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Research Article
Substantial Virulence Genes among *Brucella melitensis* Field Strains Isolated from Cattle in Egypt

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Abstract

Background and Objective: The economic losses due to brucellosis as well as its potential public health in human worldwide encourage more researches to find novel pathways for effective control methods of the disease. The objective of this study was to investigate the most prevalent *Brucella* strains obtained from cattle and their virulence genes. **Materials and Methods:** Three hundred small-holders cows in Menoufia governorate, Egypt, were screened for brucellosis using rose bengal test (RBT) and confirmed by complement fixation test (CFT). Milk samples and supra-mammary lymph nodes of serologically positive cows were collected for bacteriological isolation and identification. The obtained isolates were genotyped using PCR and their virulence genes (*omp*25, *omp*31, *man*A, *man*B, *vir*B and *znu*A) were screened. **Results:** The prevalence rate of bovine brucellosis was 15 (5%), 11 (3.6%) and 7 (2.33%) by RBT, CFT and bacteriological examination, respectively. The seven isolates were identified and genotyped as *Brucella melitensis* biotype3. Furthermore, the molecular detection of substantial virulence genes revealed that *man*A, *man*B, *omp*25 and *omp*31 genes were detected in all tested *B. melitensis* strains. Meanwhile, the *vir*B genes were detected in 4 strains and the *znu*A genes were detected in 3 strains among the isolated *B. melitensis* strains. **Conclusion:** It was concluded that *B. melitensis* biotype3 was the pre-dominant *Brucella* spp. as well as omp25, *omp*31, *man*A and *man*B were the most common related-virulence genes which assumed to play a worthy function in the pathogenesis of brucellosis.

Key words: *Brucella*, pathogenicity, PCR, virulence genes, bacteriological examination

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Brucellosis is a highly contagious zoonotic disease causing significant losses in animals. Late gestation abortion was the predominant clinical sign of *B. abortus* infection in cows, resulting in reproductive failure and decrease in milk production[1]. It was estimated that more than 300 million cattle as well as more than half a million of human infections occurred[2]. Egypt, Middle East and Mediterranean countries were endemic areas with higher prevalence rates[3]. *Brucella* invades the host cell and interferes with the different immune system mechanism through adaption to oxygen-limited conditions inside macrophages[4]. *Brucella* has a special affinity for the reticuloendothelial system and reproductive tracts of domestic and wild animals. Stillbirth in females and infertility in males are the most common complications of the brucellosis in livestock[5]. *Brucella* spp. including *B. melitensis* and *B. abortus* have similar pathogenic effects and zoonotic concern with some differences in virulence properties and host susceptibility. These pathogens carry various furtive strategies to enter into host cells then propagate and overcome the host defense mechanism[6]. In dairy animals, *Brucella* spp. replicates in the mammary gland and supra-mammary lymph nodes and continue to be excreted into the milk along with their lives[7]. A recent discovery of new a typical *Brucella* spp., with new genetic properties was recorded[8]. Therefore, it was expected that a new outbreak of brucellosis may occur in the future. *Brucella* was already exhibiting virulence factors required to form the infection due to their activation by erythritol[9]. The constant researches for *Brucella* virulence genes such as cell envelope associated genes and other virulent genes such as *omp*25, *omp*31, *man*A, *man*B, *wbk*A, *cβg*, *vir*B, *mvi*N, *znu*A and *bvf*A genes are necessary to understand their role in *Brucella* pathogenesis, characterize the *Brucella* spp., genome and have efficient control measures[10]. As a result of the brucellosis' endemic status in Egypt and the need to develop new preventive measures against brucellosis, this study was aimed for molecular detection and genotyping of the most virulence genes of local *Brucella* strains affecting cattle in Egypt.

MATERIALS AND METHODS

This study was assessed and agreed by the Animal Care and Welfare Committee Ethics, University of Sadat City, Egypt with approval number (VUSC-001-4-19).

Examined animals: The present study was carried out in Menoufia governorate, which located in Nile Delta, Egypt. A total number of 300 cows of small-holders which were not vaccinated against brucellosis were examined during the period from January-April, 2018.

Serum samples: About 10 mL of blood was collected from jugular vein of cattle. Collected samples were kept in a refrigerator overnight for serum separation then were centrifuged at 3000 rpm for 5 min. Clear sera were siphoned off and stored in cryotubes at -20°C until its use for serological studies[11].

Milk and tissue samples: From all serologically positive animals, milk samples were collected immediately before slaughtering from lactating cows. While the supra-mammary lymph nodes were collected after slaughtering in sterile containers and then both samples were transferred immediately to the laboratory for further bacteriological examination[12].

Serological analysis: Three hundred serum samples were examined by Rose Bengal Test (RBT) as a screening field test. Positive RBT samples were confirmed by complement fixation test (CFT)[13].

Phenotypical identification of *Brucella* species: Milk samples and supra-mammary lymph nodes of all serologically positive animals were sterile cultivated onto Petri dishes containing selective medium prepared using *Brucella* medium base as described (Oxiod, code CM0169) with addition of 5-10% v/v inactivated horse serum SR0035 and 1-5% w/v of a sterile solution of glucose and finally added to *Brucella* selective supplement as described (Oxoid, code SR0083). All plates were incubated at 37°C with and without 5-10% CO_2 for 5-7 successive days[11]. The typical biochemical and phenotypic identification of the suspected *Brucella* colonies (morphology, CO_2 requirements, H_2S production, urease, catalase and oxidase activity, nitrate reduction, lactose fermentation, citrate utilization, growth in presence of thionine and fuchsin dyes "1:50.000 (20 μg mL^{-1})", lysis by Tbilisi phage and agglutination with A and M anti-sera) were done[13,14].

Molecular characterization of *Brucella* species and virulence determinant genes

DNA extraction: The DNA was extracted from isolates using commercial kits (Thermo Scientific Gene Jet Genomic DNA purification Kit) (Cat nu, K0721) according to manufacturer recommendations.

Table 1: Sequence of used primers

Targets	Sequence (5'- 3')	Amplicon size (bp)	References
Brucella cell surface protein (*bcs*P)	TGG CTC GGT TGC CAA TAT CAA CGC GCT TGC CTT TCA GGT CTG	223	Mukherjee *et al.*[15]
B. melitensis IS711	AAA TCG CGT CCT TGC TGG TCT GA TGC CGA TCA CTT AAG GGC CTT CAT	731	Bricker and Halling[16] and Gupta *et al.*[17]
*man*A	TCG ATC CAG AAA CCC AGT TC CAT ACA CCA CGA TCC ACT GC	271	Khosravi *et al.*[18]
*man*B	GGC TGG TTC GAG AAT ATC CA CAA TCG CAT ACC CTG GTC TT	228	
*Omp*25	CGT ACC TCA CGG CTG GTA TT CGT ACC GGC CAG ATC ATA GT	188	
*Omp*31	GCT GCT CCT GTT GAC ACC TT GCT GAA ATC GAA CCC GTA AC	257	
*znu*A	CTG GGT CCG AGC ATG TTT AT AGG CAT CGA GTT TTT CTC CA	465	
*vir*B	CGC TGA TCT ATA ATT AAG GCT A TGC GAC TGC CTC CTA TCG TC	881	Hajia *et al.*[19]

PCR amplification: The PCR assay was performed to identify *Brucella* isolates, targeted the *bcs*P gene coding outer protein of *Brucella*, reverse the primer that targeted the *Brucella* insertion element IS711 and forward primer for *B. melitensis* and a set of duplex PCR assays that targeted six different virulence genes namely *man*A, *man*B, *omp*25, *omp*31, *znu*A and *vir*B. The sequences of each primer are presented in Table 1. All reactions were performed in total volume of 25 µL reaction mix which contained 3 µL of template DNA, 20 pmol of each primer and 1X of PCR mix (Fermentas). The analysis of PCR products was carried out using 1.5% ethidium bromide stained agarose gel.

Cycling condition of PCR assays: This was performed for BCSP and *B. melitensis* at the following conditions: (1) About 4 min initial step at 95°C, (2) Followed by 35 cycles at 95°C for 60 sec, 55°C for 60 sec and 72°C for 90 sec and (3) A final extension step at 72°C for 10 min. While PCR amplification assays for virulence genes (*omp*25, *omp*31, *man*A, *man*B, *vir*B and *znu*A) were as following: (1) About 5 min initial step at 94°C, (2) Followed by 25 cycles at 94°C for 60 sec, 58°C for 60 sec and 72°C for 60 sec and (3) A final extension step at 72°C for 10 min.

Statistical analysis: Statistical analysis was performed using Graph Pad prism 5 software Inc., La Jolla, CA.

RESULTS

Prevalence of Brucellosis in examined cattle: Out of examined 300 cows, 15(5%) and 11(3.6%) were positive with RBT and CFT, respectively. The results of bacteriological examination revealed only 7(2.33%) isolates which were identified as *B. melitensis* biotype3 "4 (1.33%) were from supra-mammary lymph nodes and 3 (1%) were from milk samples" as illustrated in Table 2.

Genotyping of *B. melitensis* strains: The obtained seven isolates were genotyped using a common *bcs*P gene and a specific gene IS711. The results indicated that all 7 tested isolates were *B. melitensis* biotype3 as shown in Fig. 1 and 2.

The molecular detection of substantial virulence genes among the isolated *B. melitensis* biotype3: Successful amplifications of 228 and 465 bp specific for *man*B and *znu*A genes, respectively were obtained (Fig. 3). Also amplicons of 188 and 257 bp specific for *omp*25 and *omp*31 genes, respectively were given (Fig. 4). Regarding to 881 and 271 bp specific bands for *vir*B and *man*A genes, respectively were obtained (Fig. 5).

Distribution of detected virulence genes among the isolated *B. melitensis* biotype3: The results of detected virulence genes revealed that *man*A, *man*B, *omp*25and *omp*31 genes were found in all seven tested *B. melitensis* strains. Meanwhile, the *vir*B genes were detected in 4 strains and the *znu*A genes were detected in 3 strains among the screened *B. melitensis* isolates. All milk samples were carried *man*A, *man*B, *omp*25 and *omp*31 genes, while the *vir*B genes were detected in 2 samples and the *znu*A genes were detected in 2 samples as well. Also, all samples from lymph nodes were carried *man*A, *man*B, *omp*25 and *omp*31 genes, while the *vir*B genes were detected in 2 samples and the *znu*A genes were detected in only 1 sample among the screened *B. melitensis* strains (Table 3).

Table 2: Prevalence of brucellosis in examined cattle

	Serological examination				Bacteriological examination*			
	RBT		CFT		Supra-mammary L.N		Milk samples	
Number of examined samples	Number	Percentage	Number	Percentage	Number	Percentage	Number	Percentage
300	15	5	11	3.6	4	1.33	3	1

RBT: Rose bengal test, CFT: Complement fixation test, *Total isolates were 7 (2.33%) strains which were identified as *B. melitensis* biotype3

Table 3: Distribution of detected virulence genes among the isolated *B. melitensis* biotype3

Strain No.	Type of sample	Virulence determinant genes
S1	Milk	*man*A, *man*B, *omp*25, *omp*31, *vir*B
S2	Milk	*man*A, *man*B, *omp*25, *omp*31, *vir*B
S3	Milk	*man*A, *man*B, *omp*25, *omp*31, *znu*A
S4	lymph nodes	*man*A, *man*B, *omp*25, *omp*31, *vir*B
S5	lymph nodes	*man*A, *man*B, *omp*25, *omp*31, *znu*A
S6	lymph nodes	*man*A, *man*B, *omp*25, *omp*31, *znu*A, *vir*B
S7	lymph nodes	*man*A, *man*B, *omp*25, *omp*31

S: Strain number

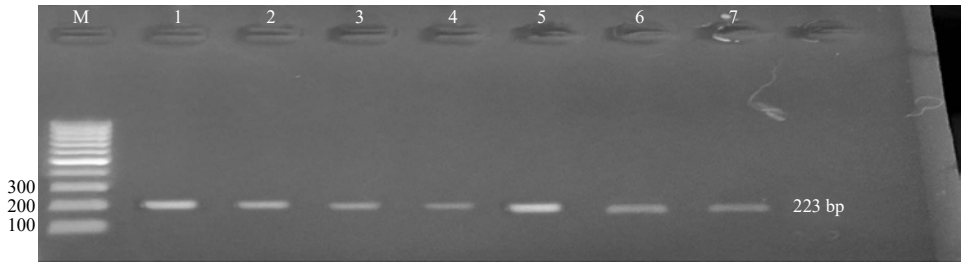

Fig. 1: PCR product showed positive amplification of *bCSP* gene of *B. melitensis* at 223 bp (common gene)
Lane M: 100 bp DNA ladder, Lane 1-7: Positive *B. melitensis* strains

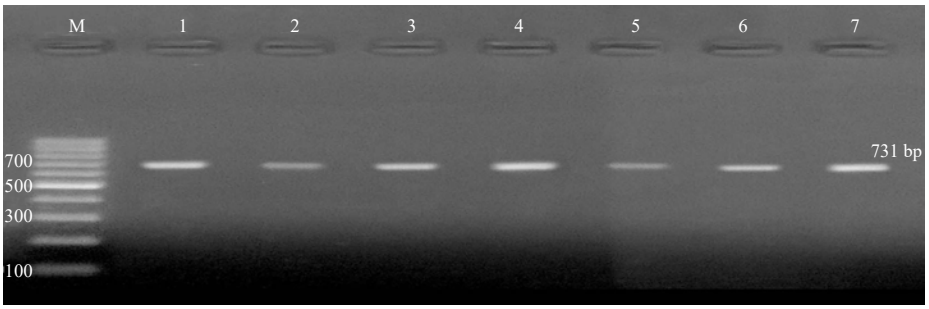

Fig. 2: PCR product showed positive amplification of IS711 gene of *B. melitensis* at 731 bp (specific gene)
Lane M: 100 bp DNA ladder, Lane 1-7: Positive *B. melitensis* strains

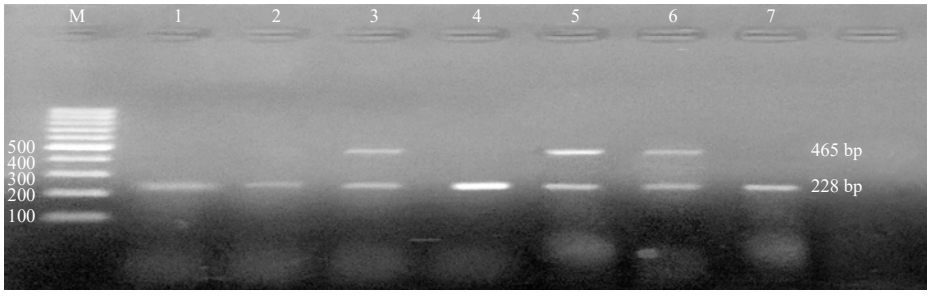

Fig. 3: PCR product; Lane M: 100 bp DNA ladder; Lanes1-7 *B. melitensis* biotype3 were positive for *man*B gene at 228 bp
Lanes 3, 5 and 6 were positive for *znu*A genes at 465 bp

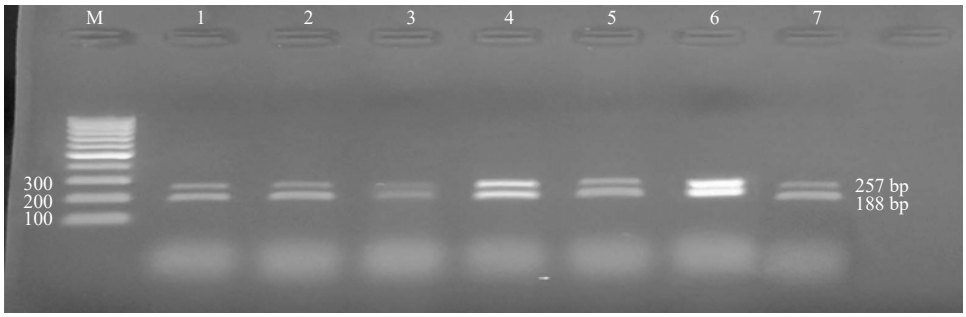

Fig. 4: PCR product: Lane M: 100 bp DNA ladder, Lanes 1-7 *B. melitensis* biotype3 were positive for *omp*25 and *omp*31 genes at 188 and 257 bp, respectively

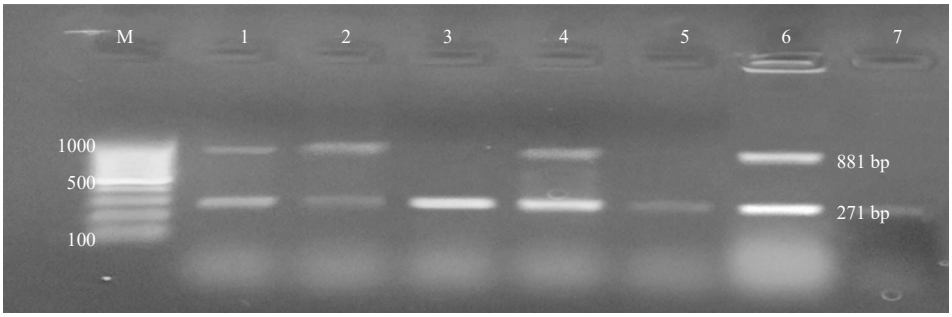

Fig. 5: PCR product: Lane M: 100 bp DNA ladder; Lanes 1, 2, 4 and 6 *B. melitensis* biotype3 were positive for *vir*B genes at 881 bp, while Lanes 1-7 were positive for *man*A genes at 271bp

DISCUSSION

Brucellosis is one of the most globally distributed diseases reported by WHO as half a million new cases reported each year[20]. Brucellosis is a bacterial zoonotic disease transmitted via various routes such as; contact directly with abraded skin or mucosa, fluids, tissues from aborted fetus[21]. The high endemic pattern of the disease in many underdeveloped countries caused large economic losses in livestock animals as well as chronic infections in human[22]. In the current study, the prevalence rates of brucellosis were 5, 3.6 and 2.33% with RBT, CFT and bacteriological examination, respectively. These results coincided with previous results which recorded that the prevalence of brucellosis in cattle was 5.3% by RBT in Bangladesh[23,24]. Furthermore, other reports demonstrated that RBT and CFT were effective methods for the detection of *Brucella* spp. antibodies[25,26]. In the Gulf area, a comparative study was applied in Sultanate of Oman where higher results were obtained (11.7 and 8.3%) positives using RBT and CFT, respectively in goats' sera[27].

The difference in the prevalence might be attributed to the difference in methodology, number of samples, examined regions and management system. The current study demonstrated seven *Brucella* isolates which were identified and genotyped as *B. melitensis* biotype3. This was supported previously by studies declared that brucellosis is endemic in Egypt particularly in the Nile Delta region and *B. melitensis* remains the more virulent strain with public health[28,29].

In the current study, results of screening the six putative virulence genes of *B. melitensis* from cattle samples detected *omp*25, *omp*31, *man*A and *man*B in all isolated strains. While the *vir*B was found in 4 strains and the *znu*A was detected in 3 isolates only. Although, *Brucellae* are deficient in some of the classical factors of ferocity and strength, such as capsules, fimbria, plasmids, exotoxins, lysogenic phages and endotoxic lipopolysaccharide, they expressed some virulent genes[30].

These virulence determinants were vital for *Brucella* spp., to live, adapt intracellularly to inappropriate conditions and resist body immune response[31]. From these substantial genes, *Omp*25/*Omp*31 were described as major surface proteins strongly contributed to the virulence of *Brucella*[32]. The outer membrane protein (*Omp*31) was a protective immunogenic component for subunit DNA vaccines of *B. melitensis* as well as a simulator for the cell-mediated immunity[33,34]. Moreover, the major function of *omp*25 depends on suppression of the tumor necrosis factor alpha (TNFα) produced by

macrophages[35]. The *vir*B type IV secretion system (T4SS) was a gene coding for an enzyme involved in the biosynthesis of LPS[36-38]. Concerning to the critical role of *man*A and *man*B and *znu*A genes among *Brucella* spp., many reports confirmed the contribution of *manB* genes in lipopolysaccharide synthesis which allowed the intracellular survival and protection against host defense[39]. Others pointed out the importance of *znu*A and *man*A genes in the virulence properties of *B. melitensis* particularly in ATP-binding mechanism[40]. As well as the presence of *znuABC* operon which is linked to Zn^{2+} ions as a catalyst for the Cu and Zn superoxide dismutase (*sodC* genes) and provided the resistance to oxidation process by phagosome[41]. The present findings were in contact with results of Naseri *et al.*[42], who detected *omp*25, *omp*31, *man*A, *man*B, *vir*B and *znu*A virulence genes in all isolated *B. melitensis* strains recovered from human patients in Iran. Nearly similar findings were obtained by another researcher who recovered that the prevalence rates of *omp*25, *omp*31, *man* A, *man*B and *znu*A genes were 87.5, 100, 87.5, 100 and 100%, respectively from human blood samples in Iraq[43]. In addition, it was detected *man*A, *Omp*25, *Omp*31, *man*B and *znu*A with different percentages 100, 100, 76.9, 38.5 and 19.2%, respectively[44]. On the other hand, many studies reported other different virulence genes as revealed that all *B. melitensis* and *B. abortus* strains were carried *vir*B2 and *vir*B5 genes[45]. Also, it was recorded that 98.1% of *B. melitensis* biotype3 were carried *vir*B genes in Egypt[46]. Despite that, it was also confirmed by a report in which *vir*B genes in 73.8% of *B. melitensis* strains isolated from goats[47]. This discrepancy in results might be attributed to the variation of sources, number of samples and prevalent strains in the examined geographic regions. Finally, due to the seriousness of bovine brucellosis and its impact on public health, further studies are needed to spot highlights on the role of these genes and others in the contagiousness of brucellosis and the ability to produce types of vaccines to control or minimize the disease incidence.

CONCLUSION

It could be concluded that *B. melitensis* biotype3 is the most predominant *Brucella* spp., among cows in Menoufia governorate. The most frequent virulence genes are *omp*25, *omp*31, *man*A and *man*B among isolates which are assumed to play a worthy function in the pathogenesis of brucellosis in this region. Moreover, it may be helpful for authorized affairs to develop a strategic plan for the prevention and eradication of this disease.

SIGNIFICANCE STATEMENT

This study discovered that *B. melitensis* biotype3 was the prevalent circulating strain among cows in Menoufia governorate, Egypt. Also, it provided a beneficial view for clarifying the substantial role of *omp*25, *omp*31, *man*A, *man*B, *vir*B and *znu*A virulence determinant genes in bovine brucellosis. This study assessed the worthy values of these genes in the virulence of *Brucella*. Further investigations were needed to evaluate other impact roles of these genes or other genes in bovine brucellosis.

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