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Detection of virulence-associated genes in *Brucella melitensis* biovar 3, the prevalent field strain in different animal species in Egypt

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Abstract

The current study involved detection of three virulence genes (*bvfA, virB, ure*) by PCR in 52 isolates of *Brucella melitensis* biovar 3, recovered from different animal species (28 sheep, 10 goats, 9 cattle and 5 buffaloes). Of the 52 *B. melitensis* strains; 48 (92.3%) isolates carried *bvfA* genes, 51 (98.1%) isolates had *virB* genes and 50 (96.2%) isolates were positive for *ure* genes. The distribution of the virulence genes is not affected by crossing the original host barriers of the animal species, as the three virulence factors (*bvfA, virB* and *ure*) detected in 28 *B. melitensis* isolates obtained from ovine species in a ratio of 26/28 (92.9%), 27/28 (96.4%) and 28/28 (100%), respectively. While 10 isolates originating from goats revealed a ratio of 10/10 (100%), 10/10 (100%) and 9/10 (90%) to the same order of virulence genes. Nearly, similar results of virulence genes detection were obtained in *B. melitensis* obtained from bovine (8/9, 9/9 and 8/9) and Buffalos (4/5, 5/5 and 5/5), respectively. The high prevalence of virulence-associated genes among the *B. melitensis* isolates detected from different animal species in Egypt indicates a potential virulence of this bacterium.

Keywords: *Brucella melitensis* biovar 3, Egypt, PCR, Virulence genes.

Introduction

Brucellosis is primarily a disease of animals that infects many domestic terrestrial mammals as well as some of the aquatic species. The pathogen transmitted from animals to humans by the ingestion of infected food products, direct contact with an infected animal or inhalation of aerosols (Schutze and Jacobs, 2011). The genus *Brucella* has traditionally been classified according to animal host preference into 6 species; *B. melitensis* (goats and sheep), *B. abortus* (cattle), *B. suis* (pigs), *B. neotomae* (desert woodrats), *B. ovis* (rams), *B. canis* (dogs), (Alton *et al*., 1988; Godfroid *et al*., 2011). Recently *B. ceti* and *B. pinnipedialis*, have been isolated from cetacean and pinniped species, respectively (Foster *et al*., 2007). *B. microti* was isolated from the common vole (Al Dahouk *et al*., 2012). Finally, *B. inopinata*, was isolated from a human breast implant (Scholz *et al*., 2010).

Although, Brucellosis is common in many developing countries, *B. melitensis* infection is considered the predominant strain in Egypt and Near East countries, not only in sheep and goats (preference host) but also in cattle, buffaloes, and camels as well as in humans (Refai, 2002; Wareth *et al*., 2014a).

Brucellosis is a major public health problem in Egypt especially in the Nile Delta region (Samaha *et al*., 2009). The clinical manifestations of brucellosis in infected humans are not pathognomonic and usually misdiagnosed with many other infectious and non-infectious diseases (Franco *et al*., 2007). *B. melitensis*, based on to its high pathogenicity and virulence, is categorized by the World Health Organization (WHO) as a risk group III. Contrary to some traditional views, *B. melitensis* remains fully virulent for human beings after infecting cattle (Hamdy and Amin, 2002; Wareth *et al*., 2014b).

Genetic and immunological evidence indicates that all members of the genus *Brucella* are closely related. Nevertheless, it has many virulence factors causing sever pathogenicity (Gandara *et al*., 2001). Differences in virulence have been observed in members of the genus *Brucella*, and the levels of virulence order shown in guinea-pigs seems to be similar to that in humans whereas, *B. melitensis* scored the high level of virulence followed by *B. suis* and *B. abortus* (Smith and Ficht, 1990).

It has been proved that mice infected with *B. melitensis* had a strong inflammatory response and prolonged splenomegaly compared with those induced by other *Brucella* strains (Crawford *et al*., 1996). *Brucella* employs a number of mechanisms for avoiding bactericidal responses inside macrophages. Unlike rough strains, smooth brucella organisms engulfed by macrophages, proved to play a role in suppressing macrophage apoptosis subsequently they have the ability to survive for longer periods inside macrophages (Pei *et al*., 2006).

Among the different gene elements responsible for virulence in *Brucellae* the *bvfA, ure*, and *VirB* are the most common factors. *Brucella* virulence factor A

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(bvfA) has been defined as being responsible for *Brucella* survival in the host cells. *BvfA* size is 11 kDa that unique to the genus *Brucella* and suggests it may play a role in the establishment of the intracellular niche.

Although *bvfA* was essential for *Brucella* virulence in both in vitro and in vivo, its particular role in virulence is still unrevealed (Lavigne *et al*., 2005). *VirB* proteins that form the type IV secretion system (T4SS) that play a role in intracellular replication are considered as one of the *Brucella* virulence factors (Delrue *et al*., 2005). One of the remarkable and important virulence factors in Brucella is the urease *(ure). Urease* is a virulence factor that plays a role in the resistance of *Brucella* to low pH conditions, both in vivo and in vitro (Sangari *et al*., 2010).

The detection of virulence genes in *B. melitensis* biovar 3, the predominant field strain in different animal species in Egypt has not been addressed. Therefore, the objective of this study is to detect and determine the presence and distribution of the common virulence-related genes in *B. melitensis* field strains isolated from sheep, goats, cattle and buffaloes in Egypt.

Materials and Methods

Bacterial strains

A total of 52 Brucella isolates recovered from clinical specimens (Table-1) of different animal species (28 sheep, 10 goats, 9 cattle and 5 isolates from buffaloes) within a period of 2 years (2015-2017). All isolates were identified and biotyped according to the methods adopted elsewhere (Alton *et al*., 1988; OIE, 2016). The identification of *Brucella* culture on the genus level was carried out based on colonial morphology, microscopic examination with modified Ziehl-Neelsen stain, and reaction with standard brucella positive and negative sera.

The smoothness of *Brucella* colonies was assessed based on colonial morphology, acriflavine test and staining the colonies with crystal violet. While biotyping of *Brucella* isolates was based on CO_2 requirement for primary isolation, production of H_2S: growth in presence of thionin (1/25.000, 1:50.000, 1:100.000) and basic fuchsin (1/50.000 and 1:100.000), urease production, reaction with mono-specific sera (A and M), catalase reaction and the usage of the following phages; Tblisi (Tb), Iz, and Weybridge - Wb (Alton *et al*., 1988; OIE, 2016). All 52 isolates proved to be *B. melitensis* biovar 3.

Template preparation

Each *Brucella* isolate cultured on Tryptic soy agar plate without addition of antibiotic was incubated at 37 C for 72 hours under 10 % CO2 tension. *Brucella* colonies were picked and suspended in 500 µl of distilled water. After mixing, the suspension was boiled for 5 min, and 300 µl of the supernatant was collected after spinning at 14,000 rpm for 10 min.

DNA extraction

DNA extraction from samples was performed using the QIAamp DNA Mini kit (Qiagen, Germany, GmbH) with modifications from the manufacturer's recommendations. Briefly, 200 µl of the sample suspension was incubated with 10 µl of proteinase K and 200 µl of lysis buffer at $56^{O}C$ for 10 min. After incubation, 200 µl of 100% ethanol was added to the lysate. The sample was then washed and centrifuged following the manufacturer's recommendations. Nucleic acid was eluted with 100 µl of elution buffer provided in the kit. The DNA concentration was determined with spectrophotometer (O'Callaghan *et al*., 1999).

Oligonucleotide Primers

Primers used were supplied from biobasic (Canada) and are listed in table (2).

PCR amplification

Primers were utilized in a 25- µl reaction containing 12.5 µl of Emerald-Amp Max PCR Master Mix (Takara, Japan), 1 µl of each primer of 20 pmol concentration, 5.5 µl of water, and 5 µl of DNA template. The reaction was performed in an *Applied Bio-system* (ABI) 2720 thermal cycler.

Analysis of the PCR Products

The products of uniplex PCR were separated by electrophoresis on 1 % agarose gel (Applichem, Germany, GmbH) in 1x TBE buffer at room temperature using gradients of 5V/cm. For gel analysis, 20 µl of the products were loaded in each gel slot. A gel pilot 100 bp plus DNA ladder (Qiagen, Gmbh, Germany), gene ruler 100 bp ladder (Fermentas, Germany) and DNA ladder H3 RTU (Genedirex, Taiwan) were used to determine the fragment sizes. The amplified products in agarose gel were visualized by ultraviolet transilluminator after gel staining with ethidium bromide stain. The gel was photographed by a gel documentation system (Alpha Innotech, Biometra). Sterile DNA-free water used as a control negative and *B. melitensis* biovar 3 reference strain (ATCC No., 23458) was used as control positive. Internal quality control samples were employed in the PCR process to ensure and exclude DNA contamination.

Results and Discussion

The pathogenicity and virulence of Brucella species is mostly attributed to their ability to survive intracellualry and to overcome unfavorable environmental conditions. *Brucella* has adapted to elude the reaction of the immune system, survive intracellular trafficking, and resist the low oxygen conditions encountered inside macrophages (Saeedzadeh *et al*., 2012).

In the current study, DNA was successfully extracted from all 52 *B. melitensis* isolates obtained from different animal species.

Table 1. distribution of *Brucella* isolates according to animal species and origin of samples.

Animal Species	Aborted foetus	L.N.	Milk	Organs tissues		Total
				Spleen	Liver	
Sheep	5	13	5	3	2	28
Goats	3	4	2	1		10
Cattle	2	1	4	1	1	9
Buffaloes	1	2	2			5
Total	11	20	13	5	3	52

Table 2. Primers sequences, target genes, amplicon sizes and cycling conditions for conventional PCR.

Target gene	Primers sequences	Amplified segment (bp)	Primary denaturation	Amplification (35 cycles)			Final extension
				Secondary denaturation	Annealing	Extension	
bvfA	ACCCTTCGTCGATGTGCTGA CCGCGCTGATTTCATCGCTG	1282	95˚C 4 min.	95˚C 1 min.	65˚C 1 min.	72˚C 1.3 min.	72˚C 10 min.
VirB	CGCTGATCTATAATTAAGGCTA TGCGACTGCCTCCTATCGTC	881	95˚C 4 min.	95˚C 1 min.	54˚C 1 min.	72˚C 1.3 min.	72˚C 10 min.
ure	GCTTGCCCTTGAATTCCTTTGTGG ATCTGCGAATTTGCCGGACTCTAT	2100	95˚C 4 min.	95˚C 1 min.	65˚C 1 min.	72˚C 1.3 min.	72˚C 10 min.

As expected the *bvf*A, *vir*B and *ure* genes assays with PCR produced amplicons of 1282, 881 and 2100 bp respectively (Fig. 1, 2 and 3). Of the 52 *B. melitensis* strains; 48 (92.3%) isolates were positive for *bvfA* gene, 51 (98.1%) isolates carried *virB* gene and in 50 (96.2%) isolates *ure* gene was detected. It is noteworthy to find that irrespective of the animal species from which *B. melitensis* was isolated, the distribution of virulence genes among the isolates was not affected by crossing the animal species host barrier.

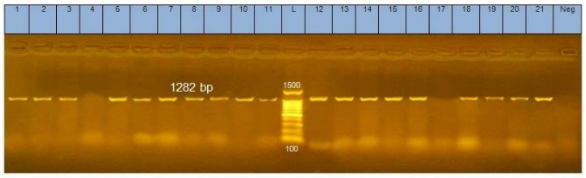

Fig. 1. Agarose gel electrophoresis image of virulence factor gene *bvfA* in *B. melitensis* isolates, where L; Marker (100bp), Negative; left lane, positive control; lane 21. All samples shown positive PCR product for the *bvfA* virulence gene except samples numbers 4 and 17 they were negative.

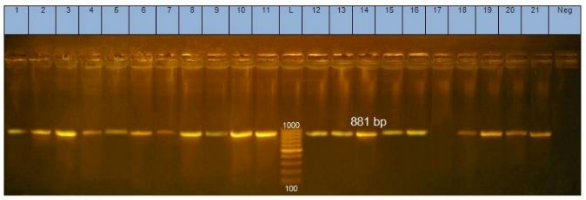

Fig. 2. Agarose gel electrophoresis image of virulence factor gene *virB* in *B. melitensis* isolates, where L; Marker (100bp), Negative control; left lane; positive control lane 21. All samples shown positive PCR product for the *virB* virulence gene except sample number 17.

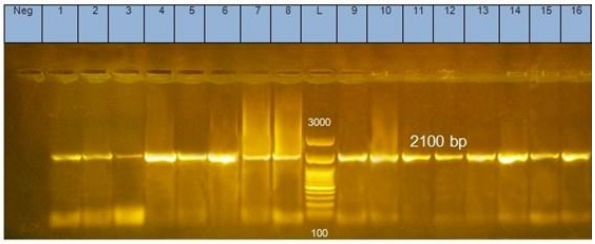

Fig. 3. Agarose gel electrophoresis image of virulence factor gene *ure* in *B. melitensis* isolates, where L; Marker (100bp), Negative control; right lane; positive control lane 1. All samples shown positive PCR product for the *ure* virulence gene.

The same levels of distribution of the three virulence genes was observed in all *B. melitensis* isolates, under test, regardless of the animal species. As the three virulence factors viz. *bvfA*, *virB* and *ure* were detected in 28 *B. melitensis* isolates originated from ovine species in a ratio of 26/28 (92.9%), 27/28 (96.4%) and 28/28 (100%), respectively. While the ratio for three virulence genes namely *bvfA*, *virB* and *ure* in 10 isolates originating from goats revealed a ratio of 10/10 (100%), 10/10 (100%) and 9/10 (90%), respectively. Nearly, similar results of virulence genes detection were obtained in *B. melitensis* obtained from bovine (8/9, 9/9 and 8/9) and Buffalos (4/5, 5/5 and 5/5), respectively (Table 3).

In this investigation, we found that 92.3% of *B. melitensis* isolates had *bvf*A genes which is similar to other studies, where Naseri *et al*. (2016) detected the *bvf*A gene in 93 % of *B. melitensis* strains isolated from human blood in Iran.

Table 3. Detection of virulence genes in *B. melitensis* isolated from different organs of different animal species.

Sample	Animal Species	Organ	Results		
			bvfA	*virB*	*ure*
1	Sheep	L.N.	+	+	+
2		L.N.	+	+	+
3		L.N.	+	+	+
4		L.N.	-	+	+
5		L.N.	+	+	+
6		L.N.	+	+	+
7		L.N.	+	+	+
8		L.N.	+	+	+
9		L.N.	+	+	+
10		L.N.	+	+	+
11		L.N.	+	+	+
12		L.N.	+	+	+
13		L.N.	+	+	+
14		Milk	+	+	+
15		Milk	+	+	+
16		Milk	+	+	+
17		Milk	-	-	+
18		Milk	+	+	+
19		Spleen	+	+	+
20		Spleen	+	+	+
21		Spleen	+	+	+
22		liver	+	+	+
23		liver	+	+	+
24		A.F.	+	+	+
25		A.F.	+	+	+
26		A.F.	+	+	+
27		A.F.	+	+	+
28		A.F.	+	+	+
29	Goats	L.N.	+	+	+
30		L.N.	+	+	+
31		L.N.	+	+	+
32		L.N.	+	+	+
33		Milk	+	+	-
34		Milk	+	+	+
35		Spleen	+	+	+
36		A.F.	+	+	+
37		A.F.	+	+	+
38		A.F.	+	+	+
39	Cattle	L.N.	+	+	+
40		Milk	+	+	+
41		Milk	-	+	+
42		Milk	+	+	+
43		Milk	+	+	-
44		Spleen	+	+	+
45		Liver	+	+	+
46		A.F.	+	+	+
47		A.F.	+	+	+
48	Buffalo	L.N.	+	+	+
49		L.N.	+	+	+
50		Milk	-	+	+
51		Milk	+	+	+
52		A.F.	+	+	+
Total		52	48	51	50
%			92.3%	98.1%	96.2%

(A.F.): Aborted foetus; (L.N.): Lymph node.

On the other hand, only 78.5% of *B. melitensis* strains originating from aborted goats, in Iran, having *bvf*A in their genomes (Derakhshandeh *et al*., 2013).

The current study show that 98.1% of the 52 local *B. melitensis* isolates had *virB* genes. This is in accordance with other studies, where *virB* genes detected in 100% *B. melitensis* strains isolated from human patients (Naseri *et al*., 2016), and disagree with the results reported by Derakhshandeh *et al*. (2013) who found *virB* genes in only 73.8% of 42 *B. melitensis* strains isolated from goats. This discrepancies may indicate that *B. melitensis* field strains prevailing in Egypt are more virulent than the strains of *B. melitensis* isolated from caprines in Iran. As, it was emphasized that the T4SS of *Brucella* encoded by the *virB* operon is a major virulence factor (Delrue *et al*., 2005).

In spite of their well-established immune-evasive behavior, *Brucella* spp. do rely on an important virulence factor for intracellular survival, the type IV secretion system (T4SS) encoded by the genes *virB1–virB12* (den Hartigh *et al*., 2008). Viable *Brucella* evades macrophage killing through *VirB*-dependent sustained interactions with the endoplasmic reticulum (ER). The role of *virB* operon for the intra-cellular survival of *Brucellae* may have two possible passways, either the *virB* operon is necessary to reach a competent intracellular replication niche or the *virB* operon is required for replication once the intracellular replication niche has been established (Saeedzadeh *et al*., 2012).

The results of the present study showed that most *B. melitensis* isolates have virulence factor gene *ure* (96.2%) in their genome. The *ure* genes has been hypothesized to play a role in the pathogenesis of disease. Other studies showed that about (100%) of *B. melitensis* strains originating from human sources having *ure* genes in their genome (Naseri *et al*., 2016) while the *ure* genes detected in 42 *B. melitensis* strains isolated from caprine species were approximately 88.09 % (Derakhshandeh *et al*., 2013).

The virulence of *Brucella* isolates was reported to be in relation with the rate of urease activity (Seleem *et al*., 2008). The microbial ureases that play a role in virulence is based on the action of multi-subunit enzymes that hydrolyze urea to form carbon dioxide and ammonia. Thus the hydrolysis of urea releases ammonium that turns the surrounding environment to the alkaline shift and facilitates intracellular survival in acidic environments.

The role of bacterial ureases in infectious disease has been recently reviewed. It was investigated that most *Brucella* species show a strong urease activity, derived from *ure1* but not from *ure2*, and this activity is responsible for the ability of *Brucella* to survive acidic environment, particularly through the transmission of the infection through ingestion or gastric route

(Bandara *et al*., 2007). This finding is substantiated by the fact that *B. ovis* is not able to infect the host by the gastrointestinal route, a fact that has been linked to lack of urease activity in *B. ovis* (Tsolis *et al*., 2009). This may refer to the role of urease activity as it is responsible for the pathogenies and virulence *Brucella* strains.

Isolated *B. melitensis* biovar 3 incorporated in this study showed fast urease activity within 30 to 75 minutes from incubation on Christensen`s solid medium (Data not shown). However, it was stated that reference *B. melitensis* strains were considered slow urea splitters but an increasing percentage of recently isolated cultures are urease positive within one hour and many are indistinguishable from *B. suis* in the rate of urease activity (Alton *et al*., 1988).

These findings may explain why *B. melitensis* induces greater acute infectivity in Fisher-344 rats, whereas *B. suis* causes chronic infectivity; and urease activity has no influence on *Brucella* infection using an intraperitoneal (IP) route (Bandara *et al*., 2013). These findings are in harmony with results obtained in the current study where 96.2% of *B. melitensis* had *ure* genes that indicating the high virulence of the local *B. melitensis* strains isolated from different animal species in Egypt.

Conclusion

A high proportion of *B. melitensis* strains recovered from clinical cases from animals were positive for the virulence genes viz. *bvfA* (92.3%) *virB* (98.1%), and *ure* (96.2%). The high prevalence of virulence-associated genes among the *B. melitensis* isolates detected from different animal species in Egypt indicates a potential virulence of this bacterium. Thereby this study offers a clear insight into the high virulence and pathogenic characteristics of *B. melitensis* biovar 3, predominating in the Egyptian region, and may be helpful to veterinary officials and public health authorities to set national campaigns for the control and eradication of this hazard.

However, further studies were needed to unveil the role of selected virulence genes and the factors responsible for expression of these genes in eliciting the massive inflammatory response that results in abortion and to elucidate the infectious cycle of this pathogen.

Conflict of interest

The authors declare that there is no conflict of interests.

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