

Genotypic variation in the *Brucella melitensis* hemagglutinin gene in vaccine strains and field isolates in Palestine

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Abstract: In Palestine, as in other countries, brucellosis is an endemic disease, even in highly vaccinated zones. Many reports suggest that a decline in vaccine productivity may be due to antigenic shifts in the circulating *Brucella melitensis*. To address this aspect, the hemagglutinin gene from *B. melitensis* field isolates was amplified by polymerase chain reaction (PCR), sequenced, digested with the *EcoRV* and *HaeIII*, and compared to the Rev.1 strain of the *B. melitensis* vaccine. From January to December 2008, 80 milk samples were collected from infected flocks, from different West Bank regions of Palestine. From these, 77.5% (62/80) were shown to be positive for specific *B. melitensis* PCR. However, from the PCR-positive milk samples, only 38 strains could be isolated by culture on Brucella agar plates. The nucleotide alignment and phylogenetic tree for the hemagglutinin gene showed a mismatch between the vaccine strain and field isolates. This is also suggested by the observation of *EcoRV* and *HaeIII* digestion profiles for the vaccine strain and field isolates. Although a limited number of isolates and genes encoding immunologically relevant proteins were analyzed, we observed antigenic divergence between the current *B. melitensis* field isolates and the vaccine strain, in particular with respect to the hemagglutinin gene. Therefore, more research will be necessary to rule out the possibility of reduced efficacy of *Brucella* whole-cell vaccines.

Key words: Brucellosis, Palestine, *B. melitensis*, bacterial isolation, PCR assay, phylogenetic analysis, *B. melitensis* vaccine strain Rev.1, molecular genotyping

Introduction

Brucellosis is a major zoonotic disease found all over the world. However, *Brucella melitensis* is particularly prevalent throughout the Mediterranean countries, parts of Africa, western Asia, and Latin America, affecting both animals and humans (1-10). Live attenuated vaccines such as *B. melitensis* strain Rev.1 have been, and continue to be, essential elements in control programs.

A pilot vaccination program for small ruminants was initiated in 1998 in Palestine.

Despite vaccination, brucellosis is still been in the area (1,11). It is possible that vaccination might have initially reduced the circulation of *B. melitensis* and that adaptation allowed the *B. melitensis* population to restore its high circulation rate. This assumption predicts a change in the makeup of the *B. melitensis* population after the introduction of vaccination, a phenomenon that has indeed been observed elsewhere (12). Therefore, we focused in the present study on the molecular characterization of *B. melitensis* strains circulating in the West Bank (Palestine) in comparison with the strain used in the vaccination.

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To address this aspect, the hemagglutinin gene from *B. melitensis* field isolates was amplified by polymerase chain reaction (PCR), sequenced, digested with *EcoRV* and *HaeIII*, and compared to a live-attenuated Rev.1 strain of *B. melitensis* vaccine.

Materials and methods

A total of 80 milk samples, collected from infected sheep and goat flocks from different West Bank regions of Palestine, between January and December 2008, were classified as seropositive, based on a positive reaction to one or both of the rose Bengal test (RBT) and the complement fixation test (CFT), according to Alton et al. (13).

The milk samples were inoculated onto *Brucella* agar plates (Oxoid Ltd. Hampshire, UK) in order to isolate *Brucella* spp., incubated at 37 °C in 10% CO₂ for 5 to 10 days, and examined daily for the presence of colonies. The observed colonies were identified as *Brucella* spp. by morphologic, cultural, and biochemical properties such as oxidase, H₂S production, urease, and CO₂ requirement (14).

Sample processing for PCR

Genomic DNA was extracted from the milk samples using the Nucleospin tissue kit (Clontech, Germany) according to the manufacturer's instructions. PCR was performed in a 50 µL volume reaction mixture containing 10 mM tris-HCl (pH 8.4), 50 mM KCl, 1 mM MgCl₂, 200 µM each deoxyribonucleotide triphosphate (dATP, dGTP, dTTP, and dCTP; Pharmacia LKB Biotechnology, Uppsala, Sweden), 0.5 U of Taq polymerase (Promega, Madison, WI, USA), and 10 nM of each primer (Syntezza, Israel). The primers (forward primer, 5' AAATCGCGTCCTTGCTGGTCTGA; reverse primer, 5' GACGATAGCGTTTCAACTTG) described by Bricker and Halling (15), were used to amplify a target sequence of 731-bp specific to IS711 gene of *B. melitensis*. PCR was performed with a master cycler (BioRad Laboratories, Inc., Hercules, CA, USA) as follows: 30 cycles of PCR, with 1 cycle consisting of 120 s at 95 °C for DNA denaturation, 45 s at 45 °C for DNA annealing, and 30 s at 72 °C for polymerase-mediated primer extension. The last cycle included incubation of the sample at 72 °C for 6 min. The PCR products were resolved by

electrophoresis on 2% agarose gel with ethidium bromide (0.5 mg/mL).

PCR and sequencing

The hemagglutinin gene from 2 of the *B. melitensis* field isolates, 1/P and 2/P, was amplified by PCR, sequenced, and compared to the Rev.1 strain of the *B. melitensis* vaccine. The primers (forward primer, 5'GAACCAGAATACGGCAAAA; reverse primer, 5' GTCGTTTCTGCGTCAACAGA), were used for this purpose. These primers were designed using Primer3 software (<http://frodo.wi.mit.edu/primer3>) and synthesized by Syntezza. PCR was performed as described above and the products were analyzed by electrophoresis in 2% agarose gels. The PCR products were purified using the MinElute PCR purification kit (Qiagen, Germany) and the inserts were sequenced using a dideoxy chain termination method on an ABI PRISM Model 301 Sequence Instrument at Bethlehem University, Bethlehem, Palestine. The alignment of the DNA sequences, phylogenetic relationships, and restriction endonuclease digestion of the *Brucella* field isolates and Rev.1 strain of *B. melitensis* vaccine were conducted using CLC Main Workbench software; version 5.6.1, 2009.

The nucleotide sequences of the 2 *B. melitensis* field isolates (1/P and 2/P) from Palestine reported here have been submitted and assigned the GenBank accession number HM345998 and HM598409, respectively.

Results

Of the collected milk samples, 77.5% (62/80) were positive for specific *B. melitensis* PCR. However, from the PCR-positive milk samples, only 38 isolates could be cultured on *Brucella* agar plates.

Polymorphism in the gene for hemagglutinin was studied in 2 field isolates and the vaccine strain was studied by DNA sequencing. Version 5.6.1 of the CLCL Main Workbench software package was used for the alignment of DNA sequences, phylogenetic, and *EcoRV* digestion relationships. The nucleotide alignment and phylogenetic tree for the hemagglutinin gene is shown in Figures 1 and 2. A remarked divergence was observed between the vaccine strain and the 2 field isolates. We analyzed the *EcoRV* digestion profiles of the sequenced

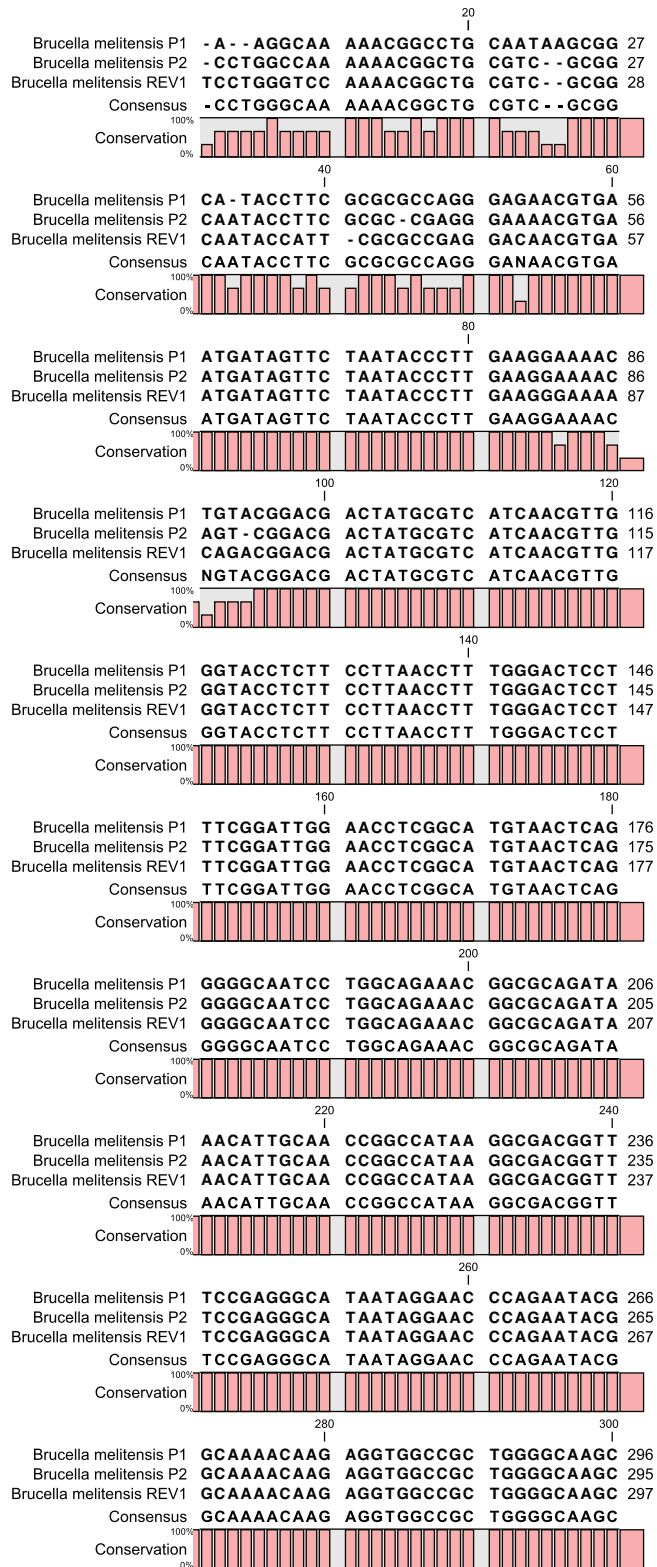


Figure 1. Partial nucleotide comparison between the hemagglutinin gene of 2 selected *B. melitensis* field isolates and the vaccine strain of *B. melitensis* Rev.1.

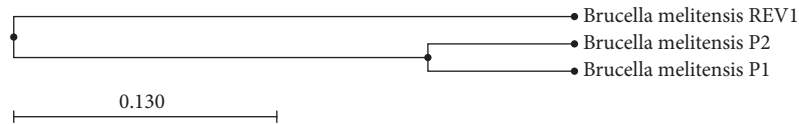


Figure 2. A distance phylogenetic tree based on the partial nucleotide sequences of the hemagglutinin gene of 2 selected *B. melitensis* field isolates (1/P and 2/P) and the vaccine strain of *B. melitensis* Rev.1. The tree was rooted using version 5.6.1 of the CLCL Main Workbench.

fragment of the hemagglutinin gene obtained from the field isolates and that obtained from the vaccine strain. As can be seen in Figure 3, *EcoRV* digestion was predicted to yield 2 DNA fragments. This was seen with the DNA from the field isolates. However, the vaccine strain Rev.1 removed the cut site of the *EcoRV*, and so no fragment was generated by the *EcoRV* digestion (Figure 3). The *HaeIII* digestion of the amplified fragments from the vaccine strain Rev.1

yielded 3 DNA fragments. In contrast, no fragments were generated by *HaeIII* digestion from the field isolates.

Discussion

The Rev.1 vaccine is quite virulent and apparently unstable, creating the need for improved vaccines for *B. melitensis* (16). It is shown that vaccination can prevent abortion; however, it probably cannot provide complete protection against infection (15,17).

Here, we reported the first study on *B. melitensis* molecular epidemiology in Palestine, showing a clear divergence between the strain used in vaccination and the field isolates. The samples used in the present study were obtained from different West Bank regions of Palestine, between January and December 2008. Of the total samples, 77.5% (62/80) proved to be *B. melitensis* by PCR, but only 47.5% (38 isolates) were culture positive. The low sensitivity of the culture method had already been reported (18,19). The higher sensitivity of the PCR assay can be explained by the fact that it detects DNA from bacteria that are damaged and/or dead; therefore, it may not be possible to isolate *Brucella* spp. by the conventional cultural techniques.

Despite the introduction of mass vaccination in 1998 in Palestine, brucellosis is currently an endemic disease with regular epidemic outbreaks. The nucleotide alignment and phylogenetic tree for the hemagglutinin gene showed a mismatch between the vaccine strain and field isolates, suggesting that it may have played a role in driving the observed high prevalence of brucellosis. This is also confirmed by the observation of a variation in *EcoRV* and *HaeIII* digest profiles for the vaccine strain and field isolates.

Although a limited number of isolates and genes encoding immunologically relevant proteins

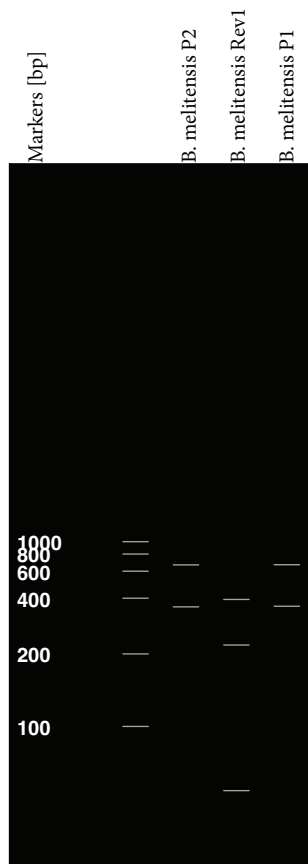


Figure 3. Agarose gel electrophoresis of *EcoRV* and *HaeIII* digest of amplified hemagglutinin gene fragments from 2 selected *B. melitensis* field isolates (1/P and 2/P) and vaccine strain Rev.1; molecular size ladder in base pairs.

were analyzed, we observed antigenic divergence between the current *B. melitensis* field isolates and the vaccine strain, in particular with respect to the

hemagglutinin gene. Therefore, more research will be necessary to rule out the possibility of reduced efficacy of brucellosis whole-cell vaccines.

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