



# The Prevalence and Molecular Distinguishing of *Brucella melitensis* Rev1 Strain among Field Isolates the *Brucella* from Sheep and Goat Milk through PCR-RFLP Analysis of *omp2* Gene Polymorphism

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## RESEARCH ARTICLE

### Abstract

The present study was undertaken to characterize the prevalence and molecular distinguishing of *Brucella melitensis* field and vaccine Rev1 strains isolated from milk among the sheep and goat population. The study was carried out on flocks of sheep and goats suffering from *Brucella* infection outbreaks. A total of 171 milk samples were collected from different districts of the West Bank, part of Palestine, and isolated on *Brucella* agar. 86 cultures were confirmed by standard biochemical methods and screened for *Brucella melitensis* 16M of IS711 element by a conventional PCR technique. All these samples were also amplified for the *omp2* gene to identify differences between field and Rev 1 strains in an infected flock. The results obtained from the *Pst*I restriction enzyme pattern revealed that only 2 (4.5%) samples have specific polymorphism accomplished with the *Brucella melitensis* Rev1 strain. All other samples were restricted according to the *Brucella melitensis* 16M polymorphism pattern and originated from nonvaccinated flocks. Despite the adverse side effects of the *Brucella melitensis* vaccine, only a well-organized whole-flock vaccination and awareness campaign may inhibit the virulence of the field strain and, subsequently, reduce the prevalence of brucellosis infection among animals and humans in Palestine.

**Keywords:** RELP, polymorphism, *Brucella melitensis*.

Received: 31 August 2022

Accepted: 13 November 2022

Published: 15 May 2023

DOI:

10.15835/buasvmcn-vm:2022.0024



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## INTRODUCTION

Brucellosis is a zoonotic disease with worldwide distribution and is evident by its visible impact on public and animal health (Dogany and Aygen, 2011; Gul and Khan, 2007). This infection is causing significant economic losses in the livestock sector due to abortion, which leads to decreasing productivity and infertility, weak offspring, reduced milk production, longer inter-calving, and losses in trade opportunities. WHO, 1999; Corbel et al., 2010; FAO, 2010). Brucellosis in small ruminants is still widespread, resulting in human illness, primarily from consumption of contaminated dairy products or occupational exposure to infected livestock, and manifested by febrile illness in humans (Sanco et al., 2000; Zvizdic et al., 2006; Saleem et al., 2010; Kaoud et al., 2010; Dogany et al., 2011).

Palestine is considered a historically endemic region and has about 5% of infected sheep and goat populations and 200 cases of human brucellosis per year. The major sources of infection are associated with direct contact with small ruminants and the consumption of their raw milk and dairy products. The control of brucellosis is linked to mass vaccination of animals, regular screenings, surveillance, and raising public health awareness. In addition, the public's consumption of pasteurized milk and dairy products is a critical issue (Awwad et al., 2018).

Brucellae are gram-negative, facultative, intracellular bacteria with the ability to survive and replicate inside phagocytes, which is the most important virulence factor in pathogenesis. It is successfully obtained through the complex structure of the cell wall and the ability to modify sugar, considered an important indication of adaptation in the vivo host environment (Reeves et al., 1994; Castaneda-Roldan et al., 2000; Letesson and De Bolle, 2004; Moreno and Gorvel, 2004; Castaneda-Roldan et al., 2004; Cardoso et al., 2006; Ficht et al., 2006; Lapaque et al., 2006; Castaneda-Roldan et al., 2006; Delrue et al., 2006; Wang et al., 2010; Xavier et al., 2010; Scholz et al., 2013). The distinction between species and biovars through the identification of specific DNA markers is a crucial epidemiological aspect with the aim of proper diagnosis, prevention, and control of the disease. The major outer membrane proteins (OMPs) of the *Brucella* genome are encoded by the genes *omp25*, *omp31*, and *omp2* locus. The *omp2* locus contains two gene copies that code for porin protein (*omp2a* and *omp2b*). These genes share a great degree of DNA homology (> 90%), but they exhibit sufficient polymorphism in all species and, therefore, are particularly useful for differential purposes and to allow distinguish between *Brucella* species and biovars. *Omp2a* and *omp2b* as loci of two nearly homologous repeated copies, indicate a greater degree of diversity in all species of *Brucella*. *Omp2a* does not have the restriction site of *Pst*I and, therefore, is not a good target for differentiation of vaccine strains with field strains in PCR-RFLP, but *omp2b* has the mentioned site for the *Pst*I enzyme and can be used successfully for differentiation of all *Brucella* vaccine and field strain infections (Ficht et al., 1988; Ficht et al., 1990; Cloeckaert et al., 1995; Cloeckaert et al., 1996; Ficht et al., 1996; Vizcaino et al., 1997; Vizcaino et al., 2000; Bardenstein et al., 2002). PCR-RFLP is one of the methods can be employed to find DNA polymorphism and perform molecular identification and typing of the *Brucella* species. *Pst*I is one of the endonuclease enzymes which can digest points of polymorphism and differentiate the 2 different restriction profiles derived from *B. melitensis* strains (Allardet-Servent et al., 1988; Halling and Zehr et al., 1990; Ficht et al., 1990; Cloeckaert et al., 1995; Ficht et al., 1996; Vizcaino et al., 1997; Cloeckaert et al., 2002; Al-Dahouk et al., 2005).

Considering the lack of epidemiologic data on sheep and goat brucellosis in Palestine and the importance of the disease, including its zoonotic potential, the aim of the present study was to evaluate the molecular prevalence and to distinguish between the vaccinal and wild strains. Indeed, epidemiological data is critical for disease control and prevention. However, the authors of the paper are not responsible for taking measures (and I doubt they can), so the goal is not to act, but to provide data.

## MATERIALS AND METHODS

### Samples collection

The West Bank, a landlocked territory in the eastern and northern parts of the Palestinian territories, was chosen for the study. The 171 milk samples from suspected sheep and goats from different districts of the West Bank were collected between March 2010 and October 2014. All milk samples were obtained from both teats of the mammary gland during routine milking and transported to the laboratory on ice at about 4°C. The milk was centrifuged at 3000 rpm for 3 minutes and the sediment was used for culture purposes.

### Isolation of *Brucella melitensis* Rev 1 strain and field strains

The DNA of vaccine strain *Brucella melitensis* Rev1 used in this study is the same Rev.1 vaccine source that has been used by the Palestinian Ministry of Agriculture since 1999. A bottle of Rev1 vaccine (Ovejero, Spain) was reconstituted with 0.5 ml of 0.9% normal saline. The solution of *Brucella melitensis* Rev1 and sediment of 171 milk samples were inoculated onto *Brucella* agar plates (Oxoid, UK) with added *Brucella* selective supplement (Oxoid, UK) and 5% of calf serum (Sigma, USA) and incubated at 37°C for 3–7 days. Colonies were investigated and identified as *Brucella* sp. by morphologic, cultural, and biochemical properties such as oxidase, H<sub>2</sub>S production, urease, CO<sub>2</sub> requirement, and ability to grow on agar by adding thiamin and basic fuchsin dye. Suspected *Brucella* colonies were diluted in 0.9% normal saline and stored at -20°C.

### PCR analysis

Defrosted bacterial suspension of *Brucella* colonies, both Rev.1 and 86 samples of DNA isolated from milk were extracted using the QIAamp viral RNA mini kit (Qiagen, Germany) according to the manufacturer's instructions. The *Brucella melitensis* 16M IS711 and *omp2* genes were confirmed in all positive cultures. Two different PCR reactions (25µl) were performed using 12.5 µl GoTaq green master mix (Promega, Germany) obtained from 0.5 U Taq polymerase, 10 mM Tris – HCl, 3 mM MgCl<sub>2</sub>, 50 mM KCl, 0.1% Triton, 400 µM of each nucleotides, 6.5 µl nuclease free PCR water and pair of primers (Synteza, Israel): 0.5 µl of each primer: IS711: forward: 5'-AAATCGCGTCCTTGCTGGTCTGA-3' and reverse: 5'-TGCCGATCACTTAAGGGCCTTCAT-3' (Garcia-Yoldi et al., 2006)

and of *omp2* gene: forward: 5'-TGGAGGTCAGAAATGAAC-3' and reverse: 3'-GAGTGCGAAACGAGCGC-5' of an *omp2* gene (Bardenstein et al., 2002) and 5 µl DNA template. PCR amplification was performed using a Master Cycler (BioRad Laboratories, Inc., Hercules, CA) the following thermal conditions: initial denaturation at 95°C for 3 min followed by 35 cycles each consisting of 30 sec of denaturation at 95°C, 45 sec of annealing at 45°C and 30 sec of extension at 72°C for IS711 (Garcia-Yoldi et al., 2006) and 20 sec of denaturation at 95°C, 1 min of annealing at 50°C and 1 min of extension at 72°C for *omp2* gene, with a final extension at 72 °C for 7 min (Bardenstein et al, 2002). DNA isolated from *Brucella melitensis* Rev1 and PCR mixture without a template, with addition nuclease-free water were used as a positive and negative control. The amplified products were examined by 2% agarose gel electrophoresis to determine the size of amplified fragment for each isolate.

#### DNA digestion by *Pst*I enzyme and 12% SDS-PAGE separation.

The protocol of restriction enzyme *Pst*I was used according to the manufacturer's instructions (Thermo Fisher Scientific, USA). A 12% polyacrylamide gel was prepared with 37.5 ml of 40% acrylamide/bis, 4.8 ml of H<sub>2</sub>O, 2.4 ml of 5xTBE buffer, 200 µl of 10% ADS, and 20 µl of TEMED. The digested DNA was separated by electrophoresis on 2% agarose gels (Promega, Germany) and 12% polyacrylamide gels. The product in polyacrylamide gels was performed by using 0.05% TBE buffer at 90V, 1.2W. DNA fragments were visualized by staining with ethidium bromide (0.5% buffer).

## RESULTS AND DISCUSSIONS

### Laboratory findings

Cultures of 86 milk samples obtained from infected small ruminants in the northern districts of the West Bank were grown on *Brucella* agar. All cultures were typical isolates of *Brucella* in morphology, colonial appearance, and growth characteristics (Figure 1). Colonies were round, glistening, smooth and mucoid, urease and oxidase positive, required no CO<sub>2</sub> and were able to grow on agar with the addition of thionine and basic fuchsin.

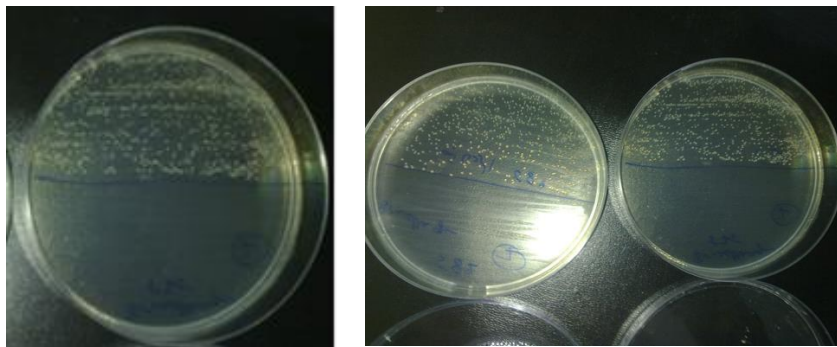


Figure 1. *Brucella melitensis* field isolate on *Brucella* agar

### PCR and RELP analysis

Genomic DNA the 86 samples of isolated bacteria was detected as positive by PCR utilizing primers specific to the IS711 gene of *B. melitensis* 16M (Figure 2).

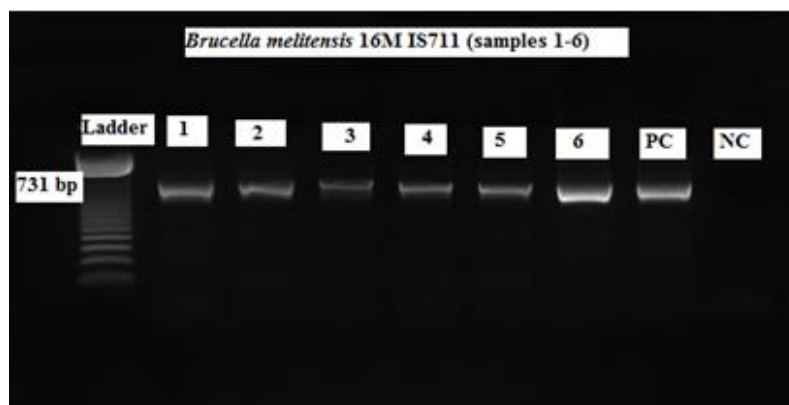


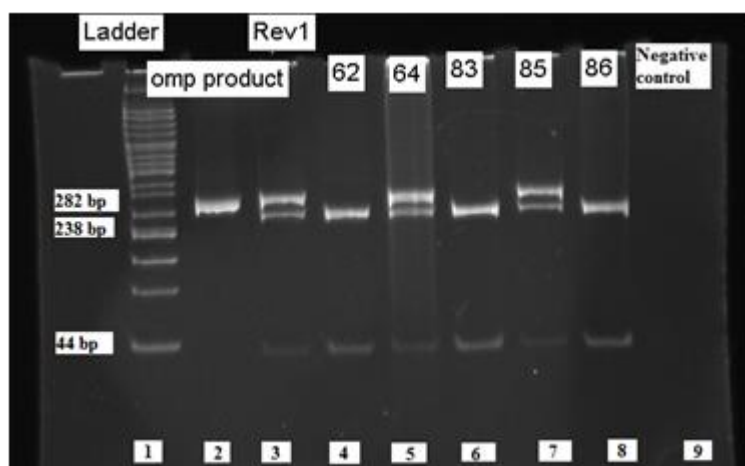
Figure 2. Agarose gel electrophoresis amplified product *Brucella melitensis* IS711 specific element with 731bp DNA fragment for 6 field isolates samples

The 86 positive *Brucella melitensis* field isolates were positive for specific *omp2* gene primers and were used in RELP analysis to differentiate the Rev 1 vaccine strain from the field strain. The overall prevalence of a *Brucella melitensis* field strain pattern was found in 84 (97.7%) samples, and only two samples (2.3%) matched with the *Brucella melitensis* Rev 1 pattern. These samples were obtained from one flock in Hebron and one from Ramallah districts (Table 1).

**Table 1.** Distribution of sample by district and flocks and *Brucella melitensis* results

Districts	No of flocks	No of milk samples			<i>Brucella melitensis</i>						
					Culture (+ve)	Field strain (RELP-PCR)		Rev1 strain (RELP-PCR)		% +ve field strain	%+ve Rev1
		Sheep	Goat	Total		(+ve)	(-ve)	(+ve)	(-ve)		
Dura	5	34	12	46	22	21	1	1	21	95.5	4.5
Hebron	9	45	25	70	40	40	0	0	40	100	0
Jericho	2	14	9	23	11	11	0	0	11	100	0
Ramallah	4	22	8	30	12	11	1	1	11	91.7	8.3
Nablus	1	2	0	2	1	1	0	0	1	100	0
Total	21	117	54	171	86	84	2	2	84	97.7	2.3

The amplification of the *omp2* gene was obtained as a single band of 282 bp in all isolates. Digestion of the PCR products from field isolates and Rev 1 with *Pst* I restriction endonuclease gave different band profiles on an agarose gel and polyacrylamide gel: *Brucella melitensis* Rev 1 has three fragments with sizes of 282, 238, and 44 bp, respectively, and DNA field isolates only two digested fragments, 238 and 44 bp, respectively. Two field isolates gave two visible bands similar to that of the vaccine strain (lanes 5 and 7). A clearly defined profile of the digested PCR products' DNA was obtained by 12% polyacrylamide gel electrophoresis. The purpose of this analysis was to visualize the smaller fragment that was not resolved by agarose gel electrophoresis. The 12% polyacrylamide gel (Figure 3) shows that, in addition to the 282- and 238-bp DNA bands, all samples produced an additional identical smaller fragment, the 44-bp.



**Figure 3.** Polyacrylamide gel electrophoresis of *Pst*I digests of amplified *omp2* gene fragments of *Brucella* Rev 1 strain and field isolates. Lines 1: Molecular size ladder (50 bp), 2 – undigested *omp2* gene; 3 – *Brucella melitensis* Rev 1 positive control with three digested bands, 4 – field isolate nr. 62; 5 – field isolate nr. 64; 6 – field isolate sample nr 83; 7 – field isolate nr. 85; 8 – field isolate nr. 86; 9 – Negative control.

*Brucella* is the most important pathogen cause abortion in sheep and goats worldwide. This bacterium causes human infection through consumption of infected milk or close contact with infected animals (Ariza, 1996; Pappas et al., 2006). The control of the disease usually depends on the implementation of a variety of activities such as vaccination, strict hygiene, and wide epidemiological investigation. The most effective plan for the elimination of the disease is the implementation of a "test and slaughter" policy, which includes the detection of infected animals by screening tests of specific serum *Brucella* antibodies followed by the elimination of positive animals (OIE, 2010, FAO, 2010; Corbel et al., 2010).

The major problem in Palestine as a third-world country that hinders the implementation of tests and slaughter policies is the lack of recourses to compensate affected owners by the government. As a result, only activities such as flock vaccinations can significantly reduce the prevalence of brucellosis to the point where eradication by test

and slaughter can be implemented. In addition, the introduction of modern molecular diagnostic techniques is essential in designing epidemiological and eradication programs.

The Rev-1 vaccine is indicated to protect small ruminants against brucellosis and to protect females from abortion in regions where the disease occurs. Lots of scientists consider the Rev 1 vaccine to be the generally preferred vaccine for controlling brucellosis, despite the adverse effect on the excretion of milk and products of abortion during the vaccination campaign. Conjunctival vaccination is safer than subcutaneous but is not safe enough to be applied regardless of the pregnancy status of animals and period of immunity. There is always a cross reaction problem in the serological examination with the impossibility of distinguishing between Rev 1 and field strains of bacteria (Blasco et al., 1997, 2006; OIE, 2009). Regarding the diagnosis of *Brucella* at this time, there exist two important molecular techniques through detection of the *rpsL* insertion element in the Rev1 strain and differentiation between banding profiles of *Brucella* species by RELP-PCR as the most promising molecular approaches with the utilization of DNA polymorphism. The RELP-PCR method is able to differentiate field infection and Rev-1 vaccine by producing different band patterns using *PstI* endonuclease (Bardenstein et al., 2002; Cloeckaert et al., 2002; Garcia-Yoldi et al., 2006; Lopez Goni et al., 2009; Mayer-Scholl et al., 2010).

Our results perform that the banding pattern of RFLP-PCR for DNA fragments obtained from *B. melitensis* Rev 1 vaccine strain and two field isolates from Hebron and Ramallah district flocks have similar *PstI* digested profiles and produce three bands, an intact 282-bp fragment from the amplified *omp2a* gene that lacks the *Pst I* site and two smaller fragments of 238 and 44 bp, the product obtained from digestion of the *omp2b* amplified fragment. In contrast, *B. melitensis* field isolates produced only two smaller fragments: a 238-bp fragment and a 44-bp fragment. This result was identical to that described in previous studies by other authors and in another country (Bardenstein et al., 2002; Salehi et al., 2006; Samadi, Unver et al., 2006; Nashwa et al., 2007; Mirnejad et al., 2013). Further epidemiological investigation revealed that these Rev 1-like flocks originated from the current vaccination flock. Additional investigation is necessary to discover the origin of infection, including horizontal transmission of infection (Banai et al., 1998; Banai et al., 2002). Previous research in Palestine has revealed a significant difference in nucleotide structure between some virulent genes, the Rev1 vaccine, and field isolates (Awwad et al., 2012). The field strains are extremely virulent and carry a high rate of virulent cell envelope genes. In addition, it has the possibility of adaptation to different environmental conditions due to a slight mutation in a *lpsB* gene that encodes production of sugar; therefore, it has the ability to modify it (Awwad et al., 2015). Despite the adverse effect of the *Brucella melitensis* vaccine, only well-organized whole-flock vaccination by the Rev 1 Elberg strain and, of course, avoiding any uncontrolled vaccination, may inhibit the virulence of the field strain and, subsequently, reduce the prevalence of brucellosis infection among animals and humans in Palestine.

## CONCLUSIONS

Detection of DNA polymorphism through RELP PCR is a very useful molecular technique in the circumstances of the Palestinian situation. Despite the adverse effects of the Rev 1 vaccine, only well-organized vaccination and extensive public awareness campaigns protect animals and humans against *Brucella* infection.

**Author Contributions:** The authors confirm contribution to the paper as follow: 1. Study conception and design, data analysis and result interpretation, manuscript preparation (Elena Awwad, Stelian Băraițăreanu, Maria Rodica Gurău and Doina Danesh), samples collection (Osama Awad and Asad Manasra), some analysis tools (Mohammad Farraj, Tamer Essawi, Israr Sabri, Kamel Adwan).

**Funding Source:** This research did not receive any specific fund.

## Acknowledgments

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

## Conflicts of Interest

The authors declare that they do not have any conflict of interest.

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