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Original article

INCIDENCE OF VIRULENCE GENES IN PREDOMINANT *BRUCELLA* STRAINS AMONG DOMESTIC ANIMALS IN EGYPT

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Summary

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To investigate the incidence of the virulence genes among predominant *Brucella* strains in infected cattle, buffaloes, sheep, goats, and camels as well as in humans in Egypt, a total of 263 samples (85 milk, 167 tissue, 11 whole blood samples), yielded 140 (53.2%) *Brucella* isolates. Confirmation of *Brucella* isolates was carried out by conventional biotyping and by PCR using IR1/IR2 primers targeting *Brucella* genus showing an amplicon of 839 bp in all *Brucella* isolates. Conventional biotyping, as well as duplex PCR of the isolated non-repetitive *Brucella* strains, identified 107 (76.4 %) as *B. melitensis* with an amplicon of 731 bp and 33 (23.6%) as *B. abortus* with an amplicon of 498 bp. Out of 87 *Brucella* strains isolated from cattle, 63 (72.4%) were *B. melitensis*. No *Brucella* isolates were obtained from 7 lymph nodes of camels or 11 human blood samples; however, DNA extraction from 7 human sera and 3 camel lymph nodes gave positive PCR yield. All these samples gave PCR products indicating infection with *B. melitensis*. The distribution of the virulence genes among 33 *B. abortus* isolates revealed that *virB* recorded the highest incidence (97%), then followed *bvfA, ure,* and *omp25* (93.9%), *wbkA* (90.9%), *manB* (87.9%) and *amiC* (84.8%). All 107 *B. melitensis* isolates had the *bvfA, virB,* and *omp25* genes, while the prevalence of *ure* was 99.1%, that of *wbkA:* 96.3%, *manB:* 95.3% and *amiC:* 94.4%. The obtained results indicated the high incidence of virulence genes among field *Brucella* strains among farm animals in Egypt.

Key words: *B. abortus, B. melitensis,* brucellosis, Egypt, virulence genes

INTRODUCTION

Brucellosis is a major zoonosis that mainly affects domestic animal species, wildlife and recently sea mammals. The disease causes severe economic losses in animals due to storms of abortions, stillbirth, retained placenta, reduction in milk production and reproductive disorders. The disease is still endemic in most countries of the world, only a few countries are declared free from animal brucellosis, however; human brucellosis is still recorded there (OIE, 2018).

Infected animals and contaminated animal products are the main sources of

human infection. Every year about a half-million of confirmed humans cases for brucellosis is reported (Pappas *et al.*, 2006). However, this incidence is underestimated as recent epidemiological investigations revealed that the actual incidence of human brucellosis was 4 times higher (Corbel, 2006). For that, brucellosis is classified among the top-seven world neglected zoonotic diseases which has social, economic, and public health impacts in developing countries. The disease in humans is manifested by undulant fever, malaise, arthritis, and spondylitis. Furthermore, in chronic patients, *Brucella* organisms have the ability to infect many vital organs leading to encephalitis and endocarditis (Al Dahouk *et al.,* 2013). International official organisations including OIE, FAO, and WHO consider brucellosis a significant public health problem (Wareth *et al.,* 2019).

In Egypt, brucellosis is endemic among humans and livestock in all governorates causing massive economic losses among livestock especially in the Delta region (Samaha *et al*., 2008) and increases the burden on the governmental budget due to the costs of setting control programmes, quarantine regulations and application of vaccination programmes. Although control programmes for brucellosis were applied in Egypt about forty years ago, and a lot of money and efforts were spent in surveillance and control of the disease, however it was not yet eradicated and the incidence is still high (Wareth, *et al.,* 2019). Besides, human brucellosis in Egypt is neglected and underestimated due to misdiagnoses (El-Metwally *et al.,* 2011). It is broadly approved that *Brucella melitensis* is the most pathogenic and important zoonotic agent causing acute intractable illness and may lead to incapacitating infection in man, followed by *Brucella abortus* (Boon *et al*., 2010).

Pathogenicity of *Brucella* spp. can be described by its ability to invade epithelial host cells and to survive and multiply intracellularly within both phagocytic and non-phagocytic cells, escaping opsonisation and engulfing by macrophages (Scholz & Vergnaud, 2013). Virulence genes enable *Brucella* organisms to practice different mechanisms for evading bactericidal responses inside macrophages (Pei *et al*., 2006). *Brucella* lipopolysaccharide (LPS) and outer membrane proteins (OMPs) are considered major virulence components of *Brucella* spp. (Lapaque *et al*., 2005). Each factor responsible for a pathogenic pattern is encoded by a gene or genes responsible for its virulence. Among the most common and important *Brucella* virulence-associated genes with impact on vital activities are *bvfA, virB, ure, wbkA, manB, omp25,* and *amiC*. Brucellae exert their pathogenicity via different virulence factors that are necessary for intracellular survival and infection (Comerci *et al*., 2001; de Jong *et al*., 2013).

Brucella virulence factor A *(bvfA)* has been proved to play a role in the survival of *Brucella* in the host cells through the establishment of the intracellular niche (Lavigne *et al*., 2005) while *virB* is considered as one of the *Brucella* virulence factors that enhance intracellular replication through affecting protein synthesis form the type IV secretion system (Delrue *et al*., 2005). One of the significant virulence factors in *Brucella* is the urease *(ure)* that enabled brucellae to withstand the acidic pH conditions (Sangari *et al*., 2010). The *wbkA* and *manB* target lipopolysaccharide (LPS), while *omp25* targets outer membrane protein (OMP),

and *amiC* gene – peptidoglycan (PG) (Awaad *et al.,* 2015).

However, several virulence-associated genes have been reported in *Brucellae* worldwide in the last decade. These virulence genes are not only responsible for the survival of *Brucella* spp. in macrophages but also protect the organisms from the killing effect of the host immune system. This finding explains the difficulty in the treatment of brucellosis. About 5 to 30% of human cases with brucellosis are prone to relapse after antibiotic treatments (Solera, 2010). The need to study additional virulence genes, particularly those targeting the LPS, OMP, and peptidoglycan (PG) in common *Brucella* strains predominant in different animal species in Egypt is of high importance.

The objective of the current work was to study the presence and distribution of the selected virulence-associated genes, namely; *bvfA, virB, ure, wbkA, manB, omp25,* and *amiC* in different *Brucella* strains among preferential domestic animal species in Egypt. The outcomes of this study are needed to highlight the role of virulence genes on the contagiousness of brucellosis and to aid in developing a vaccine candidate originating from local field strains to immunise naïve farm animals for the control of animal brucellosis and consequently, to minimise public health hazard.

MATERIALS AND METHODS

Samples

A total of 341 blood samples were collected from humans (n=23) and animals (n=318), namely: cattle (n=195), buffaloes (n=40), sheep (n=38), goats (n=24), and camels (n=21) from different localities in Egypt (Table 1). All samples were collected from animals around 2–5 years of age, located in quarantined farms or from areas with a high incidence of brucellosis. Whole blood and serum samples were collected from human inpatients in Governmental Regional Fever Hospitals, suffering from fever and with a history of contact with animals. Samples were collected from nine (9) governorates representing Upper Egypt (Aswan and Luxor), Middle Egypt (Beni-suif and Giza) and Nile Delta (Behira, El-Monofia, Sharkia, Ismailia, Garbiya). Approximately 10 mL of blood was collected from the jugular vein of animals. Blood samples were kept in a refrigerator for 2 hours for clotting and then tubes were centrifuged at 2000 *g* for 5 min to obtain clear sera, kept at

Table 1. Types and numbers of samples collected from animals and humans

Host	Serology samples	Bacteriological samples					Total samples cultured
	Serum	Milk	Whole blood	Lymph node	Spleen	Liver	
Cattle	195	58	0	67	12	12	149
Buffaloes	40	18	0	9	4	4	35
Sheep	38	4	0	22	4	4	34
Goats	24	5	0	16	3	3	27
Camels	21	0	0	7	0	0	7
Humans	23	0	11	0	0	0	11
Total	341	85	11	121	23	23	263

–20 °C until use for serological investigations and/or DNA extraction.

Precisely, a total of 263 milk, tissue, and whole blood samples were collected from seropositive animals and humans (Table 1) for isolation of the causative agent; 85 milk samples were collected from serologically positive lactating animals. A total of 167 tissue samples (121 supra mammary lymph nodes, 23 spleens and 23 livers) were collected during post mortem examination of seropositive animals slaughtered in governmental abattoirs. The remaining 11 samples were obtained from human seropositive patients.

Serological testing

All serum samples obtained from animals and humans were subjected to serological investigation using the Rose Bengal test (RBT), buffered acidified plate antigen test (BAPA), rivanol test (Riv. T), and complement fixation test (CFT). BAPA was performed according to the method adopted by Alton *et al.* (1988) and OIE (2018). The antigen used for the BAPA was purchased from NVSL/DBL, USDA. Rose bengal antigen containing 8% PCV at pH 3.65±0.05 was obtained from APHA, New Haw, UK. The RB antigen was standardised to give a negative reaction with 1/55 dilution and a positive reaction with 1/45 dilution of the standard reference serum samples that contain 1000 IU (SAT) and 1000 ICFTU (international complement fixation test units).

The rivanol test (Riv.T) was done according to Alton *et al.,* (1988). Rivanol antigen and solution (2 ethoxy 6-9 diamine acridine lactate) were obtained from NVSL/DBL, USDA. A complete agglutination at a titer of 1/25 or higher was judged as positive.

The CFT was performed according to Alton *et al.* (1988) and OIE (2018). Standard *B. abortus* antigen (AHVLA, UK) was titrated to final dilution was 1:10 in veronal buffer diluent. Positive control sera containing 595 ICFTU per milliliter were obtained from APHA, UK. Guinea pig complement and haemolysin (rabbit anti-sheep RBCs) (Lilli Dale, UK), were titrated and 3% of sheep RBCs were used and standardised in the CFT. In CFT, positive serum samples were judged as those samples having 20 ICFTU or more. Milk was collected from serologically positive animals, for the conduct of the milk ring test (MRT) and isolation trials of the causative agents.

Bacterial milk cultures were done from the cream and sediment layers after centrifugation of the samples at 2000 *g* for 15 min at 4 ^{o}C (Alton *et al.,* 1988). Trimming off the fat surrounding the lymph nodes was carried out before dipping in ethanol (95%) and momentary flaming for sterilisation of the outer surface of the lymph node, while the culture area of the spleen and liver was sterilised by a heated spatula. All culture procedures were done in duplicates on *Brucella* agar medium supplemented with the appropriate antibiotics (Oxoid) according to Alton *et al.* (1988). One of the plates was kept in a 10% CO_2 incubator and the other one was kept in an ordinary incubator at 37 ^{o}C. The colonies were noticed after the third day of incubation and the sample was considered negative for culture after 21 days (Alton *et al.,* 1988). Typing of *Brucella* isolates was carried out according to colony morphology, CO_2 requirement, H_2S production, urease activity, catalase reaction, resistance to thionin and basic fuchsin dyes at a concentration of 20 µg/mL of media, reaction with monospecific sera (A & M), and lysis by Tblizi, Iz and R phages (Alton *et al.,* 1988; OIE, 2018).

Table 2. Preparation of PCR Master Mix

Component	PCR Master Mix Volume/reaction uniplex	PCR Master Mix Volume/reaction duplex
Emerald Amp GT PCR master mix (2× premix)	12.5 μL	25 μL
PCR grade water	4.5 μL	13 μL
Forward primer (20 pmol)	1 μL	1 μL (each)
Reverse primer (20 pmol)	1 μL	1 μL (each
Template DNA	6 μL	8 μL
Total	25 μL	50 μL

Extraction of DNA

Brucella colonies were suspended in 500 μL of sterile DNA-free water and boiled at 98 °C for 5 min for killing the viable cells. The suspension was centrifuged at 14,000 rpm for 10 min and precisely 300 μL of the supernatant was collected for template preparation. DNA extraction was carried out using Mini Kit *QIAamp* (Cat. No. 51304). The PCR Master Mix for both the uniplex and duplex PCR and the quantities of all components per reaction are listed in Table 2. Oligonucleotide primers were used in the PCR for identification of the *Brucella* genus as well as differentiation of *Brucella* species to *B. abortus* and *B. melitensis,* in addition to detection of the studied virulence genes. The specific sequences were obtained from Metabion (Germany) and amplified specific products are shown in Table 3. The final products of PCR runs were separated at room temperature by electrophoresis on 1% agarose gel (Applichem, Germany, GmbH) using TBE buffer. DNA ladder of 100 bp (Qiagen, Gmbh, Germany) and gene ruler 100 bp ladder (Fermentas, Germany) were used to determine the amplified fragment sizes. PCR amplified products in agarose gel were visualised by ultraviolet trans-illuminator after gel staining with 10 mg/mL ethidium bromide stain solution. The gel was photographed by a gel documentation system (Alpha Inno-tech, Biometra). For every PCR run, a control negative (sterile DNA-free water), and a control positive (*B. melitensis* biovar 3 reference Ether strain – ATCC No. 23458 and Standard reference *B. abortus* strain 544) were used. Internal quality control of known positive and negative samples was employed in the PCR process to exclude DNA contamination.

RESULTS

The incidence of brucellosis among tested animals is illustrated in Table 4. Out of 341 tested animals by RBT, BAPA, Riv. T and CFT, 231 (67.7%), 233 (68.3%), 220 (64.5%) and 223 (65.4%) were positive respectively. Out of 122 milk samples, 85 (69.7%) were positive in MRT.

Bacterial culture of 263 samples (85 milk samples, 167 tissue samples and 11 whole blood samples) on selective Brucella media, with the addition of specific antibiotic supplements (Oxoid), yielded 140 (53.2%) *Brucella* isolates. Out of the 140 *Brucella* isolates, 46 (54.1%), 70 (57.9%), 13 (56.5%), and 11 (47.8%) were recovered from 85 milk samples, 121 supra mammary lymph nodes,

Table 3. Oligonucleotide primers sequences and cycling conditions during PCR

Target gene	Sequence	Amplified product	Reaction conditions
Brucella genus IS711[1]	IR1: GGC-GTG-TCT-GCA-TTC-AAC-G IR2: GGC-TTG-TCT-GCA-TTC-AAG-G	839 bp	Primary denaturation: 94 °C, 5 min; Secondary denaturation: 94 °C, 30 s; Annealing: 55 °C, 40 s; Extension: 72 °C, 50 s; No. of cycles: 35; Final extension: 72 °C, 10 min
B. melitensis[1]	1S711-specific primer TGC-CGA-TCA-CTT-AAG-GGC-CTT-CAT *B. melitensis*-specific primer AAA-TCG-CGT-CCT-TGC-TGG-TCT-GA	731 bp	Primary denaturation: 94 °C, 5 min; Secondary denaturation: 94 °C, 30 s; Annealing: 55 °C, 40 s; Extension: 72 °C, 45 s; No. of cycles: 35; Final extension: 72 °C, 10 min
B. abortus[1]	1S711-specific primer TGC-CGA-TCA-CTT-AAG-GGC-CTT-CAT *B. abortus*-specific primer GAC-GAA-CGG-AAT-TTT-TCC-AAT-CCC	498 bp	Primary denaturation: 94 °C, 5 min; Secondary denaturation: 94 °C, 30 s; Annealing: 55 °C, 40 s; Extension: 72 °C, 45 s; No. of cycles: 35; Final extension: 72 °C, 10 min
bvfA[2]	ACCCTTCGTCGATGTGCTGA	1282 bp	Primary denaturation: 95 °C, 5 min; Secondary denaturation: 95 °C, 1 min; Annealing: 65 °C, 1 min; Extension: 72 °C, 1.3 min; No. of cycles: 35; Final extension: 72 °C, 10 min
virB[2]	CGCTGATCTATAATTAAGGCTA TGCGACTGCCTCCTATCGTC	881 bp	Primary denaturation: 95 °C, 5 min; Secondary denaturation: 95 °C, 1 min; Annealing: 54 °C, 1 min; Extension: 72 °C, 1.3 min No. of cycles: 35; Final extension: 72 °C, 10 min
ure[2]	GCTTGCCCTTGAATTCCTTTGTGG ATCTGCGAATTTGCCGGACTCTAT	2100 bp	Primary denaturation: 95 °C, 5 min; Secondary denaturation: 95 °C, 1 min; Annealing: 65 °C, 1 min; Extension: 72 °C, 1.3 min; No. of cycles: 35; Final extension: 72 °C, 10 min
manB[3]	GGCTGGTTCGAGAATATCCA CAATCGCATACCCTGGTCTT	228 bp	Primary denaturation: 94 °C, 5 min; Secondary denaturation: 94 °C, 30 s; Annealing: 58 °C, 30 s; Extension: 72 °C, 30 s No. of cycles: 35; Final extension: 72 °C, 10 min
wbkA[4]	AATGACTTCCGCTGCCATAG ATGAGCGAGGACATGAGCTT	931 bp	Primary denaturation: 94 °C, 5 min; Secondary denaturation: 94 °C, 30 s; Annealing: 60 °C, 40 s; Extension: 72 °C, 50 s No. of cycles: 35; Final extension: 72 °C, 10 min
amiC[4]	CAATTCGCCAATCACCTTTT AATGTGCCTTCCACTTTTGC	948 bp	Primary denaturation: 94 °C, 5 min; Secondary denaturation: 94 °C, 30 s; Annealing: 60 °C, 40 s; Extension: 72 °C, 45 s No. of cycles: 35; Final extension: 72 °C, 10 min
omp25[4]	TTT CCG TGT CCA ATT ATG CTA ACCGCGCAAAACGTAATTT	701 bp	Primary denaturation: 94 °C, 5 min; Secondary denaturation: 94 °C, 30 s; Annealing: 60 °C, 40 s; Extension: 72 °C, 45 s No. of cycles: 35; Final extension: 72 °C, 10 min

[1] Bricker & Halling (1994); [2] Derakhshandeh *et al*. (2013); [3] Naseri *et al*. (2016); [4] Awwad *et al*. (2015).

Table 4. Detection of *Brucella* antibodies in blood and milk samples

Source of samples	Serological tests				Milk samples
	RBT-positive	BAPA-positive	RIV.T-positive	CFT-positive	MRT-positive
Cattle (n=195)	139	141	136	137	58
Buffalo (n=40)	28	29	27	27	18
Sheep (n=38)	29	30	27	28	4
Goats (n=24)	14	12	10	11	5
Camels (n=21)	7	7	7	7	0
Humans (n=23)	14	14	13	13	0
Total (n=341)	231	233	220	223	85
Percent positive	69.7	65.4	64.5	68.3	67.7

RBT= Rose Bengal test; BAPA= buffered acidified plate antigen test; RIV.T=rivanol test; CFT= complement fixation test; MRT = milk ring test.

Table 5. Results from the bacterial culture on different samples collected from animals and man

	Samples (number positive/total number)					Total (number; % positive)
	Milk	Lymph node	Spleen	Liver	Whole blood	
Cattle	32/58	43/67	7/12	5/12	0/0	87/149
Buffalo	9/18	6/9	2/4	2/4	0/0	19/35
Sheep	2/4	12/22	2/4	2/4	0/0	18/34
Goats	3/5	9/16	2/3	2/3	0/0	16/27
Camels	0/0	0/7	0/0	0/0	0/0	0/7
Humans	0/0	0/0	0/0	0/0	0/11	0/11
Total	46/85	70/121	13/23	11/23	0/11	140/263
% positive	54.1	57.9	56.5	47.8	0.00	53.2

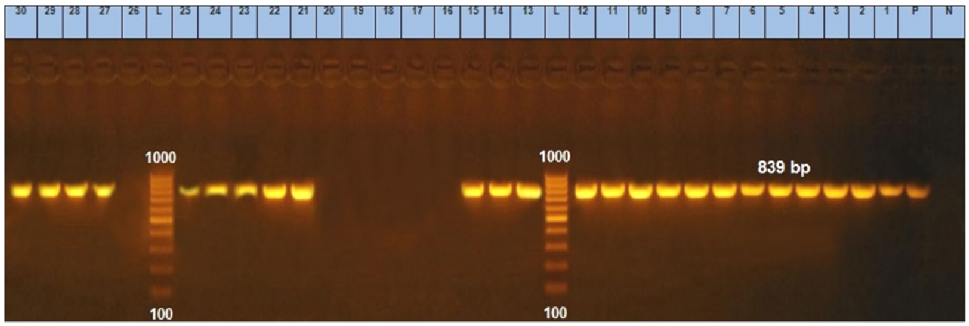

Fig. 1. Agarose gel electrophoresis image, showing uniplex-PCR products of *Brucella genus* gene IS711 (*IR1, IR2*) isolated from animal milk, lymph nodes, tissues and human sera. L: lane marker (100 bp); P: positive control, N: negative control; lanes 1–15, 21–25, 27–30: positive samples; lanes 16–20: negative samples.

23 spleens, and 23 livers, respectively (Table 5). No *Brucella* isolates were obtained from human blood samples.

All isolated *Brucella* strains were subjected to phenotypic characterisation, namely colony morphology, CO_2 require-

ment, H_2S production, urease activity, catalase reaction, growth in the presence of dyes (thionin and basic fuchsin – 20 µg/mL), reaction with monospecific sera (A & M) and lysis by *Brucella* phages (Tblizi and Iz) (Alton *et al.,* 1988; OIE, 2018). The PCR assay was applied to all isolated strains for the detection of *Brucella* DNA on the genus level (Table 5; Fig. 1). The isolated *Brucella* strains (n=140) were subjected to the duplex PCR using specific primers for identification of species level, out of them, 107 (76.4%) were identified as *B. melitensis* and the remaining 33 (23.6%) were proved to be *B. abortus* (Table 6; Fig 2). Conventional biotyping procedures classified all *B. melitensis* strains as biovar 3 and all *B. abortus* strains belonged to biovar 1.

Out of 87 strains isolated from cattle, 63 (72.4%) were proved by conventional typing as well as by duplex PCR as *B. melitensis* and only 24 (27.6%) as *B. abortus*. Out of 19 strains isolated from buffaloes, 10 (52.6%) were *B. melitensis* and 9 (47.4%) – *B. abortus*. In the meanwhile, all strains isolated from sheep (n=18) and goats (n=16) were found to be *B. melitensis* (Table 6). Although no *Brucella* isolates were obtained from 7 camel lymph nodes of or 11 human blood

Table 6. Prevalence of *B. melitensis* and *B. abortus* by duplex PCR among 140 isolated *Brucella* strains

Origin of isolated strains	No.	*B. melitensis*		*B. abortus*	
		number	%	number	%
Cattle	87	63	72.4	24	27.6
Buffalo	19	10	52.6	9	47.4
Sheep	18	18	100	–	–
Goats	16	16	100	–	–
Total number of *Brucella* strains	140	107	76.4	33	23.6
Brucella DNA extracted from human sera	7	7	100	–	–
Brucella DNA extracted from camel lymph nodes	3	3	100	–	–

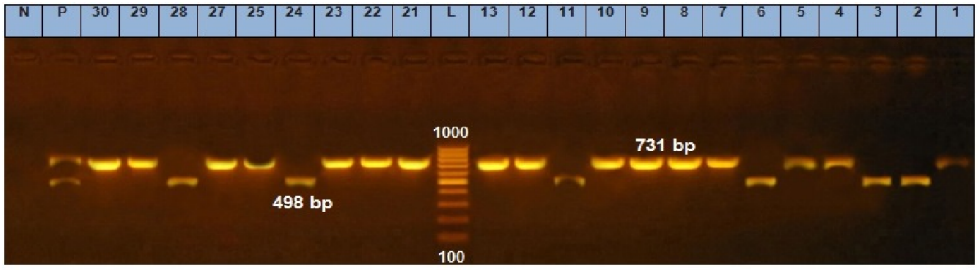

Fig. 2. Agarose gel electrophoresis image, showing duplex-PCR products of *B. abortus* and *B. melitensis* specific genes. L: lane marker (100 bp); P: positive control, N: negative control; lanes 1, 4, 5, 7–10, 12, 13, 21–23, 25, 27, 29, 30: *B. melitensis* positive samples; lanes 2, 3, 6, 11, 24, 28: *B. abortus* positive samples.

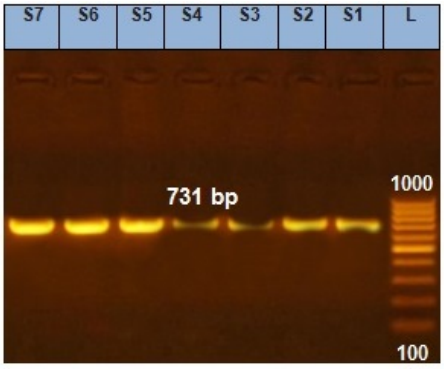

Fig. 3. Agarose gel electrophoresis image, showing duplex-PCR products of *B. abortus* and *B. melitensis* genes from human sera samples. Only *B. melitensis* positive DNA was detected in all samples (S1– S7); L: lane marker (100 bp).

samples, DNA extraction from 7 human sera and 3 DNA extracts of camel lymph nodes gave positive PCR yield. All these samples gave PCR products indicating infection with *B. melitensis* (Table 6; Fig 3).

All 140 *Brucella* strains isolated in this study, were subjected to PCR for studying the distribution of the virulence genes (Table 7 and 8; Fig. 4–10). Out of them, all 107 *B. melitensis* isolates harboured *bvfA, virB, and omp25* virulence genes, followed by *ure* gene detected in 106 (99.1%), *wbkA* detected in 103 (96.3%), *manB* detected in 102 (95.3%) and finally *amiC* gene detected in 101 (94.4%) of isolated *B. melitensis* strains (Table 7). On the other hand, the *virB* recorded the highest distribution (32/33; 97%) among the *B. abortus* isolates, followed by *bvfA, ure,* and *omp25* detected in 31/33 (93.9%) of the isolates, *wbkA* gene present in 30/33 isolates (90.9%), *manB* in 29 (87.9%) and finally *amiC* detected in 28 (84.8%) of the *B. abortus* isolates (Table 8).

DISCUSSION

Unlike other pathogenic bacteria, brucellae possess no classical virulence factors as they are lacking exotoxins, capsules,

Table 7. Prevalence of the virulence genes among 107 *B. melitensis* isolates according to animal species

	Organs	*bvfA*	*virB*	*ure*	*wbkA*	*manB*	*omp25*	*amiC*
Cattle	Milk	23	23	23	22	23	23	22
	Lymph node	29	29	29	29	29	29	29
	Spleen	4	4	4	4	4	4	4
	Liver	4	4	3	4	4	4	3
Buffalo	Milk	6	6	6	5	5	6	5
	Lymph node	3	3	3	3	3	3	3
	Spleen	2	2	2	2	2	2	2
	Liver	2	2	2	2	1	2	2
Sheep	Milk	2	2	2	2	2	2	2
	Lymph node	12	12	12	12	11	12	11
	Spleen	2	2	2	2	2	2	2
	Liver	2	2	2	1	1	2	1
Goats	Milk	3	3	3	3	3	3	3
	Lymph node	9	9	9	9	9	9	9
	Spleen	2	2	2	1	2	2	2
	Liver	2	2	2	2	1	2	1
	Total number	107	107	106	103	102	107	101
	Percent	100.0	100.0	99.1	96.3	95.3	100.0	94.4

Table 8. Prevalence of the virulence genes among 33 *B. abortus* isolates according to animal species

Organs	*bvfA*	*virB*	*ure*	*wbkA*	*manB*	*omp25*	*amiC*
Cattle							
Milk	8	9	9	8	8	9	8
Lymph node	13	13	13	14	14	13	14
Spleen	3	3	3	3	3	3	3
Liver	1	1	0	1	0	1	0
Buffalo							
Milk	3	3	3	2	2	2	1
Lymph node	3	3	3	2	2	3	2
Total number	31	32	31	30	29	31	28
Percent	93.9%	97.0%	93.9%	90.9%	87.9%	93.9%	84.8%

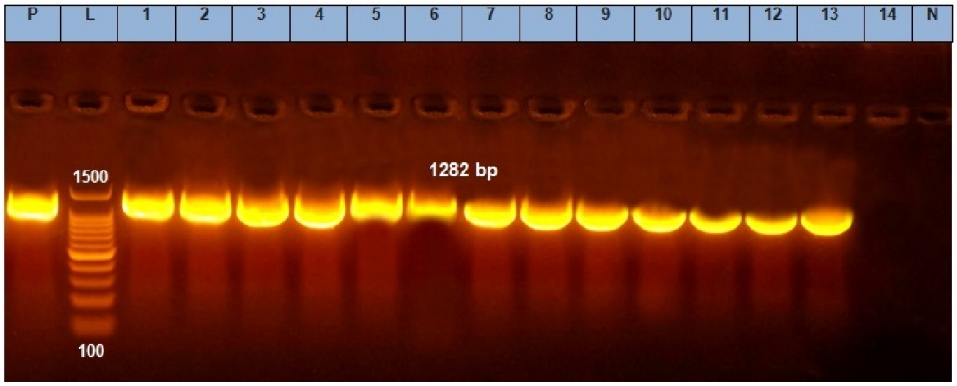

Fig. 4. Agarose gel electrophoresis image, showing uniplex-PCR products of *Brucella* virulence *bvfA* gene. Lanes 1–13: positive samples; lane 14: negative sample, L: lane marker (100 bp), P: positive control, N: negative control.

plasmids, and lysogenic phages. Moreover, *Brucella* endotoxic lipopolysaccharide (LPS) tends to be of low pathogenic affinity, being several hundred times less active and less toxic than the LPS of *Escherichia coli* (Moreno & Moriyon, 2002; Seleem *et al.,* 2008). However, *Brucella* LPS exhibits antibacterial peptide attacks through suppression of immune mediators (Lapaque *et al.,* 2005). On the other hand, the organism has the ability to adapt to inappropriate environmental conditions through escaping the opsonisation process, inhibiting host cell apoptosis, and favouring bacterial intracellular survival by avoiding the macrophages killing effect. In addition, the organism is acclimatised to oxygen-limited concentrations inside macrophages and tolerates acidic pH (Kohler *et al.,* 2002; Pei *et al.,* 2006). Nevertheless, *Brucella* organisms have many virulence factors responsible for extreme pathogenicity (Abdo *et al.,* 2011).

In the current study and for the purpose to detect humans and animals positive for brucellosis, 341 blood samples were collected and serologically tested by RBT, BAPA, Riv. T and CFT (Table 4). It is noted that the BAPA detected the highest numbers of seropositive cases –

233 (68.3%) as the relatively low acidic pH 4 enables some of the IgM to share in the reactions compared with the more acidic pH of the RBT antigen (3.65), that detected 231 cases (67.7%) (Alton *et al.,* 1988). CFT has been and is still widely used as a confirmatory test for diagnoses of brucellosis in control and eradication programmes. Both the CFT and RBT were recommended by the OIE for testing animals for international trades (OIE, 2018). Based on this recommendation, samples positive to both tests (223 or 65.4%) were considered positive and collection of samples for isolation of the causative agents was practiced as possible from these animals. Out of 122 milk samples, 85 (69.7%) reacted to MRT (Table 4). The relatively high incidence of brucellosis among animal species in this study (65.4%) is due to the fact that it targeted infected farms.

Trials for the isolation of the causative agents were carried out on serologically positive samples. Out of 263 tissues, milk, and whole blood samples, 140 (53.2%) non-repetitive *Brucella* isolates were recovered from serologically positive animal species, namely; cattle, buffaloes, sheep, and goats. The highest recovery rate was obtained from supra mammary lymph nodes (70/121; 57.9%), followed

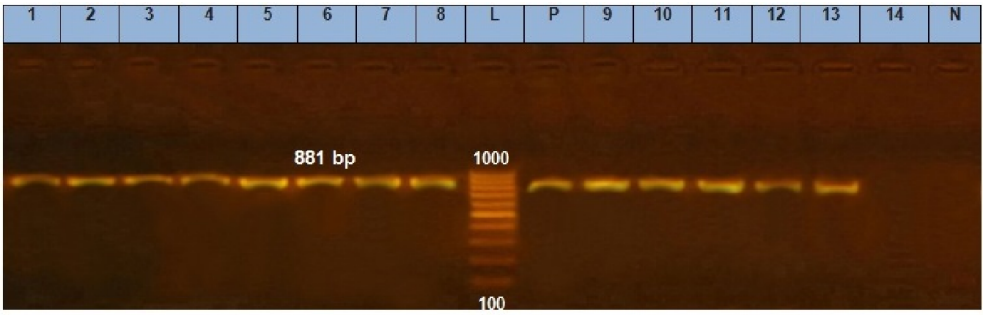

Fig. 5. Agarose gel electrophoresis image, showing uniplex-PCR products of *Brucella* virulence *virB* gene. Lanes 1–13: positive samples; lane 14: negative sample, L: lane marker (100 bp), P: positive control, N: negative control.

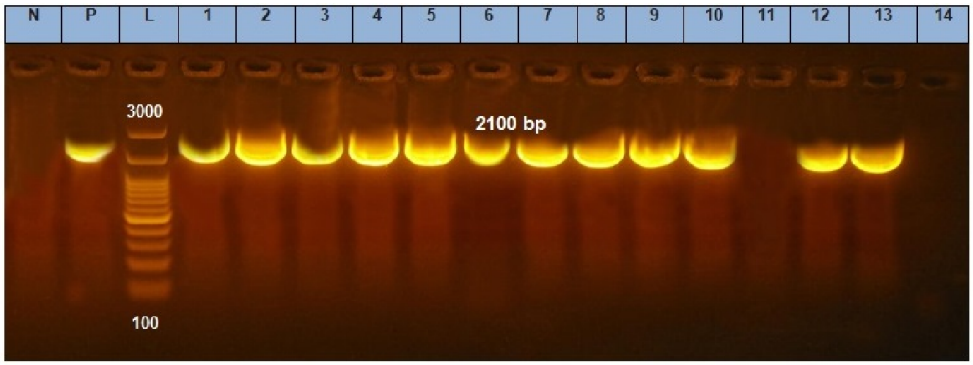

Fig. 6. Agarose gel electrophoresis image, showing uniplex-PCR products of *Brucella* virulence *ure* gene. Lanes 1–10, 12, 13: positive samples; lanes 11, 14: negative samples, L: lane marker (100 bp), P: positive control, N: negative control.

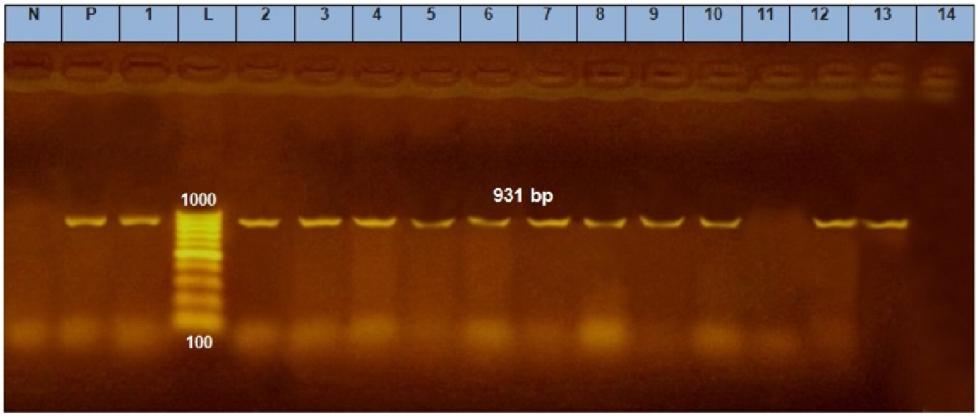

Fig. 7. Agarose gel electrophoresis image, showing uniplex-PCR products of *Brucella* virulence *wbkA* gene. Lanes 1–10, 12, 13: positive samples; lanes 11, 14: negative samples, L: lane marker (100 bp), P: positive control, N: negative control.

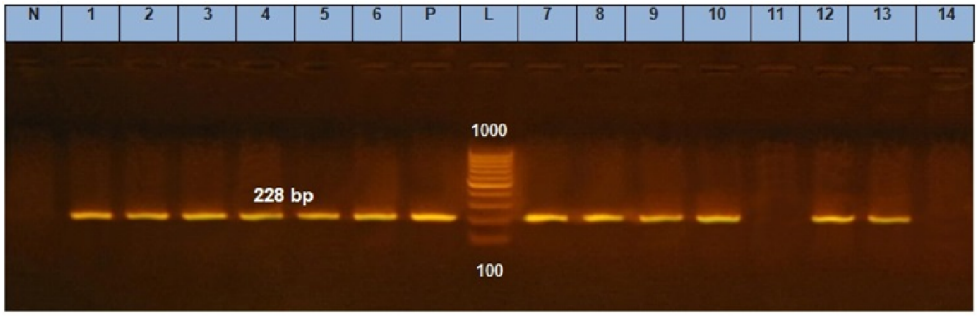

Fig. 8. Agarose gel electrophoresis image, showing uniplex-PCR products of *Brucella* virulence *manB* gene. Lanes 1–10, 12, 13: positive samples; lanes 11, 14: negative samples, L: lane marker (100 bp), P: positive control, N: negative control.

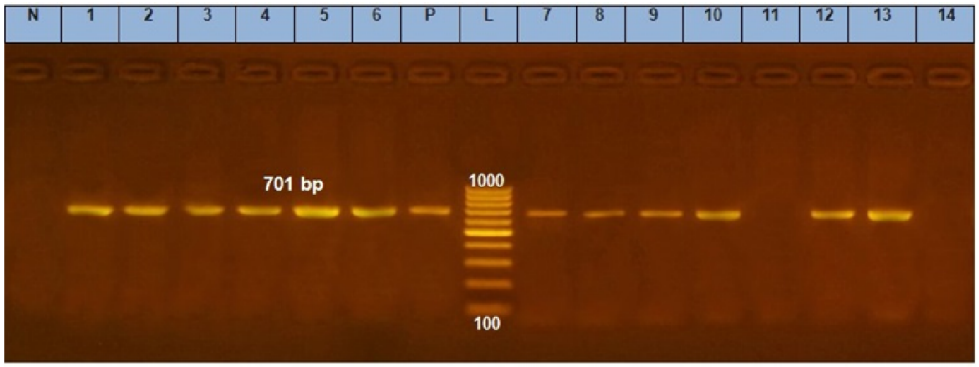

Fig. 9. Agarose gel electrophoresis image, showing uniplex-PCR products of *Brucella* virulence *omp25* gene. Lanes 1–10, 12, 13: positive samples; lanes 11, 14: negative samples, L: lane marker (100 bp), P: positive control, N: negative control.

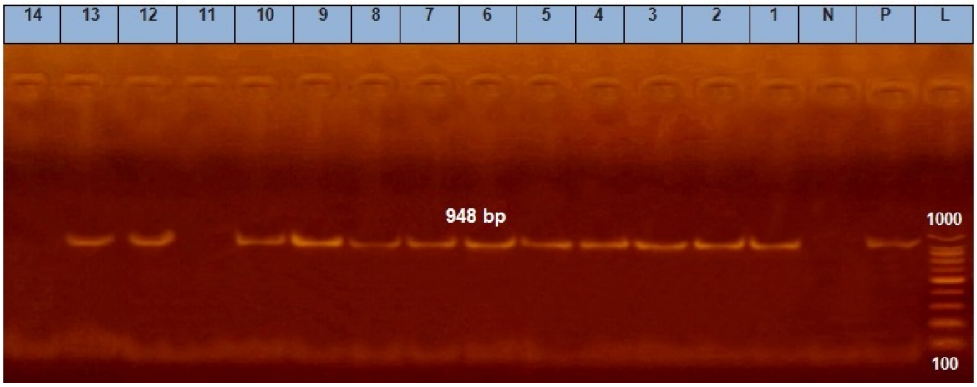

Fig. 10. Agarose gel electrophoresis image, showing uniplex-PCR products of *Brucella* virulence *amiC* gene. Lanes 1–10, 12, 13: positive samples; lanes 11, 14: negative samples, L: lane marker (100 bp), P: positive control, N: negative control.

by spleen (13/23; 56.5%), milk (46/85; 54.1%), and finally liver (11/23; 47.8%) as described in Table 5. The shedding of *Brucellae* in the milk of infected animals is intermittent and depends on the stage of infection, so the recovery rate (54.1%) of the organisms from the milk of serologically positive animals in this study, was expected and complies with this concept.

Conventional biotyping of the isolated *Brucella* strains according to adopted methods (Alton *et al.,* 1988; OIE, 2018) revealed that most of the isolated strains were proved to be *B. melitensis* biovar 3, (107/140; 76.4%) and *B. abortus* biovar 1 (33/140; 23.6%) (Table 6). *Brucella melitensis* was not only isolated from the original hosts sheep (18/18) and goats (16/16) but also from cattle (63/87; 72.4%) and buffaloes (10/19; 52.6%). Isolation of *B. melitensis* from the non-original host (cattle and buffaloes) is common in countries like Egypt where infected sheep and goats flocks are raised side by side with bovine herds, sharing the same pasture. It has been stated that *B. melitensis* was the least host-specific among other *Brucella* species and it may invade other animal species, particularly cattle (Verger *et al.,* 1989; Alton, 1990). Parallel findings were reported in Egypt by many workers, who found that *B. melitensis* was the predominant strain in different animal species and man in Egypt (Refai, 2002; Samaha *et al.,* 2008; Menshawy *et al.,* 2014, Wareth *et al.,* 2014, Hamdy & Zaki, 2018; Hosein *et al.,* 2018).

All *Brucella* strains were subjected to PCR for confirmation of conventional biotyping methods. It was found that 140 strains were identified using a specific primer (IR1/IR2) targeting the *Brucella* genus (Fig. 1). IR1/RI2 primers were more sensitive than other primers for the detection of *Brucella*-DNA in human and animal samples (Zamanian *et al.,* 2015). It was approved that DNA homology among members of the genus *Brucella* was approximately not less than 95%.

The duplex PCR technique was applied to identify *B. abortus* and *B. melitensis* species of the genus *Brucella*. It was evident that the PCR correctly identified all isolated strains (107*B. melitensis* out of 140) and the remaining 33 strains were proved to be *B. abortus* (Table 6, Fig. 2). Amplification of *Brucella* DNA

by PCR using a specific primer sequence annealing to the insertion sequence (*IS711)* region, allowed producing PCR products of variable sizes. The number of repetitive copies of the genetic element *IS711* varied from one species to another with different positioning loci in the *Brucella* genome that could be visualised and measured to identify different *Brucella* species (Al Dahouk *et al.,* 2003; Orzil *et al.,* 2016). The PCR was proved to identify *Brucella* species in 100% agreement with the conventional biotyping methods (Bricker & Halling, 1994). Our results confirmed this finding, as PCR proved to identify all *Brucella* strains on the genus and species levels similar to the biotyping procedures. However, PCR was proved to be easier, faster, inexpensive, safer, and non-infectious than traditional isolation and identification techniques. Trials for isolation of *Brucella* organisms from 11 human blood samples obtained from hospitalised inpatients failed to recover any *Brucella* isolates. This in fact may be due to the fact that all these patients were on antibiotic therapy. Human sera were subjected to DNA extraction which succeeded to identify 7 *Brucella* DNA samples (Table 6). These samples were amplified by PCR using genus and specific primers and it was found that they had DNA of *B. melitensis* species. It is noteworthy to mention that no *Brucella* organisms were recovered from the lymph nodes of the 7 seropositive camels, however *Brucella* DNA was extracted from 3 of them and all were classified by duplex PCR as *B. melitensis*. PCR proved to be more sensitive and superior to traditional isolation and identification techniques, since *B. melitensis* DNA tended to persist in the serum of infected humans and animals for long periods. The same finding was obtained by many workers (Zerva *et al.,* 2001; Castaño & Solera, 2009; Hamdy *et al*., 2017). Isolation of *B. melitensis* from infected cattle is a serious problem as a result of the relatively large volume of contaminated milk produced by infected cattle, in addition to the contamination of the surrounding environment with huge numbers of viable *Brucellae* as a result of abortion, retained placenta and uterine exudates (Corbel, 2006). *Brucella melitensis* particularly biovar 3, is endemic in several parts of the world including the Mediterranean and Middle Eastern countries (Young, 2000; FAO, 2010; Greco *et al.,* 2018).

Virulence of *Brucella* spp. is mainly due to its ability to survive intracellularly in host cells. *Brucellae* have the ability to attack reticuloendothelial cells of the host, leading to infection (Ackermann *et al.,* 1988). Once *Brucella* spp. invade the host cells, they are capable with the aid of virulence factors to survive within phagocytic cells (Carvalho Neta *et al.,* 2010). Virulence genes are vital not only for the survival and intracellular adaptation of *Brucella* spp. in host cells but also for their resistance to body immune response (Saeedzadeh *et al.,* 2013). Among virulence factors of *B. melitensis* are cell envelope proteins that are responsible for the initial survival inside macrophages (Godfroid *et al.,* 2000; Moreno & Moriyon, 2002; Xavier *et al.,* 2010).

All 140 *Brucella* strains isolated in this study were subjected to the PCR assay to study the incidence and distribution of the virulence genes *bvfA, virB, ure, wbkA, manB, omp25* and *amiC* (Table 7 and 8, Fig. 4–10). On the other hand, the distribution of the same virulence genes in the 107 *B. melitensis* isolates revealed that *bvfA, virB, and omp25* were detected in all isolates (100%), followed by *ure* gene detected in 106 (99.1%), *wbkA* detected in

103 (96.3%), *manB* detected in 102 (95.3%) and finally *amiC* gene detected in 101 (94.4%) of *B. melitensis* strains (Table 7).

Brucella virulence factor A (*bvfA*) was detected in all *B. melitensis* strains (100%) and in 93.9% of *B. abortus*. The *bvfA* gene is a small size periplasmic protein unique to the genus *Brucella* with no match in the gene bank (Lavigne *et al.,* 2005). This factor shares in the establishment of the intracellular niche and plays an essential role in the virulence of *Brucella* spp. (Lavigne *et al.,* 2005).

The *virB* gene was detected in 100% and 97% of *B. melitensis* and *B. abortus* strains isolated from Egypt, respectively (Tables 7 and 8, Fig. 5). The *virB* gene encodes many factors enhancing intracellular survivability of *Brucella* organisms into host cells (Celli *et al.,* 2003). In *Brucella* spp. the *virB* type IV secretion system (T4SS) is an important virulence factor for intracellular survival of the organism, through a gene coding for an enzyme involved in the biosynthesis of LPS that is responsible for immune evasive behaviour (Delrue *et al.,* 2001; den Hartigh *et al.,* 2008). Besides, the *virB* is required for the survival of *Brucella* spp. within macrophages, hence, mutants of *Brucella* organisms devoid of the *virB* genes were unable to replicate intracellularly (Roux *et al.,* 2007; den Hartigh *et al.,* 2008). *In vivo* infection studies on lab animals confirmed the role of the *Brucella* T4SS for the viability and persistence of the organism inside murine cells (Hong *et al.,* 2000).

In the current study, *ure* virulence factor was detected in 93.9% and 99.1% of *B. abortus* and *B. melitensis*, respectively (Tables 7 and 8, Fig. 6). The *ure* gene encodes the urease enzyme, which hydrolyzes urea to produce carbonic acid and two molecules of ammonia. The ammonia molecules protonate to form ammonium, causing neutralisation of the surrounding acidic environment. Thus, the degradation of urea facilitates survival in an acidic environment (Cruz-Ramos *et al.,* 1997). Recent experimental studies on BALB/c mice infected with *B. abortus* through the oral route, which is the major route of infection in human brucellosis, indicated that urease protected the organisms during their existence in the acidic medium of the stomach (Bandara *et al.,* 2007; Sangari *et al.,* 2007).

Similar results were obtained from the analysis of 52 *B. melitensis* strains revealing that 48 (92.3%) isolates carried *bvfA* genes, 51 (98.1%) – the *virB* gene, and 50 (96.2%) – *ure* gene (Hamdy & Zaki, 2018). However, another study has demonstrated that the virulence factor *virB* was detected in only 4 out of 7 bovine *B. melitensis* isolates in one dairy farm in Monofia governorate (Ramadan *et al.,* 2019). Although both studies were done in Egypt, the discrepancies between the results may be attributed to the fact that the present study was done on *Brucella* isolates collected from different localities – Upper Egypt, Middle Egypt and Nile Delta regions of the country representing 9 governorates.

The incidence of the *wbkA* and *manB* virulence genes in *B. melitensis* isolates was 96.3% and 95.3% respectively and in *B. abortus* isolates: 90.9% and 87.9% respectively (Table 7 and 8, Fig. 7, 8). The *wbkA* and *manB* genes play a role in intracellular survival and intracellular modulatory activity of *Brucellae* in host cells, besides; they are proved to protect the organisms from the host's defense mechanism (Lapaque *et al.,* 2005). The *wbkA* gene encodes mannosyl-transferase and *manB* gene encodes phosphorus-

mannomutase, both being involved in the LPS synthesis in brucellae. Lipopolysaccharides are the most significant virulence factor that enables *Brucella* organisms to survive inside macrophages and other cells of the reticuloendothelial system by the incidence of the O-side chain on the LPS of smooth strains (Cloeckaert *et al.,* 2003). The smooth brucellae can escape the immune defense mechanism of the host by avoiding factors released from dead cells during apoptosis (Jimenez de Bagues *et al.,* 2005). The presence of such genes in the *Brucella* genome indicates their virulence. The obtained results in this study were in harmony with results obtained by other workers who found that *wbkA* and *manB* were found only in all smooth *Brucella* isolates (Caron *et al.,* 1996). However, both *wbkA* and *manB* genes were not detected in 4 and 5 *B. melitensis* strains (Table 7 and 8). This finding may be ascribed to the concept that smooth LPS of *Brucella* has many atypical features, relatively low toxicity for macrophages. The obtained results were in accordance with this concept, as *Brucella* isolates obtained in this study were isolated from animals in chronic stages of infection and with a known history of brucellosis. Our results are in agreement with another study that detected *wbkA* and *manB* in 8 *B. melitensis* isolates obtained from human patients in Babylon Hospital, Iraq (Razzaq *et al.,* 2014). Similar findings were reported for the incidence of the virulence factors *virB, ure, bvfA, omp25, wbkA* and *manB* among 57 *B. melitensis* strains isolated from inpatient human blood samples in Hamadan province, Iran – the *ure, bvfA, omp25, wbkA,* and *manB* were detected in all *B. melitensis* isolates (100%) and the *virB* gene were detected in 53/57 (93%) of the same *Brucella* strains (Naseri *et al.,* 2016).

In the current study, both *omp25* and *amiC* genes were detected in 100% and 94.4% of *B. melitensis* isolates and 93.9% and 84.8% of *B. abortus* isolates (Table 7 and 8, Fig. 9, 10). *Brucella* outer membrane contains two components that account for virulence including the LPS and the outer membrane proteins (OMPs) (Lory & Tai, 1984). Both components are important for explaining the differences in virulence and host specificity of *Brucella* spp. (Ratushna *et al*., 2005). It was approved that *omp25* gene is responsible for the virulence of *Brucellae* through encoding outer membrane proteins (omp25) of human macrophages that inhibit the release of tumor necrosis factor-alpha (Jubier-Maurin *et al.,* 2001; Edmonds *et al.,* 2002). However, other researchers detected *omp25* and *amiC* in 100% of 80 *B. melitensis* strains isolated from sheep and goats in Palestine, while the *wbkA* and *manB* genes were detected in 95% of the isolated strains (Awaad *et al.,* 2015). These results coincide with the results obtained from *B. melitensis* in the current study indicating that the virulence genes were more predominant not only in *B. melitensis* strains isolated from Egypt but also in the other *B. melitensis* strains isolated from the Mediterranean Region.

CONCLUSION

The obtained results revealed high incidence and frequency of the studied virulence genes, namely; *bvfA, virB, ure, manB*, *wbkA, omp25* and *amiC,* among *B. abortus* and *B. melitensis* field strains isolated from different animal species in Egypt. The potential risk of these biohazard virulent strains reflects the contagiousness of the disease in animals in Egypt and constitutes a real threat to public health. The public health and veteri-

nary authorities in the country should launch an effective control programme to control and prevent the spread of this hazard.

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