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Detection of virulence genes (*bvf*A, *vir*B and *ure)* in *Brucella melitensis* isolated from aborted fetuses of sheep and goats

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ABSTRACT

Background and Objectives: *Brucella,* causative of brucellosis, has some potential virulence factors involved in *Brucella* replication and its strategies to circumvent the immune response. One of them is the *virB* gene that encodes the type *IV* secretion system proteins (T4SS) involved in intracellular replication of organism. *Brucella* virulence factor A (*bvf*A), and urease (*ure*) has also been described as being implicated in survival, and virulence in the hosts. The aim of this study was to investigate the *B. melitensis* virulence factor genes among *Brucella* isolated from aborted fetuses of sheep and goats in Fars province, southern Iran.

Materials and Methods: A total of 42 isolates of *B. melitensis* isolated from aborted fetuses between 2005-2011 in Fars province of Iran was used in this study. PCR assay was performed in order to detect the *virB, bvfA,* and *ure* genes using specific primers.

Results and Conclusions: The frequency of *bvf*A, virB, and *ure* genes was 78.50%, 73.80%, and 88.09% among all isolates respectively. The results of the present study showed that most *Brucella* isolates from this region have virulence factors genes (*vir*B, *bvf*A, *ure*) in their genome, and most *B. melitensis* had *ure* genes that has been hypothesized to play a role in the pathogenesis of disease.

Keywords: *Brucella melitensis*, Virulence genes (*bvf*A, *vir*B and *ure)*, Sheep, Goat, Fars province, Iran

INTRODUCTION

Members of genus *Brucella* are facultative intracellular pathogens responsible for brucellosis, worldwide zoonoses (1). *B.melitensis*, *B.abortus*, and *B.suis* cause brucellosis in humans, leading to chronic stages of the disease that can be manifested as orchitis, spondylitis, arthritis and debililitating illness known as undulant fever (2). The pathogenesis of brucellosis is due to its ability to adapt to the environmental conditions encountered in its intracellular explicative niche including low levels of nutrients and oxygen, acidic pH and reactive oxygen intermediates (3). Smooth *Brucella* inhibits host cell apoptosis, favoring bacterial intracellular survival by escaping host immune surveillance, while rough *Brucella* mutants (*B*. *melitensis* and *B*. *ovis* are two exceptions) induce macrophages (4). *Brucella* uses a number of mechanisms for avoiding or suppressing bactericidal responses inside macrophages. Molecular characterization of intracellular survival process of *Brucella* is important as it will provide guidance for prevention and sth. control (5, 6). VirB proteins that forms the type *IV* secretion system (T4SS) and that are involved in intracellular replication are considered as one of the *Brucella* virulence factors (7, 1). The T4SS in *Brucella* is typed by the *vir*B operon encoding 12 proteins. The mechanism of assembly of factors secreted by T4SS in *Brucella* is still unknown. However, the similarity with the well-studied plant pathogen *Agrobacterium tumefaciens* suggest that

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Table 1. Oligonucleotide primers used in the PCR assay.

genes	Primer sequences (5' - 3')	annealing temperature	Reference
bvfA	F: ACCCTTCGTCGATGTGCTGA R: CCGCGCTGATTTCATCGCTG	65°C	(13)
virB	F: CGCTGATCTATAATTAAGGCTA R: TGCGACTGCCTCCTATCGTC	54°C	(12,19)
Ure	F: GCTTGCCCTTGAATTCCTTTGTGG R: ATCTGCGAATTTGCCGGACTCTAT	65°C	(15)

Brucella uses in it for translocation of virulence factors into mammalian cells (8-9). It is clear that VirB proteins forming the type IV secretion system are involved in *Brucella* virulence and intracellular replication (10-12). In addition to this secretion system, *Brucella* virulence factor A (*bvf*A) has also been described as being implicated in *Brucella* survival in the host. *Brucella* virulence factor A (*bvf*A) is a small 11 kDa periplasmic protein that unique to the genus *Brucella* (13) and suggests it may play a role in the establishment of the intracellular niche (6). Although *Bvf*A was essential for *Brucella* virulence in both *in vitro* and *in vivo*, its assumed role in virulence is still unknown (13). The other *Brucella* virulence factor is urease (*ure*). The *Brucella* urease are interesting candidates to consider as they are important *Brucella* virulence factor. Urease is a virulence factor that plays a role in the resistance of *Brucella* to low pH conditions, both *in vivo* and *in vitro*. *Brucella* contains two separate urease gene clusters, *ure1* and *ure2*. Although only *ure1* codes an active urease, *ure2* is also transcribed, but its contribution to *Brucella* biology is unknown (14). *Brucella* contains two urease operons, both located in chromosome I. The proposed role of *Brucella* urease in inhibition of phagosome acidification by ammonia release was not observed (15).

The aim of this study was to investigate *Brucella* virulence factor genes among *B.melitensis* isolates from aborted fetuses of sheep and goats, in Fars province, Iran.

MATERIALS AND METHODS

Bacterial strains. A total of 42 isolates of *B. melitensis* (41 isolates biovar 1, One isolate biovar 2) recovered from clinical specimens from 2005 - 2011 used for the PCR assay.

DNA preparation. A loopful of colonies of each isolate on agar plate was picked and suspended in 200 µl of distilled water. After vortexing, the suspension was boiled for 5 min, and 50 µl of the supernatant was collected after spinning at 14,000 rpm for 10 min. The DNA concentration of the boiled extracts was determined with spectrophotometer (12).

PCR assay. PCR amplifications were performed in a final volume of 25 µL in PCR tubes. The reaction mixtures consisted of 2 µL of the DNA template, 2.5 µL 10x PCR buffer (75 mM Tris-HCl, pH 9.0, 2 mM $MgCl_2$, 50 mM KCl, 20 mM $(NH_4)_2SO_4$, 1 µL dNTPs (50 µM), 1 µL (1U Ampli Taq DNA polymerase), 1 µL (25 pmol) of forward and reverse primers**,** (Table 1) and the volume of the reaction mixture was completed up to 25 µL using distilled deionized water. PCR program for amplification of *bvf*A consisted of initial denaturation at 95°C for 4min, 32 cycles of application with denaturation at 95°C for 1min, annealing at 65°C for 1min, extension at 72°C for 1.30 min and final extension of the incompletely synthesized DNA at 72°C for 10 min in the BioRad thermal cycler (MJ Mini, BioRad, USA). This procedure was carried out for amplification of *vir*B and *ure* genes with annealing temperatures described in Table 1. The PCR products were analyzed in 2.0% agarose gels containing 0.5 µg /ml of ethidium bromide and subjected to electrophoresis in a 1X TAE buffer. Gels were visualized under UV light and documented using Uvitec System DOC-008. XD (EEC). A molecular weight Marker with 100 bp increments (100 bp plus ladder, Vivantis, Malaysia) was used as a DNA standard.

RESULTS AND DISCUSSION

DNA was successfully extracted from all 42 *B. melitensis* isolates. As expected the *bvf*A, *vir*B and *ure genes* assays produced amplicons of 1282, 881 and 2100 bp respectively (Fig. 1). Of 42

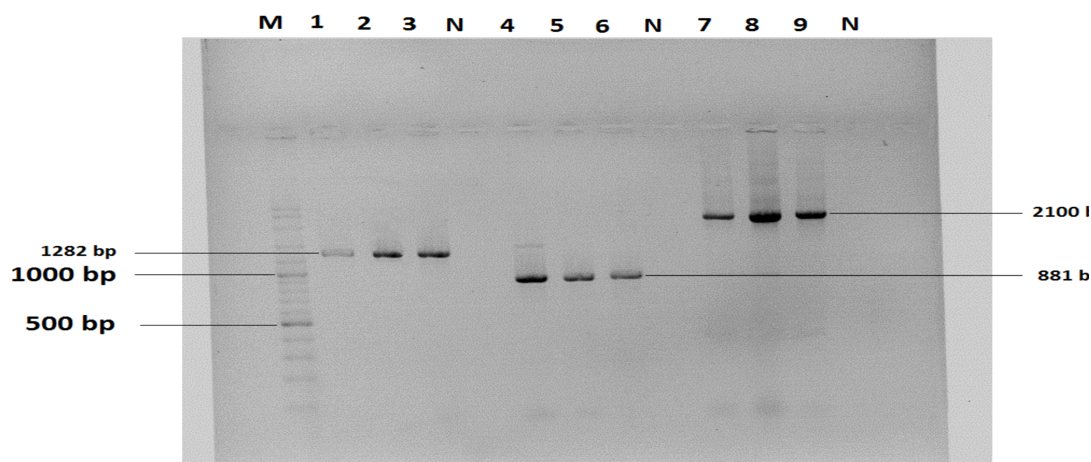

Fig. 1. Agarose gel electrophoresis of PCR product of three *brucella* virulence genes. Lane M, 100 bp ladder, lanes 1- 3: PCR PCR product of *ure* gene: 9-PCR product of *virB* gene, lanes 7: 6-product of *bvfA* gene, lane N, Negative control, lanes 4

B. melitensis isolates; 33 (78.5%) isolates had *bvf*A genes, 31(73.8%) isolates had *vir*B genes and 37 (88.09) isolates had *ure* genes.

The pathogenicity of *Brucellae* is due to its amazing ability to adapt to the environmental conditions encountered intracellularly. *Brucella* has evolved to avoid induction of immune system, interfere with intracellular trafficking, resist respiratory burst and adapt to oxygen-limited conditions encountered inside macrophages (6). In many regions of Iran *B. abortus* biovar 3 still remains the dominant biotype (16), however, *B. melitensis* biotype 1 is the predominant infective biotype in sheep and goats in Iran (17). The results of the present study showed that most *B. melitensis* isolates from Iran have virulence factors genes (*vir*B, *bvf*A, *ure*) in their genome, and most *B.melitensis* isolates had *ure* genes that has been hypothesized to play a role in the pathogenesis of disease. Other studies show most *Brucella* isolates exhibit potent urease activity that has been hypothesized to play a role in the pathogenesis of disease (18). Furthermore, some isolates, such as the well-known laboratory strain *B. abortus*, are urease negative, while they seem to retain most of their pathogenic potential (15). Whole-genome sequencing revealed the presence of a second urease operon (*ure2*) with all the genes potentially active in *B. suis* and *B. melitensis*. On the other hand, *B. abortus* had two frameshift deletions in *ureE2*, and *B. ovis* had deletions in *ureG2* and *ureT* (15). These data support our results that 88.09% of *B. melitensis* had *ure* gene.

Our results show that 73.8% isolates had *virB* genes. This is in accordance with other studies; O'Callaghan *et al*. (1990), described the presence of a *vir*B region of *B. suis* containing 11 genes highly similar to the 11 *vir*B genes of *Agrobacterium tumefaciens* and an extra ORF 12 that shares homology with an adhesion of *Pseudomonas fluorescens* (12). Sieria *et al*. (2000) suggested that putative effector molecules secreted by this T4SS determine routing of *B. abortus* to an endoplasmic reticulum-related replication compartment. The requirement of an active *vir*B operon for intra-cellular survival of *Brucellae* may have two possible explanations: (i) the *vir*B operon is essential to reach a competent intracellular replication niche or (ii) the *vir*B operon is needed for replication once the intracellular replication niche has been established (19). Delrue *et al*. (2004) reported that the T4SS of *Brucella* encoded by the *vir*B operon is a major virulence factor (20).

In this investigation we found that 78.5% isolates of *B.melitensis* had *bvf*A genes which is similar to other studies; Lavigne et al. (2005) reported that *Brucella* virulence factor A (*bvf*A) is a small 11 kDa periplasmic protein unique to the genus *Brucella* with no homologues in GenBank and no conserved domains or structural features was reported (13). It is suggested that it may play a role in the establishment of the intracellular niche (6). Although *bvf*A was essential for *Brucella* virulence in both *in vitro* and *in vivo*, its assumed role in virulence is still unknown (13). It is concluded from the study most *B. melitensis* isolates from Iran have virulence factors genes (*vir*B, *bvf*A, *ure*) in their genome, and most *B. melitensis* isolates had *ure* genes that has been hypothesized to play a role in the pathogenesis of disease.

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