

EPIDEMIOLOGIC APPROACHES OF BRUCELLOSIS THROUGH PHYLOGENETIC ANALYSIS PATTERN IN PALESTINE

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Abstract

*Brucellosis is an endemic zoonotic disease of the small ruminants in Palestine. The previous study revealed that the *lpsB* gene (responsible for the biosynthesis of mannosyltransferase) of *Brucella melitensis* has a degree of genetic diversity. Despite these variations, the bacteria keep its virulence and ability to produce abortion and human infection. The aim of this study was to identify the evolutionary relationships between 18 field strains of *Brucella melitensis* isolated from herds from West Bank, and to compare them with strains from different countries, using phylogenetic analysis, in order to characterize their epidemiological pattern. The sequencing of the *lpsB* gene of the 18 field strains has been performed with ABI PRISM 3500 Genetic Analyzer. The alignment and the phylogenetic analysis between field isolates, *Brucella melitensis* reference strain and Rev 1 strains were done with CLC Workbench version 6.1.5 software. The analysis revealed that all field isolates were closely related to each other: isolates from neighboring districts have had close variations, but the strongest relationship was among strains from flocks of the same village. The isolates from some flocks were closer to *Brucella melitensis* Rev 1, suggesting the exposure or infection of these with the vaccinal strain. Viewed in a global context, the West Bank isolates were phylogenetically closest to the Spanish isolates. This study highlights the input of the phylogenetic tree analysis as an epidemiological tool for the investigation of *Brucella* infection.*

Key words: brucellosis, zoonosis, phylogenetic tree, alignment

1. INTRODUCTION

Brucellosis is zoonotic disease and an important public health problem worldwide, mainly in Mediterranean countries (Doganay & Aygen 2003; Gul & Khan 2007; Refai 2002). The genus *Brucella* include 11 species of intracellular, gram-negative bacteria (Mayer-Scholl 2010; Pappas 2010; Scholz & Vergnaud 2013), the most important of them, for the public health, being *Brucella melitensis*. Due to the close homology between the *Brucella* species/strains, some researchers propose to consider all the *Brucella* as single species, who is grouping different biovars (DeVecchio et al. 2002; Whatmore 2009). The infections produced by *B. melitensis* in small ruminants are still frequent, resulting in human illness, primarily by consumption of contaminated dairy products or as professional exposure to infected livestock (Saleem, Boyle & Sriranganathan 2010; Kaoud et al. 2010; Doganay & Aygen 2003; Zvizdic et al. 2006; Scientific Committee on Animal Health and Animal Welfare 2001). This disease causes significant economic losses by decreasing productivity in reason of the abortions, of the weakness of offspring, of the diminishing of milk production and of the loss of trade opportunities (Food and Agriculture Organization 2010). In humans, the disease manifested as well as flu and if not diagnosed and treated promptly and effectively, it can become chronic and affect multiple body systems (Food and Agriculture Organization 2010; Saleem, Boyle, & Sriranganathan 2010; Scientific Committee on Animal Health and Animal Welfare 2001; World Health Organization 1999; Zvizdic et al. 2006).

The ability of sugar modification to allow survive, replicate and avoid apoptosis intra phagocytes, is essential virulence factor of pathogenesis (Gomes-Cardoso et al. 2006; Castaneda-Roldan et al. 2006; Delrue et al. 2001, 2004; Lapaque et al. 2006; Letesson & de Bolle 2004; Moreno & Gorvel 2004; Reeves 1994; Scholz & Vergnaud 2013; Wang et al. 2010; Xavier et al. 2010).

Animals are the main source for the transmission of this pathogen, therefore, several eradication programs of brucellosis, using vaccination of the animals, surveillance studies to identify the infected farm animals, the slaughter of animals and the limitation of movement in the regions where the infection has been detected, have been launched in many countries, but the success of these programs still has not reached the required level (Corbel 2006; Food and Agriculture Organization 2010).

The information on the prevalence of *Brucella* - species, biovars, genotypes/strains - in endemic areas may shed new light on the epidemiology of *Brucella* infection, on the species/biovars circulating, as well as on the efficacy of the control policy. The understanding of the *Brucella* epidemiology is critical for refining the control of brucellosis, in countries with limited resources, who cannot implement the same policy as in high-income countries (Corbel 2006; Gul & Khan 2007; Kaud 2010). Therefore, there are simple structural properties of phylogenetic trees can distinguish communicable disease outbreaks in endemic areas, can identify sources and chains of transmission, when possible using genome data alone, and can be done during an outbreak to support his management (Colijn & Gady 2014; Hall 2011).

The Palestine economy is mainly based on the agriculture, crop and animal husbandry as well (Ministry of Agriculture in Palestine 2014). The farmers are exposed to a broad spectrum of pathogens and some of them become diseased. In this respect, more systematic studies will be required to establish the epidemiological situation of brucellosis and the significance of the strains/biovars circulating in the livestock of Palestine. These data could guide the decision makers in Palestine to select the suitable alternative for future control strategies and eradication of brucellosis, at the animal and humans.

2. MATERIALS AND METHODS

2.1. Samples collection and bacterial isolation

All milk samples were obtained from animals during their routine milking time. The cream and sediment mixtures of milk were assessed after centrifugation of samples (10 mL) at 2000g, 4°C for 20 minutes, then were spread onto *Brucella* agar plates (Oxoid, UK), incubated at 37°C for 2 to 7 days and plates were daily examined for the colony presence and identification according to standard methods. In this study were used the suspensions of 18 pure isolates of *Brucella*, from the different geographical sites, for PCR identification and sequencing [Table 1].

2.2. Primer design and PCR identification

The primers used in this study for *lpsB* gene were designed using Primer3 software (<http://frodo.wi.mit.edu/primer3>). As positive control, has been used 200µl of bacterial suspension of *Brucella melitensis* Rev.1 vaccine (Ovejero, Spain) submitted to DNA extraction procedure as prescribed by the commercial kit QIAamp RNA Mini Kit (Qiagen, Germany). PCR was performed in 25 µL volume master mix (Promega, Madison, WI, USA) containing 10 mM tris-HCl (pH 8.4), 50 mM KCl, 1 mM MgCl₂, 200 µM each deoxyribonucleotide triphosphate (dATP, dGTP, dTTP, dCTP) and 0.5 U of Taq polymerase, and 10 nM of each primer (Syntezza, Israel). The primers (forward: 5'-TTCAGCTCATTCCATTGCAG-3'; reverse: 5'-CGGATTATCTTCCTGCGAAA-3') were used to amplify a target sequence of 978-bp specific to the *lpsB* gene of *B. melitensis*. PCR was performed in a thermocycler (BioRad Laboratories, Inc., Hercules, CA, USA) as follows: 30 cycles of PCR, with 1 cycle consisting of 120 s at 95 °C initial DNA denaturation, 30 s at 60 °C for DNA annealing, and 30 s at 72 °C for polymerase-mediated primer extension with final extension at 72°C for 5 min. 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).

Table 1. *Brucella melitensis* field isolates used in this study

No of isolate	Host	District	Village	Gene Bank Accession no.
BM - flock 1	sheep	Hebron	Yatta	KU980160
BM - flock 2	sheep	Jericho	Jericho	KU980145
BM - flock 3	sheep	Jenin	Qabatiya	KU980146
BM - flock 4	goat	Jenin	Qabatiya	KU980147
BM - flock 5	sheep	Hebron	Dura	KU980148
BM - flock 6	sheep	Bethlehem	Ta'amra	KU980149
BM - flock 7	goat	Hebron	Za'atara	KU980150
BM - flock 8	goat	Jericho	Al-Auja	KU980151
BM - flock 9	sheep	Hebron	Dura	KU980152
BM - flock 10	sheep	Hebron	Za'atara	KU980153
BM - flock 11	sheep	Ramallah	Qubbiya	KU980154
BM - flock 12	goat	Salfit	Bidyia	KU980155
BM - flock 13	sheep	Nablus	Beit Furiq	KU980156
BM - flock 14	sheep	Hebron	Hebron	KU980157
BM - flock 15	sheep	Jericho	Al-Auja	KU980158
BM - Rev 1(16)	vaccine	-	-	-
BM - flock 17	sheep	Jericho	Jericho	KU980159
BM - flock 18	sheep	Jericho	Al-Auja	KT357498
BM - flock 19	sheep	Hebron	Yatta	KT357499

2.3. Sequencing of PCR product

The PCR products were purified using the MinElute PCR purification kit (Qiagen, Germany) according to the manufacturer's recommendations and the sequencing was performed by ABI PRISM 35003500xL 8-channel Genetic Analyser (ABI, USA).

The sequencing was done in three steps. In the first one, it was done PCR running using Big Dye labeling for the differentiation of nucleotides sequence, as follow: 2µl RR, 2µl 5x sequencing buffer, 4µl of nuclease free water (kit BigDye terminator v3.1, ABI, USA), 3.2 pmol reverse primer, and 1µl of purified PCR products.

The amplification was carried out under thermal condition described below: 1 min at 96°C for initial DNA denaturation, followed by 25 cycles consisting of 10s 96°C for DNA denaturation, 5s 50°C for annealing DNA and 4 min. at 60°C for extension. The second step, the purification of sequencing products was performed using DyeEx 2.0 Spin kit (Qiagen, Germany), according to manufacturer instructions. In the third step, the samples were sequenced into the ABI PRISM 3500 Genetic Analyser.

2.4. The alignment and phylogenetic analysis

The alignment of the DNA sequences and the phylogenetic relationships of the *Brucella* field isolates, compared with the reference strain and the vaccine strain Rev.1, were conducted by using CLC Main Workbench software, version 5.6.1, 2009.

To determine genetic distances, it was used the UPGMA model of the phylogenetic tree software. The nucleotide sequences of the 18 *B. melitensis* field strains from Palestine, reported here, have been submitted to GenBank, who assigned them with the accession numbers [Table 1].

3. RESULTS

3.1. The alignment of sequences

The alignment of sequences of *Brucella melitensis* - 18 field strains and the reference strain - shows a different degree of variation [Figure 1].

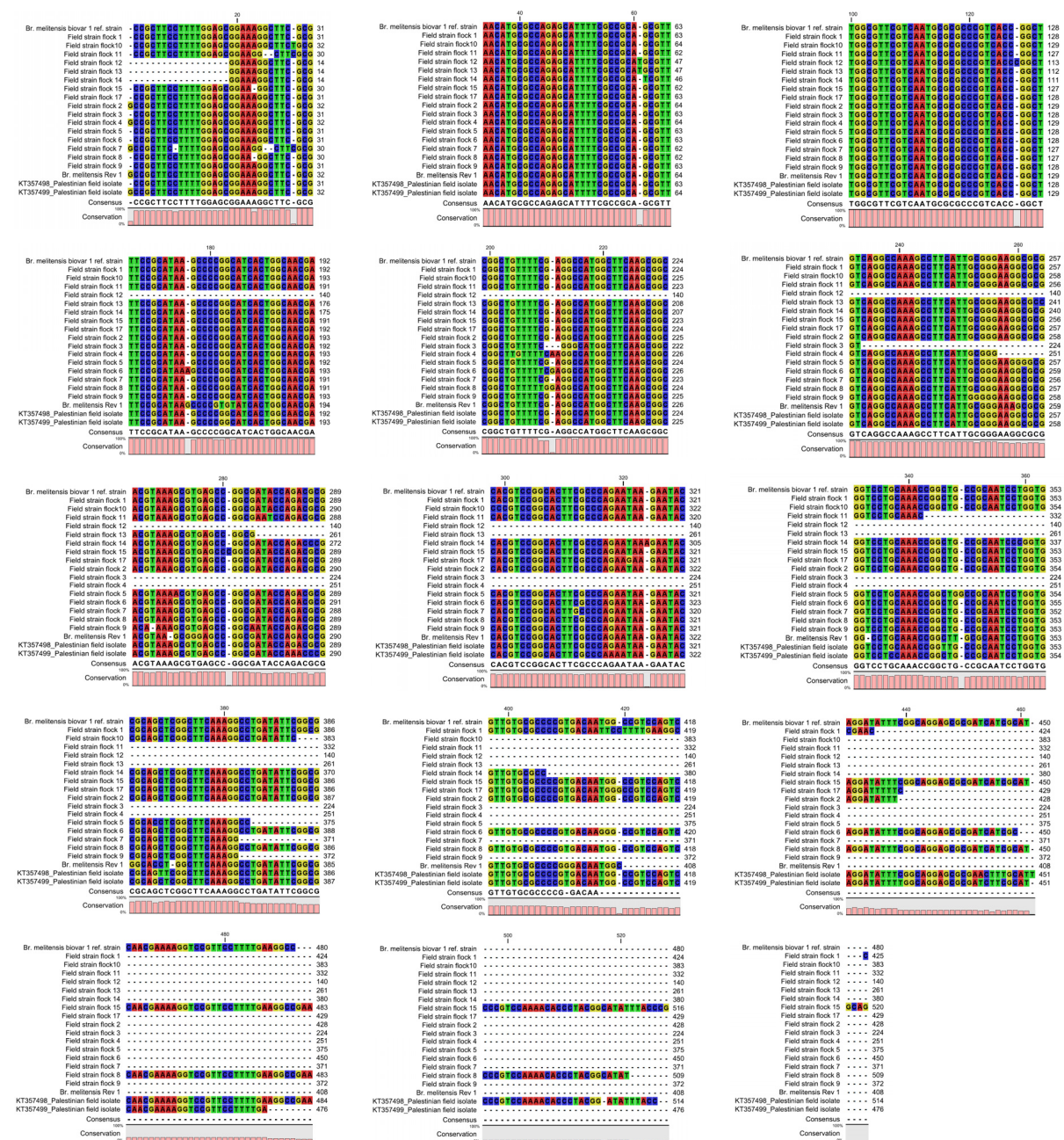


Figure 1. Multiple sequences alignment of the 18 fields isolates the *lpsB*-like gene, of the reference strain *Brucella melitensis* 16M biovar 1 and of the Rev 1 strain. Several substitutions, insertions, deletions and frameshifts of the *lpsB*-like gene.

3.2. Phylogenetic tree analysis pattern

All samples have been originated from one ancestor. Samples of flocks 9, 5, 10, 7, 2, 11, 8, 5, 4, 3 are located on same branches and originated from same villages. Samples of flocks 2, 17, 1, 6 are closer to *Brucella melitensis* Rev 1 strain [Figure 2].

The Palestinian isolates of *Brucella melitensis* revealed that their sequences have relationship to the Spanish isolates, and also to isolates from Cyprus, Turkey, Israel, and India [Figure 3].

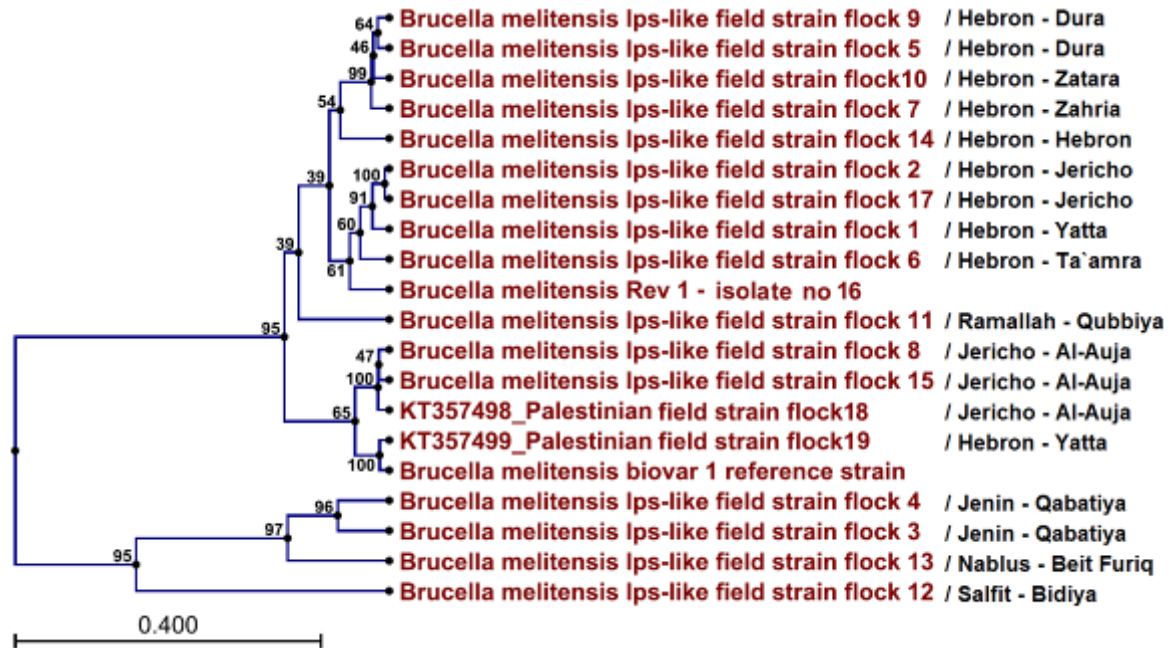


Figure 2. Phylogenetic relationship of *Brucella melitensis* -field strain isolates, between them and the reference strain *Brucella melitensis* and the Rev 1 vaccine strain.

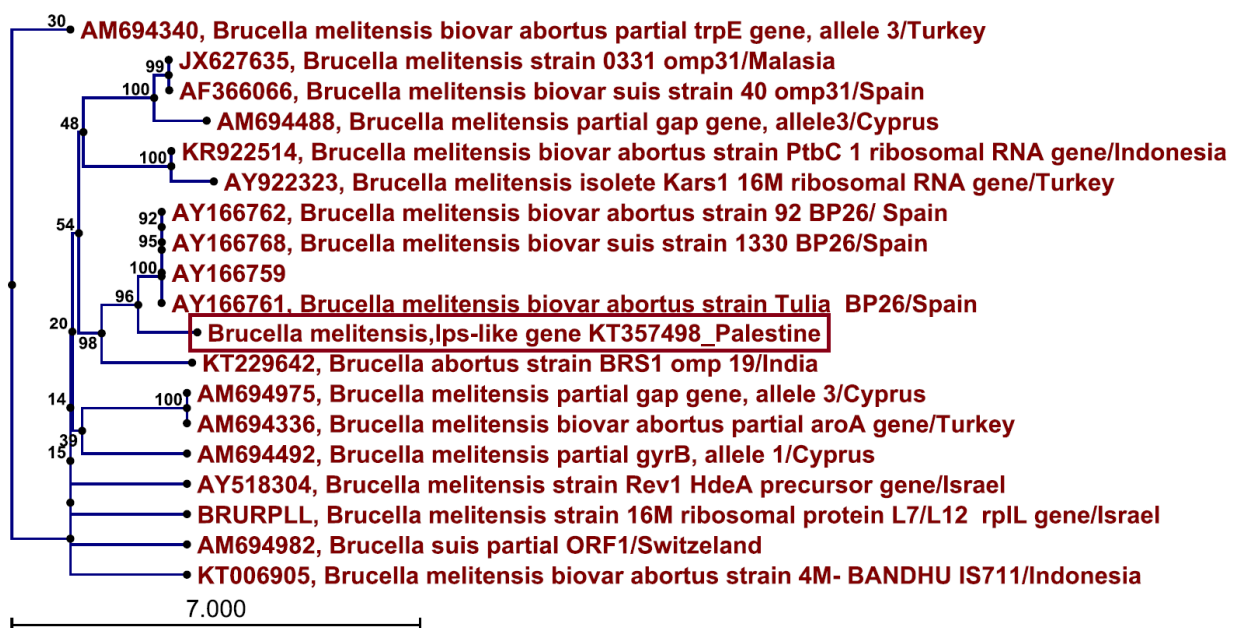


Figure 3. The phylogenetic relationship of the *Brucella melitensis*- Palestinian field strains, with the isolates from other countries.

4. DISCUSSION

The accurate diagnosis and the epidemiological surveillance of the brucellosis, as well as the taxonomy of the genus *Brucella*, have clearly benefited from the appearance and development of molecular analysis.

The genetic homology and diversity between the isolates and the reference strain, comparing it with GenBank data, are able to indicate the relationship of the field strains and the data global recorded.

The straight homology with isolates from other countries, registered the wide geographical distribution of this clade. Therefore, the restrictive criteria for animals' trade from neighboring countries must be in force, to enhance the rate of success of the control policy for *Brucella melitensis* disease in Palestina. The geographical location is an external risk factor, predicting the relationships between isolates; consequently, isolates from neighboring regions tending to be more closely related to each other (Bamaiyi et al. 2012; Corbel 2006; Gul & Khan 2007; Hall 2011; Mahmod 2014; Mirnejad et al. 2012; Muendo et al. 2012; Mujizi 2015; Rajagunalani et al. 2013; Saitou & Nei 1987; Tien et al. 2015).

In some cases, geographical distance, it is less relevant for isolates with some level of variations and is irrespective whether they originated from man or animals, due to factors such as transhumance, mutation, breed and species variability (Al-Garadi et al. 2011; Nagalingam et al. 2012; Sayan et al. 2009).

The phylogenetic tree in our study contains a lot of information about the inferred evolutionary relationships among a set of field isolates.

The phylogenetic mapping analysis of the 18 field isolates of *B. melitensis* revealed a high level of homology, despite the presence of some degree of diversity, suggesting the same phylogenetic origin.

The strains sampled from the same village (the flocks 9, 5, 4, 3, 2, 17) express closer similar genetic variation, the phylogenetic analysis locating them on the same "tree" branches (the flocks 9, 5, 4, 3, 2, 17) [Figure 2]. This study shows also that strains from the same source (origin, geographic location) are evolving over a similar pattern, achieving similar variations - quite identical.

The strains isolated from the herd's number 2, 17, 1, 6 had been closer to *Brucella melitensis* Rev 1 strain, bringing up the probability of the infection due to vaccine [Figure 2]. In conclusion, the slight variation degree of the field strains of *Brucella melitensis* does not alter its virulence and ability to produce a storm of abortion in ewes, and human infections.

The Palestinian isolates of *Brucella melitensis* revealed that their sequences have closest relationship to the Spanish isolates, and are also very close to isolates from Cyprus, Turkey, India, and Israel [Figure 3]. Corroborating this evidences with our above mentioned observation that the strains from the same source (origin, geographic location) are evolving over a similar pattern, we can assume a common ancestral evolutionary origin of the Palestinian field strains and isolates from Cyprus, Turkey, India, and Israel.

Despite the intensive vaccination program using the *Brucella melitensis* Rev 1 vaccine, in Palestine, the brucellosis remains a significant problem: the extremely high rate (95-100%) of cell envelope virulence-associated genes among Palestinian isolates, the ability of some of genes, like *lpsB*, to mutate and survive in different environment condition, without losing their virulence, seem to explain the unsatisfactory results of the vaccination program (Awwad et al. 2015).

It is possible that vaccinations initially reduce the circulation of *B. melitensis*, but the adaptive mutations allow the *B. melitensis* population to restore its high circulation rate. Also, environmental stress might contribute to the change in the makeup of the *B. melitensis* (Awwad et al. 2015; Bardenstein et al. 2002). Therefore, in Palestine only with a sustained mass vaccination program would be reduce the circulation of the field strains.

Phylogenetic analysis of *Brucella sp* contribute to better understanding of geographical transmission patterns and gives a reliable tool to be used by authorities, in order to implement control measures of outbreak, to prevent the spread of the disease among the human and animal population.

5. CONCLUSION

The phylogenetic analysis of Palestinian isolates of *Brucella melitensis* revealed the closest relationship to the Spanish isolates, and also with the isolates from Cyprus, Turkey, Israel, and India.

The slight variation degree of the Palestinian field strains of *Brucella melitensis* does not alter its virulence and ability to produce animal and human infections.

Phylogenetic analysis of the field isolates is a highly useful tool for the monitoring of brucellosis and for the assessment of the control policy.

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