Electronic Physician (ISSN: 2008-5842) http://www.ephysician.ir
October 2015, Volume: 7, Issue: 6, Pages: 1381-1386, DOI: 10.14661/1381

Genetic characterization of the wboA gene from the predominant biovars of Brucella isolates in Iran

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Type of article: Original

Abstract

Introduction: *Brucella* spp. are gram-negative, facultative intracellular bacteria pathogens responsible for brucellosis, a zoonotic disease that can cause abortion, fetal death, and genital infections in animals and undulant fever in humans. Lipopolysaccharide (LPS) is known as a major virulence factor of *Brucella* spp. The wboA gene is capable of encoding a glycosyltransferase that appears to play a major role in LPS biosynthesis. Hence, the characterization of this gene can help in the clarification of the pathogenicity of *Brucella* spp.

Methods: This study was carried out at Razi Vaccine and Serum Research Institute in 2011. Briefly, the wboA gene in *B. abortus* biovar 3 and *B. melitensis* biovar 1, the predominant biovars in Iran, were amplified by using two pairs of specific primers. Polymerase chain reaction (PCR) products were cloned into a thymine–adenine (TA) cloning vector and transformed into an E. coli DH5α before being sequenced. Multiple alignments of identified sequences were performed, with all wboA sequences deposited in the GenBank sequence database.

Results: This study showed that a mismatch has occurred in B. melitensis biovar 1; this biovar is predominant in Iran. In contrast, the wboA gene from *B. abortus* biovar 3 was similar to that of other *B. abortus* variations.

Conclusion: The comparison and alignment of the wboA gene of native *Brucella* strains in Iran to all wboA sequences deposited in GenBank revealed that the wboA gene has changed in the long term; hence, because of its unique nucleotide pattern, the gene can be used for specific diagnosis of *B. abortus* and B. canis.

Keywords: *Brucella* , wboA gene , LPS , *B. abortus*

1. Introduction

The *Brucella* genus consists of facultative intracellular, non-motile, aerobic, gram-negative coccobacilli bacteria that create brucellosis disease, a zoonotic disease that causes abortion, fetal death, and genital infections in animals, and it is one of the most important zoonotic diseases with respect to public health concerns (1). *Brucella* belongs to class 2 *Alphaproteobacteria*, order Rhizobiales, family Brucellceae, and genus *Brucella* (2). The genus according to host specificity and pathogenicity is composed of 10 recognized species, six of which are considered the classic species,

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Received: July 06, 2015, Accepted: August 25, 2015, Published: October 19, 2015
iThenticate screening: July 22, 2015, English editing: August 01, 2015, Quality control: September 26, 2015

Brucella abortus (seven biovars), *B. melitensis* (three biovars), *B. suis* (five biovars), *B. canis*, *B. ovis*, and *B. neotomae*. The four species newly isolated from sea mammals are *B. ceti*, *B. pinnipedialis*, *B. microti*, and *B. inopinata* (3, 4). *B. melitensis*, *B. suis*, *B. abortus*, and (rarely) *B. canis* are responsible for human infections, with *B. abortus* having the widest geographic distribution (4-6). Many countries have taken steps to control disease related to *B. abortus*, but regions still exist where the infection is present in animals and therefore may be transmitted to humans (7). The major means of *B. abortus* transmission to humans (i.e., brucellosis) include direct contact with infectious animals' tissues, inhalation of aerosolized droplets, and consumption of unpasteurized dairy products (8). *B. abortus* biovar 1 is reported as the most frequent biovar in comparison to other varieties, and it contributes to brucellosis in many countries across the world. In Mediterranean countries, *B. melitensis* biovar 3 has been identified as the most frequent biovar (9). In Iran, the results of epidemiologic studies have shown that *B. abortus* biovar 3 and *B. melitensis* biovar 1 are the dominant biovars (10). Brucella's outer membrane, as with other gram-negative bacteria, is an asymmetrical lipid bilayer composed of lipopolysaccharide (LPS), proteins, and phospholipids (PLs); LPS and PLs are located in the outer and inner membrane, respectively (11, 12). Depending on its structure, LPS can be smooth (S-LPS) or rough (R-LPS). These two types of LPS have many functions, including substrate transportation and genetic exchanges, and they also are considered to be a determining factor in virulence of the pathogen. Specifically, S-LPS (because of its external position) has an important role in many host–pathogen interactions, and it is the immunodominant antigen of Brucella (13, 14). LPS is composed of two connected portions, i.e., the lipid A-core and the o-polysaccharide. The details of the structures of Brucella vary among the different bacteria. O-polysaccharide (due to compositional subunits) can be either homopolymeric or heteropolymeric (15). Brucella species with a smooth phenotype (*B. abortus*, *B. melitensis*, *B. suis*, *B. neotomae*) have an O-side chain in their LPS structure, while naturally rough phenotypes (such as *B. canis* and *B. ovis*) lack the O-polysaccharide side chain.

The genes involved in O-polysaccharide synthesis are located in two genetic regions, wbo and wbk, which have activity in polysaccharide synthesis and translocation, respectively (16). The wboA gene is capable of encoding a glycosyltransferase (wboA-wboB) that is important for O-antigen synthesis of the Brucella (17, 18). Studies have shown that the wboA gene is conserved in B. canis (rough phenotype) as well as in other Brucella species with a smooth phenotype (18, 19). Since LPS is known to be a major virulence factor of *Brucella* spp, characterization of the gene involved in LPS biosynthesis can help demonstrate the mechanism of the pathogenicity of *Brucella* as well as demonstrate its level of virulence. Furthermore, this characterization can clarify the relationship between genotype and phenotype and their use in differential diagnosis as well as approaches in the development of novel recombinant vaccine.

2. Material and Methods

2.1. Research design and setting

This research was conducted at Razi Vaccine and Serum Research Institute of Iran in 2011. The bacterial strains used in this study are listed in Table 1. All *Brucella* strains were grown on *Brucella* broth and/or agar media at 37 °C for 72 h. The colonies were checked for purity, and the species and biovar were characterized using standard procedures.

Table 1. *Brucellal* strains used in this study

Species	Species Biovars	Host or source	Geographic origin	Strain or Accession No.
B. abortus	3	Cow milk and aborted fetuses	Isfahan (Iran)	HQ845203.1
B. melitensis	1	Cow milk	Golpyegan (Iran)	JF261627.1
B. abortus	1	Cattle	England	Reference strain: 544 (= ATCC 23448)
B. melitensis	1	Goat	USA	Reference strain: 16M (= ATCC 23456)

2.2. DNA Extraction and PCR

The bacterial suspension was centrifuged at 2500 g for 5 min, and the pellet was resuspended in 200 µl of phosphate buffer solution, and DNA was extracted using a QIAamp DNA Mini Kit (Qiagen) according to the manufacturer's instructions. The extracted DNA was evaluated by gel electrophoresis using a 1.0% agarose gel (20). The wboA

gene was amplified using primers F: 5- GAGTAGACACGGGAAATC-3 and R: 5- GATAAACACGCCGAGCTT-3. The primers were designed using oligo software based on the wboA sequence available in the National Center for Biotechnology Information (NCBI) database. Amplification consisted of an initial denaturation step of 5 min at 94 ^{o}C followed by 30 cycles at 94 ^{o}C for 30 s, 55 ^{o}C for 30 s , 72 ^{o}C for 90 s, and with a final extension at 72 ^{o}C for8 min. After confirming the size of the product, the product was purified using PCR product Purification Kit according to the manufacturer's instructions (Qiagen).

2.3. Cloning and sequencing

PCR products were cloned using an Ins TA clone PCR Cloning Kit (Thermo Scientific) according to the manufacturer's instructions. The transformation was performed using competent E. coli DH5 α cells. Colony screening was done for ampicillin resistance. The plasmids that contained the wboA gene were extracted using the high purity plasmid isolation kit (Roche) and sequenced.

2.4. Amino acid sequence alignment

The alignment of Iranian native biovars' sequences with the others *Brucella* species including *B. abortus, B. melitensis, B. suis, B. canis, B. microti, B.pinipeialis,* and *B.ctei*. were performed using MegAlign (DNASTAR, Inc.) software.

3. Results

The nucleotide sequence of wboA gene belonging to *B. abortus* biovar 3 and *B. melitensis* biovar 1 (predominant species in Iran) were submitted to GenBank with accession numbers HQ845203.1 and JF261627.1, respectively. Comparative nucleotide and deduced amino acid sequence of the wboA among all *Brucella* species and predominant biovars in Iran showed that the wboA gene is highly conserved, but with a low number of point mutations. The wboA sequense analysis of *B. melitensis* biovar 1 in Iran showed a nucleotide substitution in position 335 that was unique in comparison with the other Brucella species. This nucleotide mutation caused an amino acid change as a missense mutation (aspartic acid to glycine) (Figure 1).

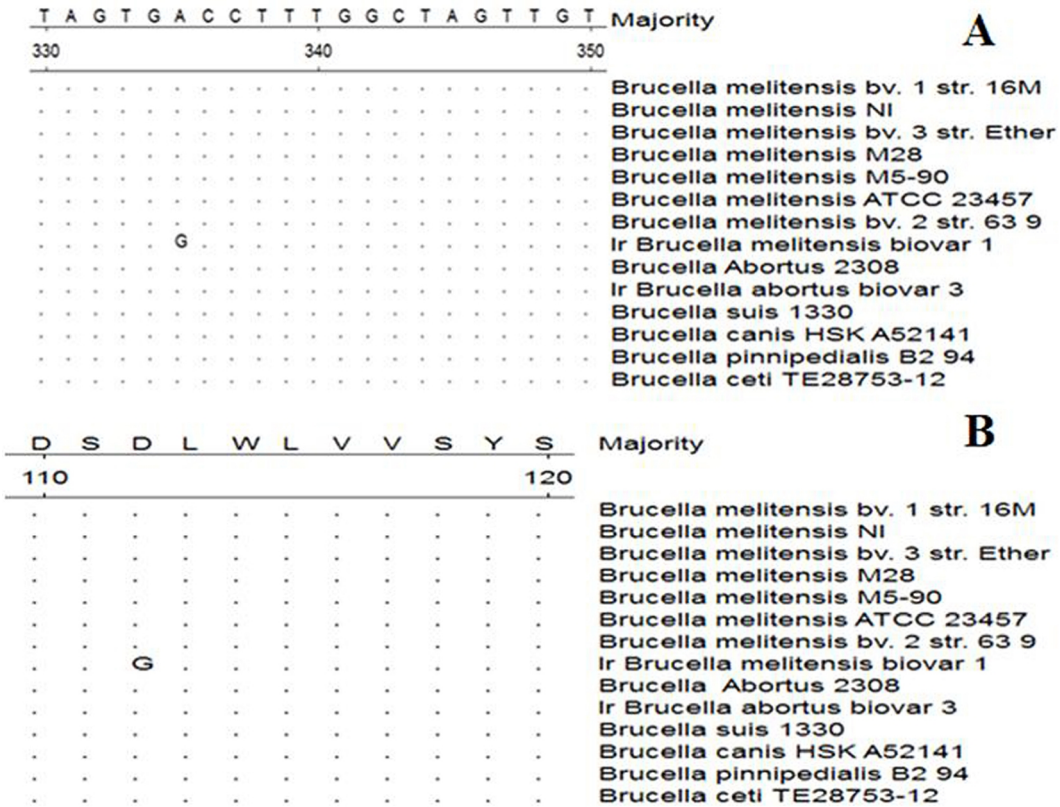

Figure 1. (1-A), wboA nucleotic subsituation in *Brucella melitensis* biovar; (1-B), Variation in glycosyltransferase amino acid sequence in *Brucella melitensis* biovar 1

The nucleotide sequence of the wboA gene from *B. abortus* biovar 3 (another predominant biovar) was similar to all *B. abortus* variants submitted to GenBank. Alignment of the wboA sequences among all *Brucella* species revealed unique patterns, such as the presence of a single nucleotide polymorphism at position 987, which can be used for specific differentiation of *B. abortus* from other *Brucella* species (Figure 2), and two nucleotide substitutions in position 1182 that also caused a missense mutation (tryptophan to tyrosine). This specific substitution pattern is suitable for discriminating *B. canis* from other *Brucella* species (Figure 3).

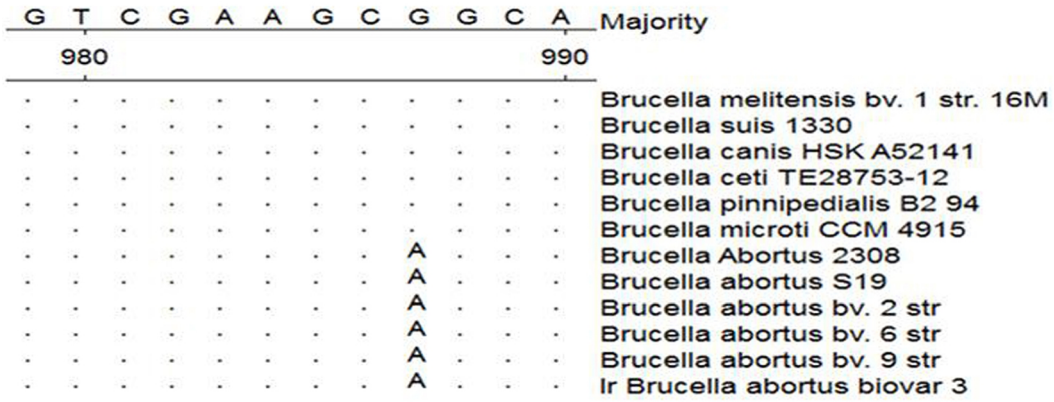

Figure 2. *Brucella abortus* Specific pattern in the wboA gene

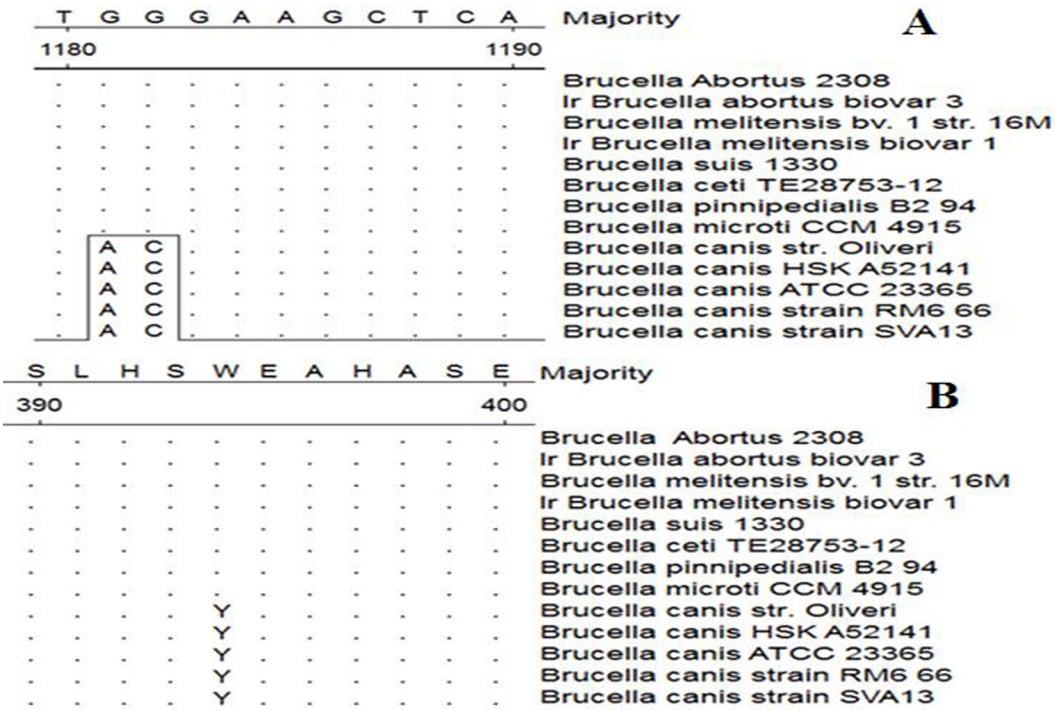

Figure 3. (3-A), *Brucella canis* Specific pattern in the wboA gene; (3-B), Variation in glycosyltransferase amino acid sequence in *Brucella canis*

4. Discussion

The wide range of different *Brucella* species makes Brucella a subtle model for the investigation of pathogenic bacteria. *Brucella* spp. belong to alpha-2 proteobacteria that have a nucleotide sequence of two circular chromosomes with more than three mega base pairs. Whole-genome comparative analysis showed more than 94% identity at the nucleotide level (6, 10, 21, 22). The O-polysaccharide in LPS structures has polymers of sugar groups; a large family of polymerase enzymes, called glycosyltransferases (GTs), forms glycoside bonds between

sugar residues. The presence of these enzymes in a eukaryotic and prokaryotic cell has been established. Based on crystal structure, GTs are divided into two super families, i.e., GTA and GTB. A mutation in genes encodes the O-polysaccharide due to the conversion of the smooth phenotype to the rough phenotype. Previous studies have shown disruption of wboA in Brucella species that have a smooth phenotype, such as *B. abortus*, *B. melitensis*, and *B. suis*, which the can be converted to the rough phenotype. These mutated strains were not able to produce O-polysaccharide in an LPS structure (15-17, 23, 24). The wboA is a suitable candidate for increasing the coverage provided by brucellosis vaccine strains and decreasing the side effects produced by similar antibodies in response to the wild and vaccine strains in test and slaughter programs (18, 19). In *Brucella* spp., the wboA gene is composed of 1,233 base pairs that encode glycosyltransferase enzyme. This enzyme acts to polymerize O-polysaccharide. In this study, the wboA sequences for the dominant *Brucella* biovars in Iran (*B. abortus* biovar 3 and *B. melitensis* biovar 1) were characterized and aligned with all sequences of the wboA that belonged to Brucella species in a comparative analysis. The results showed a unique nucleotide mutation in *B. melitensis* biovar 1 that caused an amino acid substitution. This strain originally was isolated from cow's milk; one interesting point is that penicillin sensitivity was present as a phenotypic feature. This is a novel report of genetic changes in wboA and phenotypic differences based on the wboA sequence. The phylogenetic tree exhibited considerable genetic diversity in *B. melitensis* biovar 1. Atypical features, such as sensitivity to dye and penicillin in *B. melitensis* biovar 1, have been reported in previous studies. Variations of bacteria porins (outer membrane proteins in gram-negative bacteria) in the LPS structure have been mentioned as the causative agent of these phenotypic characteristics (25). The nucleotide sequence of the wboA gene in *B. abortus* biovar 3 was the same as that for all *B. abortus* wboA sequences deposited in GenBank, confirming the genetic conservation of wboA in *B. abortus* strains. The novelty of the present study was the presentation of the wboA gene as a genetic element for detection of *B. abortus* and *B. canis*. The results revealed that the point mutation of the wboA gene in native *B. melitensis* is correlated with changes in phenotype features.

5. Conclusions

Unique nucleotide substitution in wboA sequence of *B. melitensis* bivar 1 resulted in missense mutation. Regarding the function of the wboA gene in O side chain biosynthesis, it may be involved in colony morphology variations.

Acknowledgments:
The present research was conducted as a Master's of Science thesis at Razi Vaccine and Serum Research Institute of Iran. The authors thank all of the staff members of the Brucellosis Department and the Biotechnology Department at the Razi Vaccine and Serum Research Institute of Iran-Karaj.

Conflict of Interest:
There is no conflict of interest to be declared.

Authors' contributions:
All authors contributed to this project and article equally. All authors read and approved the final manuscript.

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