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Cell Envelope Virulence Genes among Field Strains of *Brucella melitensis* Isolated in West Bank Part of Palestine

Elena Awwad[a,b], Kamel Adwan[d], Mohammed Farraj[c], Tamer Essawi[c], Issa Rumi[b], Assad Manasra[b], Stelian Baraitareanu[a], Maria Rodica Gurau [a], Doina Danes[a]*

[a]*Facultaty of Veterinary Medicine, University of Agronomic Sciences and Veterinary Medicine, Bucharest, 050097, Romania*
[b]*Central Veterinary Laboratory, General Directorate of Veterinary Services and Animal Health, Ramallah, Palestine*
[c]*Master Programs in Clinical Laboratory Science (MCLS), Birzeit University, Birzeit, Palestine*
[d]*Department of Biology and Biotechnology, An-Najah National University, Nablus, Palestine*

Abstract

Despite the conjunctival vaccination program against Brucellosis in Palestine for more than 15 years, brucellosis continues to be a big problem concern, with increased incidence widely noted. *Brucella melitensis* is a facultative intracellular bacterium that replicates within macrophages. Recent reports showed that certain cell envelope associated genes of the *B. melitensis* is associated with its environmental stress adaptation and intracellular survival. Identification and characterization of these genes are essential to implementing efficient disease control and prevention systems. The aim of this study is to identify the cell envelope virulence associated genes of *B. melitensis* strains in Palestine. A total of 80 non-repetitive *B. melitensis* isolates were obtained from milk samples collected from infected small ruminants in the northern districts of the West Bank, Palestine during 2013 and 2015. The milk were cultured on *Brucella* agar (Oxoid) then incubated at 37^{0}C for 3 to7 days. Positive cultures were identified by standard biochemical methods and confirmed by IS*711* and Rev 1 *rpsL* gene PCR. All samples were negative for vaccine strain Rev 1 *rpsL* gene-PCR. The 80 *B. melitensis* under investigation were tested by multiplex PCR for 19 cell envelope virulence associated genes. Interestingly, our results revealed that all the 80 isolates were positive for the following genes: *lps*B, *lps*A *rfb*D, *wbp*L, *pgm omp*19, *omp*25, *omp*10, *mgt*A, *upp*S, *ami*C and *dac*F, while the other genes: *wbd*A, *wbpZ*, *pmm*, *wbk*A, *per*A, *man*B and *wpb*W were detected in more than 95% of the isolates.

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Keywords: Brucella melitensis; cell envelope associated genes; lipopolysaccharide, outer membrane protein; peptidoglycan

* Corresponding author. Tel.: +4-021-401-1122; fax: +4-021-401-1122.
E-mail address: danes.doina@gmail.com

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1. Introduction

Brucellosis is a zoonotic disease produced by *Brucellae,* Gram-negative bacteria that cause major worldwide economic losses due to infection of livestock (Seleem et al., 2010; Corbel et al., 2006; Ariza et al., 1996; Pappas et al., 2006; Young et al., 2000; Sauret et al., 2002). The pathogenesis of the resulting disease, called brucellosis, is mostly linked to the ability of *Brucella* to survive and replicate intracellular in host cells by expressing several cell envelope molecules that contribute to the control of the intracellular trafficking of the pathogen. Recent reports showed that certain cell envelope associated genes of the *B. melitensis* is associated with its environmental stress adaptation and intracellular survival (Scholz et al., 2013; Lapaque et al., 2006; Castaneda-Roldan et al., 2006; Wang et al., 2010; Xavier et al., 2010; Cardoso et al., 2006; Delrue et al., 2006; Letesson et al., 2004; Moreno et al., 2004; Reeves et al., 1994).

Although several *Brucella* cell envelope associated genes has been reported in last years, no article has documented the molecular characterization of the cell envelope virulence associated genes of the circulating *B. melitensis* population in Palestine. Essential role in epidemiological studies, management of the outbreaks and control programs have the characterization of the cell envelope virulence associated genes.

The present study is focused on the cell envelope associated genes of *Brucella melitensis*, one of the nominal species distinguished within the genus by their surface features and preferential host range, and which have been studied in more detail because of their greater importance in animal and human health.

2. Research Methods

2.1. Sample collection

A total of 80 non-repetitive *B. melitensis* isolates were obtained from milk samples collected from infected small ruminants in the northern districts of the West Bank, Palestine during 2013 and 2015. Milk samples were cultured on *Brucella* agar (Oxoid, CM0169) with added *Brucella* selective supplement (Oxoid, SR0083) and 5% of calf serum (Sigma, C8056) then incubated at 37^0C for 3 to7 days.

2.2. PCR analysis

Genomic DNA of both Rev.1 and *Brucella* field isolate was extracted using the QIAamp viral RNA mini kit (Qiagen, Hilden, Germany, 52906) according to the manufacturer's instructions. The Rev.1 DNA used in this study is the same Rev.1 vaccine source that has been used by the Palestinian Ministry of Agriculture since 1999. Positive cultures of the 80 milk samples were confirmed by IS711-PCR and differentiated from Rev 1 vaccine strain by *rps*L-PCR. Genomic DNA was amplified using IS711 primers specific for *Brucella melitensis* and *rps*L primers specific for Rev.1 vaccine strain (Table 1). PCR reaction (25µl) used 12.5 µl GoTaq green master mix obtained from Promega, M2173 (0.5 U Taq polymerase, 10 mM Tris – HCl, 3 mM MgCl2, 50 mM KCl, 0.1% Triton, 400 µM of each nucleotides), 6.5 µl nuclease free PCR water (Qiagen, Hilden, Germany, 129114), 0.5 µl of each primer (Syntheza Company) and 5 µl DNA template. PCR amplification was performed using a Master Cycler (BioRad Laboratories, Inc., Hercules, CA) as the following thermal conditions: initial denaturation at 95ºC for 2 min. followed by 35 cycles each consisting of 30 sec. of denaturation at 95°C, 45 sec. of annealing at 60°C for IS711, and 56°C for *rps*L Rev 1 primers and 30 sec. of extension at 72°C, and final extension at 72 °C for 5 min. The amplified products were examined in 2% agarose gel electrophoresis to determine the size of amplified fragment for each isolate.

Table 1. Sequences of primers for Brucella IS711 and *rps*L genes used in the present study

Primer types	Sequence (5'-3')	Amplified product (bp)
***B. melitensis* - specific primer**		
IS 711 - Forward	AAATCGCGTCCTTGCTGGTCTGA	731
IS 711 - Reverse	TGCCGATCACTTAAGGGCCTTCAT	
Vaccine strain Rev 1 - specific primer		
rpsL Rev1-Forward	CAGGCAAACCCTCAGAAGC	752
rpsL Rev1-Reverse	GATGTGGTAACGCACACCAA	

Source: Lopez Goni, 2009; Garcia-Yoldi, 2006; Gerrit et al., 2005

2.3. Identification virulence genes by PCR

The primers used in our study were designed with Primer3 software (http://frodo.wi.mit.edu/primer3) and synthesized using Syntheza. The target genes and primer sequences are detailed in Table 2. The PCR assays were conducted in 25 µL of reaction mixture comprising 12.5µl of GoTaq master mix (Promega, M2173) which contain 1X reaction buffer, 3.0 mM MgCl2, 400 µM each deoxyribonucleotide triphosphate and 0.5 U of Taq DNA polymerase and 200 nM of primers (Syntheza). For duplex PCR assays, an additional set of primers was added to the reaction mixture. The PCR program followed by 30 cycles of denaturation for 30 sec. at 94°C, annealing for 30 sec. at 60°C, and extension for 30 sec. at 72°C, then a final extension step of 5 min. at 72°C. The PCR products were analyzed by electrophoresis on a 2% agarose gel.

Table 2. Virulence associated genes and primers used in detection of virulence-associated genes of *B. melitensis*

Gene	GenBank accession No	Gene location	Primers sequence (5′ to 3′)	Amplified product (bp)
Lipopolysaccharide (LPS)				
lpsB	**BMEI0509**	529370-530275	Forward: TTCAGCTCATTCCATTGCAG Reverse: CGGATTATCTTCCTGCGAAA	978
*wbd*A	**BMEI0997**	1037374-1038874	Forward: TTTGCTTCGCATTTTCACTG Reverse: AACCCAGGCCAATTAGATCC	793
*lps*A	**BMEI1326**	1377527-1379693	Forward: GTTCAGCAGCGATGAAAACA Reverse: TATCTGTCAGCGCAACAAGG	911
*wbp*Z	**BMEI1393**	1448164-1449298	Forward: TTTGCCCATTCAGATTGACA Reverse: CCCGAAATTTTGATGGTCAC	783
pmm	**BMEII0899**	1452116-1453430	Forward: CTCCGGGAACTTCCTTAACC Reverse: CTTATGCAATGGCTCCCAAT	903
*wbk*A	**BMEI1404**	1456730-1457789	Forward: AATGACTTCCGCTGCCATAG Reverse: ATGAGCGAGGACATGAGCTT	931
*rfb*D	**BMEI1413**	1461049-1462138	Forward: TACCTTGCGGAATTGCTTCT Reverse: CAGTCGCGTAATGAGTCCAA	632
*per*A	**BMEI1414**	1462106-1463249	Forward: GGAACGGTGGCACTACATCT Reverse: GGCTCTCTGTGTTCCGAGTT	716
*wbp*L	**BMEI1426**	1470324-1471332	Forward: TAATTCCCGCTATCCTCGTG Reverse: GCAGGCGATCAGGATTGTAT	734
pgm	**BMEI1886**	1936919-1938510	Forward: GTTTTCCAGCAGCCCAATTA Reverse: CATGCCGATACCGAGTTTTT	939
*man*B	**BMEII0899**	941010-942434	Forward: GGTCGGCGTTTATCAACATT Reverse: TGTCATCCGAAGATTCCACA	833
*wpb*W	**BMEII0900**	1030249-1030834	Forward: GGCGATTCATCCTGTCTGTT Reverse: CAAAGGAAAGTGCACAGCAA	639
Outer membrane protein (OMP)				
*omp*19	**BMEI0135**	130096-130630	Forward: TGATGGGAATTTCAAAAGCA Reverse: GTTTCCGGGTCAGATCAGC	550
*omp*25	**BMEI1249**	1297525-1298167	Forward: TTTCCGTGTCCAATTATGCTA Reverse: ACCGCGCAAAACGTAATTT	701
*omp*10	**BMEII0017**	15808-16189	Forward: TGGAGAGCATGGACATGAAA Reverse: AAACCAGTGCGCAATTTACC	324
Peptidoglycan (PG)				
*mgt*A	**BMEI0271**	284236-284872	Forward: CGCTCAGGATATTGGTGGTT Reverse: TGGCGTGTTTATTTCACACAA	658
*upp*S	**BMEI0827**	284236-284872	Forward: CGCTCAGGATATTGGTGGTT Reverse: TGACCAGCGGGAGTCTTATC	700
*ami*C	**BMEI1056**	1097649-1098822	Forward: CAATTCGCCAATCACCTTTT Reverse: AATGTGCCTTCCACTTTTGC	948
*dac*F	**BMEII0350**	362276-363833	Forward: TAGAACAAGGCCGGTTTCAG Reverse: GTGGTCAGGAACGGTGAGAT	993

Source: Delrue et al., 2004; NCBI, updated October, 2015.

3. Results and Discussion

3.1. Laboratory Findings

Cultures obtained from the 80 milk samples collected from infected small ruminants in the northern districts of the West Bank were grown on *Brucella* agar (Figure 1). Colonies were round, glistening, smooth and mucoid. All cultures on *Brucella* agar medium were typical isolates of *Brucella* in morphology, colonial appearance and characteristics of growth. The isolated strains were oxidase positive and urease positive.

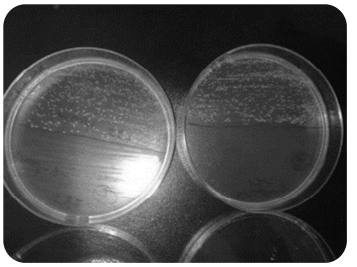

Figure 1. *Brucella melitensis* on *Brucella* specific agar

3.2. PCR Analysis

All the 80 positive milk samples detected by bacterial isolation were also detected as positive by the by PCR utilizing primers specific to IS711 gene of *B. melitensis* 16M. *B. melitensis*-specific DNA fragments with 731 bp were amplified and no DNA was observed in negative control samples (Figure 2a).

To differentiate the *B. melitensis* field isolates from Rev 1vaccine strain *rps*L-PCR was also done. No *rps*L-PCR positive result was observed in field isolates (Figure 2b).

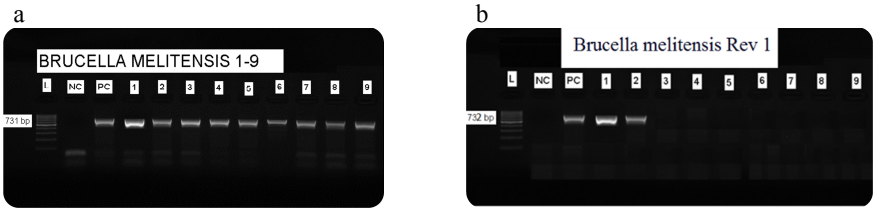

Figure 2. (a) *Brucella melitensis* 16 M IS711 ; (b) Gene rpsL: L-ladder, NC-Negative control, PC- Rev1 vaccine Hipra company (Spain), 1- Rev1 vaccine Ovejero company (Spain), 2- Rev 1 vaccine Jovac company (Jordan), 3-9-field samples of *Brucella melitensis*

We screened all 80 *B. melitensis* isolates for each of the 19 genes associated with virulence (Figure 3). Twelve of the 19 genes tested (lpsB, lpsA rfbD, wbpL, pgm omp19, omp25, omp10, mgtA, uppS, amiC and dace) were found in all of the isolates. The remaining seven genes (wbdA, wbpZ, pmm, wbkA, perA, manB and wpbW) were found in more than 95% of the isolate (Table 3).

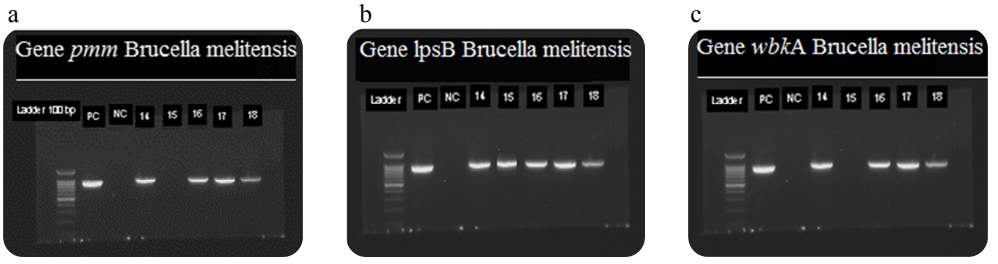

Figure 3. (a) Gene *pmm* of *Brucella meitensis*: Ladder, PC-Positive control, NC-Negative control, 14 sample positive, 15-samples negative, 16,17,18-sammples positive ; (b) Gene *wbk*A *of Brucella melitensis*: Ladder, PC-positive control, NC-Negative control, 14-sample positive, 15-sample negative, 16,17,18-samples positive ; (c) Gene *lps*A of *Brucella melitensis*: Ladder, PC-positive control, NC-Negative control, 14,15,16,17,18 - positive samples

Table 3. Prevalence of virulence genes in the *B. melitensis* isolates

Gene	Positive strains	
	No.	%
lpsB	80	100
wbd	79	99
lpsA	80	100
wbp-lipoO	79	99
pmm	78	98
wbk	79	99
rfbD	80	100
perA	76	95
wbpL	80	100
pgmO	80	100
manB	76	95
wbpW	77	96
omp19	80	100
omp25	80	100
mgtA	80	100
omp10	80	100
uppS	80	100
amiC	80	100
dacF	80	100

Brucellosis in small ruminants is mainly caused by *Brucella melitensis*, although this pathogen may also infect cattle and other ruminants (Cloeckaert et al., 2005; Ariza, 1996; Pappas, 2006). This pathogen, which has three different biovars, is endemic in several parts of the world, particularly biovar 3 in Mediterranean and Middle Eastern countries (Koneman, 2006; FAO, 2010; Young, 2000). The pathogenic potential of *B. melitensis* is highly dependent on its ability to enter and survive within host cells. Cell envelope proteins is another virulence factor of *B. melitensis* that contributes to initial survival of bacteria in macrophages (Brambila-Tapia et al., 2014; Razak et al., 2014; Xavier et al., 2010; Starr et al., 2008; Chain et al., 2005; Moreno et al., 2004; Rajashekara et al., 2006; Ugalde et al., 2003; Godfroid et al., 2000). Interaction between pathogens and hosts initiates a dynamic cascade of signals that lead to the change of gene expression patterns in the cell envelope proteins, which results in either colonization or elimination of pathogen in hosts. To our knowledge, up to now, no article has documented the molecular characterization of cell envelope virulence associated genes in the circulating *B. melitensis* strains in Palestine.

This characterization has a pivotal role in epidemiological studies, management of the outbreaks and implementing control and preventive measures. Despite the introduction of mass vaccination in 1998 in Palestine, data from the present study indicate an extremely high rate (95-100%) of the 19 cell envelope virulence associated genes tested among our isolates. Recent reports showed that certain cell envelope associated genes of the *B. melitensis* is associated with its environmental stress adaptation and intracellular survival. Thus, one could argue that it is possible that vaccination or the environmental stress might contribute to the change in the makeup of the *B. melitensis* in Palestine. Therefore, more research will be necessary to rule out this assumption.

4. Conclusion

Cell envelope virulence associated genes has clearly emerged as a serious problem with *B. melitensis* in Palestine. By analogy on the basis of experiences in other parts of the world, this problem is likely to grow in the future. Thus, more aggressive microbiological and infection control policies are necessary to prevent the further spread of these microorganisms.

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