

Isolation, identification and molecular characterization of *Brucella* strains isolated from small ruminants in the West Bank, Palestine

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SUMMARY

The seroprevalence of brucellosis in the small ruminant (sheep and goat) population from infected flocks was investigated in the West Bank (Palestine). 36.8% (129/350) and 32.3% (113/350) of animals were found to be positive by the Rose Bengal test (RBT) and the complement fixation test (CFT) respectively, the agreement score between the 2 methods being 89.1%. The presence of *Brucella* directly assessed by bacterial isolation and PCR assay in milk samples from 80 seropositive animals was confirmed in 47.5% (38/80) and in 77.5% (62/80) of samples, leading to a moderate agreement score between the 2 methods (70.0%). The phylogenetic analysis of a putative open reading frame (ORF) in the haemagglutinin gene revealed that the current *B. melitensis* isolates were genetically close to those from different Mediterranean countries (France, Spain and Cyprus) and particularly to the Israel strain. This first report of DNA sequencing of *Brucella* strains in small ruminants in Palestine would greatly help to implement control and preventive measures for brucellosis.

Keywords: Brucellosis, small ruminants, Palestine, serological test, *B. melitensis*, bacterial isolation, PCR assay, phylogenetic analysis.

RÉSUMÉ

Isolement, identification et caractérisation moléculaire des souches de *Brucella* chez les petits ruminants en Palestine (West Bank)

La séroprévalence de la brucellose en Palestine (West Bank) a été évaluée au sein de la population de petits ruminants (moutons et chèvres) issus de troupeaux infectés : 36,8 % (129/350) et 32,3 % (113/350) des animaux se sont révélés séropositifs, respectivement par le test au Rose Bengale (RBT) et la fixation du complément (TFC), l'accord entre les 2 méthodes étant de 89,1 %. La présence des *Brucella* dans les prélèvements de lait provenant de 80 animaux séropositifs a été confirmée dans 47,5 % (38/80) des cas par isolement bactérien et dans 77,5 % (62/80) par PCR, l'accord entre ces 2 méthodes restant modéré (70,0 %). L'analyse phylogénétique d'un des cadres possibles de lecture ouverte du gène codant pour l'hémagglutinine (HA) a révélé que les isolats de *B. melitensis* réalisés au cours de cette étude étaient génétiquement proches de ceux obtenus dans différents pays méditerranéens (France, Espagne et Chypre) et plus particulièrement de la souche israélienne. Ce premier travail de séquençage de l'ADN des souches de *Brucella* chez les petits ruminants en Palestine devrait aider à la mise en œuvre d'un contrôle et de mesures préventives de la brucellose.

Mots clés : Brucellose, petits ruminants, Palestine, test sérologique, *B. melitensis*, isolement bactérien, PCR, analyse phylogénétique.

Introduction

Brucellosis, especially caused by *Brucella melitensis*, is recognized as a significant public health challenge, with a major economic and financial burden in countries where the disease remains endemic [6-8, 13, 20]. In the Mediterranean region as well as in Middle East, parts of Africa, Western Asia and Latin America, *B. melitensis* infections in small ruminants are still widespread, resulting in significant human illness, primarily from consumption of contaminated dairy products or from occupational exposure to infected livestock [6-8, 11, 13, 15, 18-21].

In small ruminants, contagious abortion, reduced fertility and economic losses in terms of milk are the major impacts. *B. melitensis* is enzootic in different regions of Palestine and is the main cause of abortion in small ruminants [3, 10]. Brucellosis in Palestine is mainly diagnosed based on clinical signs, serological and bacteriological investigations. Up to now, no article has documented the molecular characterization

of the circulating *B. melitensis* population in Palestine. This characterization has a pivotal role in epidemiological studies, management of the outbreaks and implementing control and preventive measures. The present study aimed to obtain a snapshot of *Brucella* infections in the West Bank (Palestine), to explore the prevalence of these strains in small ruminants of infected flocks and to molecularly characterize *B. melitensis* strains circulating in Palestine.

Materials and Methods

ANIMAL SAMPLES AND SEROLOGICAL ANALYSES

A total of 350 sera and 80 milk samples collected from infected flocks in the West Bank, Palestine between January and December 2008, were classified as seropositive based on a positive reaction to one or both of the following tests: 1) rose Bengal test (RBT); and 2) complement fixation test

(CFT) carried out according to ALTON *et al.* [2]. All milk samples were obtained from seropositive animals.

BACTERIOLOGICAL ANALYSIS OF MILK SAMPLES

The cream and sediment mixtures from each of the 80 milk samples from seropositive small ruminants were assessed after centrifugation of milk samples (10 mL) at 2 000g, 4°C for 20 minutes. Cream and sediment were then spread onto *Brucella* agar plates (Oxoid Ltd., Hampshire, UK), incubated at 37°C in 10% CO₂ for 5 to 10 days then plates were daily examined for the colony presence and identification according to standard methods adapted by QUINN *et al.* [17].

PCR ASSAY AND SEQUENCING

Genomic DNA was extracted from milk samples and from positive milk cultures using the Nucleospin tissue kit (Clontech, Germany) according to the manufacturer's instructions.

PCR assay 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, dCTP, Pharmacia LKB Biotechnology, Uppsala, Sweden), 0.5 U of Taq polymerase (Promega, Madison, WI, U.S.A.), and 10 nM of each primer (syntezza, Israel). The primers were genus-specific primer pairs designed to amplify the IS711 gene of the genus *Brucella* (Table I). The cycling condition included an initial denaturation step at 95°C for 120 seconds and then templates were cycled 35 times (30 seconds of denaturation at 95°C, 45 seconds of annealing at 51°C and 30 seconds of extension at 72°C) with a final extension step at 72°C for 6 minutes by a Master Cycler (BioRad Laboratories Inc., Hercules, CA). The positive control contained DNA isolated from Rev.1 strain of *B. melitensis* as the template, and the negative control consisted of sterile water instead of the DNA template. The PCR products were resolved by electrophoresis on 2% agarose gel with ethidium bromide (0.5 mg/mL).

Two of the *B. melitensis* field isolates (1/P and 2/P) were used to amplify and sequence a putative ORF in the haemagglutinin (HA) gene. The oligonucleotide primers used for this purpose are listed in Table I. After PCR assay (as described above) and agarose 2% gel electrophoresis, the PCR

products were purified using the MinElute PCR purification kit (Qiagen, Germany) and the inserts were sequenced by a dideoxy chain termination method on an ABI PRISM Model 301 Sequence Instrument at Bethlehem University, Bethlehem, Palestine.

The alignment of DNA sequences and the comparisons with other sequences were performed using the version 2.2.23 of BLAST. The phylogenetic relationships between the *Brucella* isolates and selected reference *Brucella* originated from different Mediterranean countries were conducted using the CLC Main Workbench software (version 5.6.1, 2009). The nucleotide sequences of the two *B. melitensis* field isolates (1/P and 2/P) from Palestine reported here have been submitted to the GenBank accession number and were assessed as HM345998 and HM598409, respectively.

Results

Among the 350 sera from infected flocks in the West Bank (Palestine) tested in the present study for detecting *Brucella* antibodies, 129 (36.8%) and 113 (32.3%) were positive for RBT and CFT, respectively (Table II). A comparison between the RBT and CFT results showed that 102 animals (29.1%) were positive in both tests and 210 (60.0%) were negative in both tests, leading to an agreement score (number of similar results with both tests) of 89.1%.

By testing milk samples obtained from 80 serologically positive animals, all the 38 (47.5%) positive ruminants detected by bacterial isolation were also detected as positive by the PCR assay (Table III). Furthermore, 24 (30.0%) additional milk samples (that were negative for the bacterial isolation) gave positive results with the PCR assay, and the frequency of *Brucella* spp. in milk samples from seropositive small ruminants determined by this method reached 77.5%. Consequently, the agreement score between the bacterial isolation and the PCR assay was moderate (70.0%). Moreover, *B. melitensis* was identified in 42 PCR-positive milk samples, leading to a high prevalence (67.7%) - of this specie - among *Brucella* spp. in milk samples.

The DNA sequence of the haemagglutinin gene ORFs from field isolates 1/P and 2/P were identical to the recently identified sequence of type strain of *B. melitensis* (ATCC 23457, GenBank access no: CP001489), confirming that the isolates belonged to the genus *Brucella*. The results of a maximum likelihood phylogenetic analysis by creating a cluster

Primer types	Oligonucleotide sequences (5'-3')	Amplified product
<i>Brucella</i> - primer	Forward: G GTTGTAAAGGAGAACAGC	600 bp
	Reverse: GACGATAGCGTTTCAACTTG	
<i>B. melitensis</i> -specific primer	Forward: AAATCGCGTCCTTGCTGGTCTGA	731 bp
	Reverse: TGCCGATCACTTAAGGGCCCTTCAT	
HA-ORFs (DNA sequence)	Forward: GAACCAGAATACGGCAAAA	2 000 bp
	Reverse: GTCGTTTCTGCGTCAACAGA	

HA: haemagglutinin gene.

TABLE I: Sequences of primers for *Brucella* used in the present study.

tree of the current isolate with other GenBank-accessible gene sequences of *B. melitensis* isolated from several Mediterranean countries are shown in figure 1. This tree sorts the *Brucella* sequences studied here into three lineages, named 1, 2 and 3. Although the tree is technically branching, the shortness and suboptimal support of those branches in the lineage n° 1 suggest a higher average of similarity between France / Spain / Israel / Palestine (isolates 1 and 2) and Cyprus *Brucella* strains; they appear to have radiated explosively. This tree has also showed, as expected, that the strain with an accession number AY518304 which belongs to Israeli strains is the closest relative to the *Brucella* field isolates in the present study.

Discussion

Brucellosis, particularly caused by *B. melitensis*, is considered as one of the major zoonosis in Palestine. The Palestinian Brucellosis Control Program started in 1998, adopting the strategy of a mass vaccination of sheep and goats (all ages and both sexes) via the conjunctival route using full dose Rev.1 vaccine, but despite this, the sheep and goat prevalence remained at approximately 4.8% and the flock/herd prevalence at 46.3% in 2005 [7]. In the present study, serological RBT and CFT tests showed different sensitivities (36.8% and 32.3%, respectively). It is interesting to note the discrepancies with the serological results, reflecting by a moderate agreement score (89.1%): indeed, 102 (29.1%) and 210 (60.0%) sera samples were simultaneously positive and negative respectively, in both tests whereas 11 samples (3.1%) were RBT-negative and CFT-positive and 27 (7.7%) were RBT-positive and CFT-negative; despite these discrepancies between the 2 serological methods observed in the present study and in others [4, 14] and the probable overestimation of the brucellosis seroprevalence throughout the

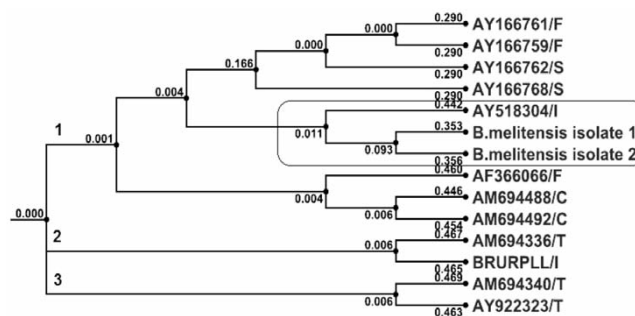


FIGURE 1: A distance phylogenetic tree based on the partial nucleotide sequences of the ORFs sequences of haemagglutinin gene of two selected *B. melitensis* field isolates and GenBank-accessible gene sequences of *B. melitensis* isolated from several Mediterranean countries (I, Israel; F, France; S, Spain; C, Cyprus; T, Turkey). Horizontal distances are proportional to the genetic distances. The tree is rooted by the version 5.6.1 of CLCL Main Workbench.

RBT, the results evidenced a high brucellosis seroprevalence in small ruminants and confirm in one hand that the disease is endemic in Palestine and should not to be underestimated and on the other hand, that it was impossible to detect all infected animals using a single serological test [4, 12].

Because the positive detection of specific DNA, as well as the bacteriological isolation of *Brucella*, is a certain indication of the presence of the pathogen, the feasibility of molecular assays as diagnostic tools for brucellosis was evaluated in the present study. The PCR assay has allowed the infection detection in 77.5% of milk samples from seropositive animals, while *Brucella* species could be isolated in only 47.5% samples. The low sensitivity of the culture method has already been reported [5, 9]. The higher sensitivity of the PCR assay can be explained by the fact that it detects DNA from bacteria that are damaged and nonviable and therefore impossible to isolate by conventional cultures. Interestingly, 22.5% (18/80)

		RBT		Total
		positive	negative	
CFT	positive	102 (29.1%)	11 (3.1%)	113 (32.3%)
	negative	27 (7.7%)	210 (60.0%)	237 (67.7%)
Total		129 (36.8%)	221 (63.1%)	350

TABLE II: Frequencies of serum anti-brucella antibodies detected by RBT (Rose Bengal Test) and CFT (Complement Fixation Test) in 350 cattle from infected farms.

		Bacterial isolation		Total
		positive	negative	
PCR assay	positive	38 (47.5%)	24 (35.0%)	62 (77.5%)
	negative	0 (0.0%)	18 (22.5%)	18 (22.5%)
Total		38 (47.5%)	42 (52.5%)	80

TABLE III: Detection of *Brucella* spp. in milk samples by bacterial isolation and PCR assay recovered from 80 serologically positive cows.

of milk samples from seropositive small ruminants were PCR negative in this study. This discrepancy could be related to a lack of sensitivity of the PCR technique, to serologically false results or also to an earlier contact with bacteria sufficient to induce a specific and persistent humoral immune response whereas the pathogen has been eliminated from the organism. However, because the natural immunization in sheep and goats was in fact often coupled to a latent infection without a total elimination of the *brucella* and because of the occurrence of frequent serological cross-reactivity with other bacteria such as *Yersinia enterocolitica* O:9, *Francisella tularensis*, *Escherichia coli* O:157, *Salmonella urbana* group N, *Vibrio cholerae* and *Stenotrophomonas maltophilia* [1, 9, 16], it is highly likely that a great number of these samples were indeed false positives for brucellosis.

In the present study, the phylogenetic analysis (figure 1) strongly suggested that the two Palestinian *Brucella* field isolates and the *Brucella* isolates from the Israel, France, Cyprus and Spain are closely related. In addition, as expected, the Israeli strain identified as AY518304 was the most strongly similar to the 2 Palestinian *B. melitensis* strains (1/P and 2/P isolates), confirming anterior brucella contamination of sheep and goats from Israel to Palestine.

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