sequences had similar nucleotides with previous *Omp31* gene sequences of *B. melitensis*, namely biovar 1, 2, and 3 in the NCBI (i.e., AF076290.2, AF366061.1, and AF366062.1). Each of these *Omp31* sequences had one open reading frame of the genome fragment, as expected for sequences within the *Omp31* gene ORF. The high similarity hits for the BLAST p on UniProtKB of the deduced *Omp31* protein sequence showed that the *Omp31* sequence of Iranian *B. melitensis* isolates used in this study had similar protein to the previous *Omp31* gene sequences of *B. melitensis* (i.e., *biovar* 1, 2, and 3 in the NCBI). A phylogenetic tree of the 723nt *Omp31* gene sequences generated by the maximum likelihood method, based on distance methods, is shown in figure 1, where branches with bootstrap values of <70% collapsed. In this tree, nine known global species of *Brucella* spp. are resolved as expected. Totally, 14 Iranian *B. melitensis* sequences clustered together as a monophyletic grouping with

bootstrap support of 63, and they were most closely related to the *B. melitensis* references isolates. This phylogenetic placement strongly suggested a monophyletic origin of the *B. melitensis* in different Iranian animal and human hosts.

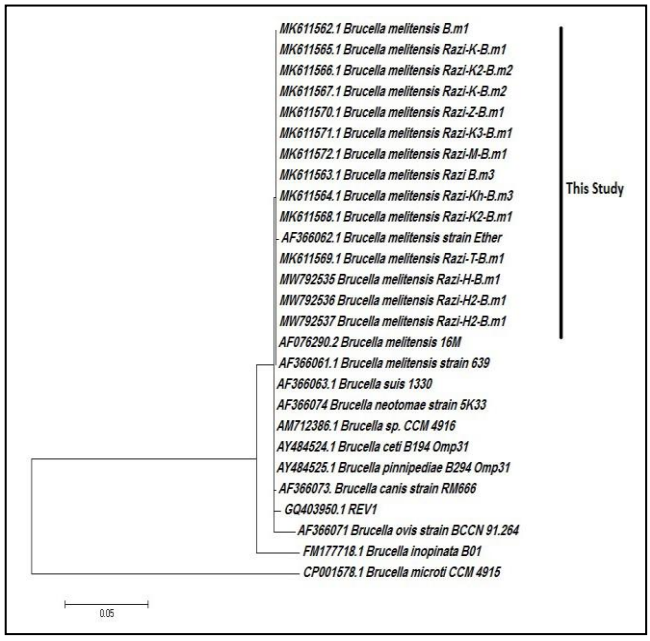

Figure 1. The Phylogenetic relationship of the Iranian *Brucella melitensis* isolates and other reference strains using maximum likelihood method in Mega 6 software. A Phylogenetic tree based on the *Omp31* gene. A scale bar shows 0.01 substitutions per sequence position.

4. Discussion

B. melitensis is a Gram-negative, non-encapsulated, intracellular bacterium that can infect different animals and human species. The findings of DNA-DNA hybridization investigations showed that the genus *Brucella* is a highly homogeneous group with more than 90% DNA homology for all species (20). Previously, the diagnosis of species and biovars was conducted according to differential phenotypic tests, including metabolic properties, dye sensitivity, phage typing, characterization of LPS antigens, CO_2 requirement, and hydrogen sulfide production (21). In addition, several approaches have been taken to find polymorphism regions of *Brucella* DNA that would enable the molecular typing of *Brucella* spp. and could differentiate their species and different biovars (10).

In this regard, the *Omp* genes, such as *Omp2a*, *Omp2b*, *Omp25*, and *Omp31* in the *Brucella* spp. have attracted growing attention as they exhibit sufficient polymorphism to allow differentiation between *Brucella* spp. (20, 22, 23). The results revealed that out of 146 samples being tested, 14 human and animal samples (9.5%) were infected with *B. melitensis* and replicated the *Omp31* gene. A homology search between Iranian *Omp31* genes and other sequences reported in the NCBI Gen Bank revealed 100% sequence similarities with different biovars of *B. melitensis*. Our findings are in agreement with the results of other studies that reported a remarkable degree of similarity (97%) among the *Omp31* sequences of *Brucella* spp. (4, 10). The results of *Omp31* PCR was compared with *IS711* PCR, which showed similar sensitivity for the molecular detection of *B. melitensis*. However, the replication of *Omp31* in this study indicated that 14 tested isolates were most likely *B. melitensis* because of the absence of the *Omp31* gene in *B. abortus*. The findings were also in line with previous results documenting the *Omp31* gene as an applicable gene for differentiating *Brucella* spp. through the PCR-RFLP tests (22, 23). Moreover, the presence of 240 conserved regions in *Omp31* amino acid sequences in all the Iranian isolates of *B. melitensis* can support the development of *Omp31*-based PCR for diagnosing *B. melitensis*-induced brucellosis (12). The phylogenetic analysis in this study also reported that *Omp31* from all the Iranian isolates of *B. melitensis* was arranged in the same cluster with the other *Omp31* sequences of *B. melitensis*. The strong similarity of the *Omp31* genes among *B. melitensis* may be due to the high degree of genetic relatedness among these species. Furthermore, the alignments of Iranian *Omp31* amino acid sequence with those of *B. melitensis* ATCC 23457, *B. melitensis* M5-90, *B. melitensis* M28, and *B. melitensis* Rev1 strains showed

100% homology with *B. melitensis* M28 and *B. melitensis* M5-90 Omp31 amino acid sequences, while the *Omp31* amino acid sequence of *B. melitensis* Rev1 showed 99.5% homology, indicating a common ancestral evolutionary origin of these two isolates. These results are in agreement with other findings related to *B. melitensis Omp31* nucleotide sequence analysis revealing the high conservation of *Omp31* in *B. melitensis* isolates (4, 10, 24).

This study tried to investigate the *Omp31* gene sequences of Iranian *B. melitensis* isolates by phylogenetic analysis. The Iranian *B. melitensis* isolates from different hosts (i.e., cow, goat, camel, sheep, and human), in 10 different provinces, had the same nucleotide sequence in the same cluster with other reported *B. melitensis* for the full length of the *Omp31* gene, indicating low genetic diversity despite broad geographic dispersion. Therefore, it seems that this gene is more important in the diagnosis of *B. melitensis* than it is in phylogenetic analysis, and it can be used as a universal method with a diagnostic value in different hosts, regardless of their geographic dispersion. However, for the discrimination and identification of strains, it is better to use other differentiating techniques, such as comparative genome analysis. This may suggest that *B. melitensis* transfers long distances from sheep to other animal hosts and humans in Iran. Nevertheless, very limited information is available regarding the actual transmission pathways of *B. melitensis* in different hosts. Regarding the high rate of *B. melitensis* infection in Iranian small and large ruminants, larger-scale molecular investigations with high numbers of *B. melitensis* isolates will be important for diagnostic purposes, vaccine candidate evaluations, investigation of transmission patterns, and phylogenetic relationships of *B. melitensis* in different hosts. Since there was no difference among the *B. melitensis* isolates by the *Omp31* gene in this study, other methods, such as VNTR-PCR and MLST, are also suggested for *Brucella* differentiation.

Authors' Contribution

All authors have made substantial contributions to the conception and design of this study (M. D., A. B., E. A. and S. D. H.), data analysis (M. D. and S. D. H.) and data interpretation procedures (M. D., A. B. and S. D. H.), drafting the article (M. D. and S. D. H.), revising it critically for important intellectual content (M. D., A. B., E. A. and S. D. H.), and final approval of the version to be submitted (M. D., A. B., E. A. and S. D. H.).

Conflict of Interest

The authors declare that they have no conflict of interest.

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