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Risk factors and Molecular genotyping of *Brucella melitensis* strains recovered from humans and their owned cattle in Upper Egypt

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ABSTRACT

Brucellosis is a zoonosis that has a devastating impact on the economy and public health, particularly in the Middle East, including Egypt. This study aimed to define risk factors associated with brucellosis in humans and in their cattle in Fayoum governorate - Upper Egypt. Also, molecular genotyping of recovered *Brucella* isolates from human cases and their cattle to assess the potential cross-species transmission in the study region. Data were obtained via double matched case–control studies for brucellosis in humans (106 cases and 160 controls) and in their cattle (78 cattle cases and 105 cattle controls). The results of multivariate regression analysis revealed that predictors of human brucellosis were animal-related occupations (OR 2.1, *P* 0.02), previous infection in other household members (OR 3.2, *P* 0.007), eating home-made soft cheese (OR 2.3, *P* 0.03), and exposure to cattle abortions (OR 6.9, $P < 0.001$). For cattle, predictors of brucellosis were maturity ≥2 years of age (OR 2.9, *P* 0.01), ≥2 animals reared by the same household (OR 3.7–6.9, $P \leq 0.001$), and recent abortion (OR 15.2, *P* 0.01). Twelve *Brucella* isolates were recovered from eight human cases (7.5%, 8/106) and four cattle cases (6.2%, 4/65). All isolates were *B. melitensis* biovar 3. Analysis of the *IS711* gene sequence revealed complete homology (100%) between isolates. Six virulence genes were utilized for virutyping: *virB* (100%), *omp25* (100%), *amiC* (100%), *ure* (91.7%), *wbkA* (91.7%), and *bvfA* (75%). Virutyping revealed four virutypes: V1 (lack *bvfA*, 16.7%), V2 (harbored all genes, 66.7%), V3 (lack *wbkA*, 8.3%), and V4 (lack *wbkA* and *ure*, 8.3%). Repetitive extragenic palindromic PCR (REP-PCR) typing revealed two REP types. Combined REP-PCR/virulence genotyping revealed five different genotypes (G1–G5) for the detected isolates and a unique genotype for the reference strain (G6, *B. melitensis* bv3 Ether). Human and cattle isolates from the same household had matched genotypes. In conclusion, there were widespread risk factors among the cases studied. Health education for high-risk groups is essential for disease prevention, and combined REP-PCR/virulence genotyping is a quick tool for traceability, particularly in developing countries endemic with brucellosis as Egypt.

1. Introduction

Brucellosis is a zoonotic disease with a detrimental global impact on economy and public health. There are 5–12.5 million human cases of brucellosis each year, and the disease is considered to be one of the most widespread zoonoses worldwide [1]. The highest rates are found in the Middle East, including Egypt, where there is inadequate control of the disease in animals and where high-risk practices such as consumption of raw milk and dairy products are common [2,3]. Brucellosis may have a serious impact on humans, potentially resulting in either disability or mortality in certain complications [1,3]. The disease may last for years, and treatment requires patient compliance with a costly and prolonged therapy [3]. Brucellosis also has a devastating impact on animal health and productivity; it may cause reproductive disorders (late-stage abortion, stillbirth, and retained placenta), and reduction in milk production by up to 30% [4].

The main risk factors for human brucellosis are the exposure to the infected animals or the consumption of their raw products as milk and

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cheese [1,2]. However, there can be indirect contributions from other risk factors through their influence on either rates of animal exposure or raw product consumption. For example, socio-demographic characters such as occupation type may alter the rate of animal exposure. Similarly, regional customs and traditional beliefs may affect the attitude toward consumption of raw dairy products [5–7]. Importantly, these risk factors vary from region to region within countries and between different countries, contributing to variation in the prevalence of brucellosis in different geographical niches [6].

Epidemiological traceability is a key step in identifying high-risk sources of the causative agents. Molecular-based tracing is greatly favored because of the highly specific link between the pathogens and their potential sources. Multiple Locus Variable Number Tandem-Repeat Analysis (MLVA) and whole genome single nucleotide polymorphism -based typing (WGS-SNP) are gold standard tools for inter- and intra-species discrimination of *Brucella* isolates worldwide [8,9]. However, these procedures are expensive and are not readily available, particularly in developing countries such as Egypt. Alternatively, several studies have employed more cost effective discrimination approaches, such as IS*711* gene sequence variations, PCR restriction fragment length polymorphism (PCR-RFLP), and Repetitive element sequence-based PCR (rep-PCR) [10–12]. However, the high genetic similarity between *Brucella* species can occasionally reduce the efficiency of these tools in discriminating between isolates of same species, especially when these tools were used individually [10].

In Egypt, brucellosis is highly endemic, with its prevalence varying in many regions of the country [13]. Three zoonotic *Brucella* species have been reported in Egypt: *B. melitensis*, *B. abortus*, and *B. suis*. The *B. melitensis* is the predominant species in livestock and humans [9,13,14]. The annual incidence of human brucellosis in the country is between 0.5/100,000 and 70/100,000, depending on the region of study [15–17]. The prevalence of brucellosis in Egypt among animals is between 0.2% and 20%, depending on the region and the species [13,18,19]. In Egypt, cattle are the most common household-reared species [20], and most cattle breeders consume the milk of their own cattle and use it for home-made dairy products [7]. The Fayoum governorate, in Upper Egypt, has recorded the highest annual incidence of human brucellosis (70/100,000) [16]; however, information on risk factors for infection among humans and animals are scarce. Therefore, the aim of this study is to identify the risk factors for human brucellosis and the risk factors for cattle brucellosis through double matched case–control studies (humans and cattle) in Fayoum governorate. Additionally, molecular genotyping of *Brucella* isolates from human and cattle cases to assess the potential transmission of this zoonotic disease in the study region.

2. Methods

2.1. Study Area

Fayoum, which is one of the governorates of North Upper Egypt, is located southwest of Cairo [29°21′48″N 30°44′45″E (Fig. 1)] and has an area of 1827 km^2 and a population of 3,362,413 [21]. Fayoum is an agriculture governorate, with many residents living in rural dwellings and keeping livestock at home. The governorate ranks 20th of 27 governorates in Egypt's human development index [22]. This poor situation correlates with socio-demographic factors such as high illiteracy rates, poverty, and strong traditional beliefs related to the rural community [22].

2.2. Study Design

We conducted double matched case–control studies (for humans and for cattle) in Fayoum governorate from April 2019 to September 2020.

2.2.1. Humans Matched Case–Control Study

We calculated the sample size using the Epitools website (available at: https://epitools.ausvet.com.au/casecontrolss), with a 95% confidence interval, 80% power, a 30% estimated exposure rate for controls, and an assumed minimum odds ratio of 2.2. We obtained an estimated number of 212 (106 cases and 106 controls) for cases and controls. However, to ensure that we had adequate numbers to cover all tested variables, we increased the number of controls to 160 (approximate rate of 1.5 controls to each case). To reduce selection bias, we matched cases and controls for residency, age, and gender. Distribution of cases and controls per district are illustrated in Fig. 1.

Cases comprised patients with brucellosis admitted to Fayoum fever hospital in Fayoum city. We defined a case by both clinical diagnosis of brucellosis and laboratory confirmation. Suspected cases with clinical presentation of brucellosis were first screened using a Rose Bengal test (RBT), which was conducted as described before [23]. The positive RBT individuals were then confirmed using a Standard Tube Agglutination Test (SAT). The SAT was conducted as described before [23] using the titer of ≥1:160 as a cutoff for a positive case. We defined a human control as being a close neighbor of the case (mainly the same street) for at least one year, had no history of brucellosis, and had no clinical criteria of brucellosis. The controls were also subjected to RBT and SAT screening, and only negative individuals were included in the study.

The study data were collected by interviewing participants using a pre-piloted structured questionnaire. The questionnaire included questions regarding socio-demographic characteristics such as residence, age, sex, education, occupation, and the family histories of brucellosis

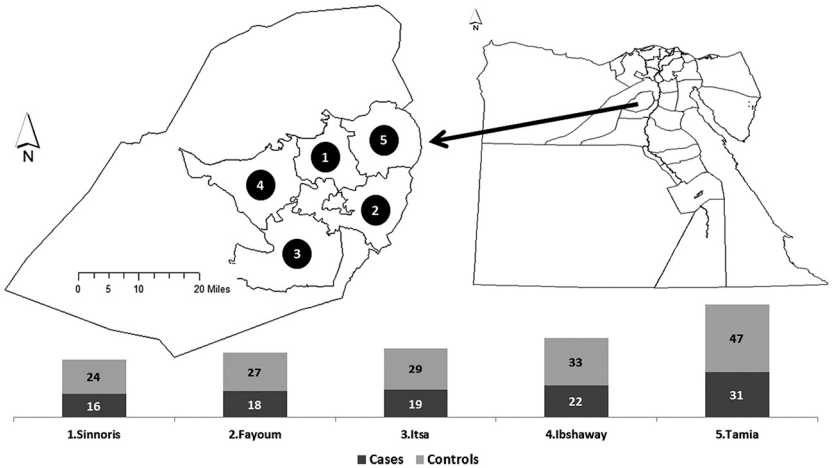


Fig. 1. Distribution of human cases and controls per district.

cases among households within the past six months. Furthermore, there were questions regarding animal contact and consumption of animal products. Finally, we recorded the data regarding the clinical presentation of the cases.

To assess the role of household cattle in direct transmission of *Brucella* pathogens to their owners, we examined all the household cattle owned by human cases and controls. Criteria for positive brucellosis in cattle included individual cattle being seropositive for both RBT and a complement fixation test (CFT). The CFT was conducted as described previously [24], and it was considered positive at a cutoff point of ≥20 ICFTU/ml.

2.2.2. Cattle Matched Case–Control Study

We conducted a cattle case-control study to define the predictors for cattle brucellosis among tested animals. All the tested household-kept cattle (regardless of their human owners who are not relevant in this analysis) were divided into cattle cases (seropositive for RBT and CFT) and cattle controls (seronegative for RBT and CFT) that were matched by the same geographical region. A total of 183 cattle were included in the case-control study. The number of cattle cases was 78 animals and the number of cattle controls was 105 animals with 1:1.3 case to control ratio. We recorded information regarding age, sex, number of cattle reared per household, and abortions during the last six months.

2.3. Case–Control Data Analysis

2.3.1. Human Case–Control Study Analysis

The differences in proportions of variables between cases and controls were tested using a Pearson chi square test. Socio-demographic variables such as education and occupation were analyzed as categorical variables. Education categories were illiterate (no education), Moderate (pre-university education), and high (university or equivalent education). Occupation categories were occupation with potential animal contact (included farmers, farm workers, butchers, and vets), and occupation with no animal contact (included employees, skilled workers, housewives, and unemployed). Notably, separation between housewives and unemployed did not alter the significance of these variables so they were combined in the no animal contact category.

We further conducted a multivariable regression model to define the risk factors associated with brucellosis case among humans. The multivariable regression analysis was conducted over three steps. First step: initial odds estimation by univariate logistic regression testing to all variables that showed $P < 0.2$ between cases and controls in chi-square test. We excluded the number of household cattle variable from next step as it was limited to cattle breeders. Second step: Testing the collinearity between each pair of variables using the phi coefficient and chi square. In the case of significant collinearity ($P < 0.05$) between a pair of variables, the most biologically plausible variable was selected for the final multivariable analysis. Third step: selection of significant variables and correction of odds ratio using multivariable logistic regression. A stepwise manual selection approach was used for the selection of variables with $P < 0.05$ in the final model and for exclusion of susceptible cofounders. Any variable that resulted in significant change in odds ratio when added to the model was considered cofounder and was excluded from the analysis.

2.3.2. Cattle Case–Control Study Analysis

We defined cattle brucellosis predictors using the multivariate regression model described before (2.3.1.). We excluded cattle gender variable, as males were not represented in positive cases.

2.4. Brucella Isolation, Identification, and Molecular Confirmation

For humans, we cultured the blood samples of all human brucellosis cases (106 cases) using a lysis concentration technique, as described previously [25]. For cattle, we collected 147 milk samples (65 from cases and 82 of controls, 50 ml per animal) under aseptic conditions. We centrifuged milk samples for 10 min at 6000 g and then used the cream layer both alone and mixed with sediment for cultivation as described before [23]. Identification of *Brucella* pathogens at the genus, species, and biovar levels was done as described before [23]. Molecular confirmation of *Brucella* species was conducted using species-specific primers (Metabion, Steinkirchen, Germany; Table S1) targeting the *IS711* gene (AMOS-PCR) as described previously [26]. We used the *B. melitensis* bv 3 reference strain Ether (ATCC 23458) as a positive control.

2.5. Molecular Genotyping of Detected Brucella melitensis Isolates

We performed molecular discrimination for the 12 detected *B. melitensis* isolates through *IS711* gene sequence based phylogenetic typing, virutyping, and Repetitive extragenic palindromic PCR (REP-PCR) typing.

2.5.1. IS711 Gene Sequencing and Phylogenetic Analysis

We purified the *IS711* PCR products from AMOS-PCR using a QIAquick gel extraction kit (Qiagen, Valencia, CA), and then carried out sequencing (both directions) using a Big dye Terminator v3.1 cycle sequencing kit (Applied Biosystems, Foster City, CA) in an Applied Biosystems 3130 genetic analyzer (Applied Biosystems) according to the manufacturer's protocol. We confirmed the nucleotide sequence identity using the BLAST 2.2 program (National Center for Biotechnology Information; NCBI). The accession numbers of the detected 12 isolates (F1–F12) are shown in Fig. 2. Multiple sequence alignment with other sequence homologies from GenBank was conducted using the ClustalW program v 1.83 [27]. The phylogenetic tree was constructed using the FastME [28], which is available on the website https://ngphylogeny.fr/.

2.5.2. Virutyping

Virutyping of detected isolates was based on variations in virulence patterns using six virulence genes: *virB*, *ure*, *omp25*, *amiC wbkA*, and *bvfA* (Supplementary Table S1).

2.5.3. REP-PCR Typing

We performed REP-PCR as described previously [10]. The REP-PCR based dendrogram was constructed using the Dice coefficient and the unweighted pair group method with arithmetic mean using GelJ software v.2.0 [29].

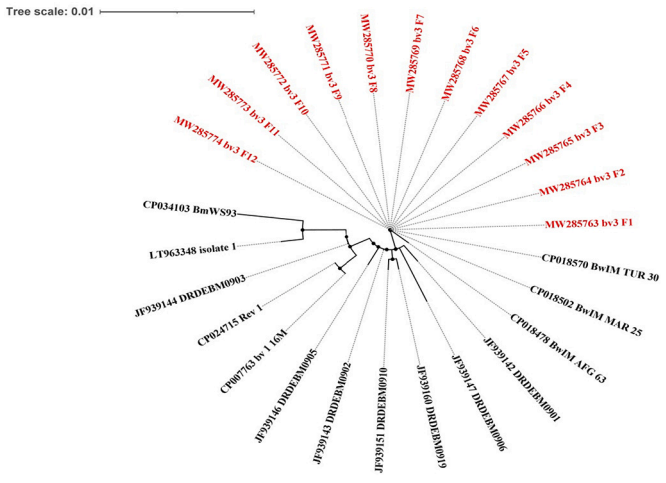


Fig. 2. Phylogenetic tree based on *IS711* gene sequence.
*Red color defines the GenBank accession numbers of the *B. melitensis* bv3 isolates recovered in this study. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

2.6. Statistical Analysis

Univariate regression, multivariate regression, and other statistical analysis were carried out using SPSS v19 (IBM, Armonk, NY), with significance considered at $P \leq 0.05$.

2.7. Ethical Approval

For human participant sampling, the study protocol and procedures were approved by the scientific research ethics committee, Faculty of Medicine, Fayoum University, Egypt (No. 158/79). Before sample collection, we obtained an informed consent from each participant after describing the research purpose. For cattle sampling, the protocol was approved by the research ethics committee for experimental and clinical studies, Animal Health Research Institute, Egypt (No. 165714).

3. Results

3.1. Characteristics of Human Brucellosis and Cattle Brucellosis Cases

The number of serologically confirmed human cases included in this study was 106. The socio-demographic characteristics of the cases and their cattle-related practices are shown in Table 1. The clinical manifestations of the cases were fever (100%), fatigue (88.7%), night sweating (80.2%), arthralgia (64.2%), headache (57.5%), spondylitis (55.7%), and meningitis (0.9%).

The seropositive cattle cases in this study were 78 (42.6%, 78/183). All seropositive cattle were owned by some of the human cases. All seropositive cattle were females, and most of them were over two years of age (85.9%, 67/78). Around one third of seropositive cattle were reared alone (35.9%, 28/78). Abortion was recorded in 9 of the seropositive cattle (11.5%, 9/78) and one of the seronegative cattle (0.9%, 1/105).

3.2. Risk Factors for Human Brucellosis in Study Region

3.2.1. Risk Factors Associated With Socio-Demographic Variables

The results of univariate logistic regression analysis revealed that higher odds of brucellosis were associated with individuals who were either illiterate (OR 4.3, $P < 0.001$), worked in an occupation related to animals (OR 5.03, $P < 0.001$), or had a household member infected with brucellosis within six months or less (OR 6.1, $P < 0.001$) (Table 2). Furthermore, family history of brucellosis was collinear with several risk practices including occupation, cattle breeding, and raw milk consumption (Phi coefficient 0.2–0.4, $P < 0.001$). Family history and occupation variables were significant risk factors for cases compared to controls in the final multivariable model (OR 2.1–3.2, P 0.02–0.007) (Table 2).

3.2.2. Risk Factors Associated With Animal Contact Practices

Household cattle rearing and exposure to abortion in cattle were significantly associated with brucellosis cases (OR 6.9–11.7, $P < 0.001$) in the univariate regression analysis (Table 2). In participants who kept cattle at home, the odds for brucellosis cases increased with the increase in the number of reared household cattle (OR 4.1–8.1, P 0.03–0.05). Abortion exposure was collinear with household cattle breeding (Phi coefficient 0.4, $P < 0.001$) as participants who kept cattle at home had greater exposure to cattle abortion than did others (45.7% in household cattle breeders vs. 10.2% in others). Risk of exposure to abortion exhibited the highest odds for brucellosis cases in the multivariable regression analysis (OR 6.9, $P < 0.001$) (Table 2).

3.2.3. Risk Factors Associated With Dairy Products Consumption

Drinking raw milk and consuming home-made soft cheese were significantly associated with brucellosis cases in univariate regression model (OR 2.3–4.4, P 0.005 – <0.001) (Table 2). However, raw milk consumption was not significant ($P > 0.05$) in multivariable analysis.

Table 1
Socio-demographic characteristics of the human participants and their animal related practices.

Variable		Category	Cases (N = 106)		Control (N = 160)		X^2	P
			No.	%	No.	%		
Socio-demographic	Residence	Rural	86	81.1	129	80.6	0.01	0.9
		Urban	20	18.9	31	19.4		
	Age	15–29	50	47.2	75	46.9	0.002	0.9
		30–44	31	29.2	47	29.4		
		≥45	25	23.6	38	23.8		
	Gender	Male	87	82.1	131	81.9	0.002	0.9
		Female	19	17.9	29	18.1		
	Marital state	Yes	78	73.6	128	80	1.5	0.2
		No	28	26.4	32	20		
	Education	Illiterate	49	46.2	36	22.5	19.3	<0.001
		Moderate	40	37.7	70	43.8		
		High	17	16.03	54	33.8		
	Occupation with animal contact	Yes	75	70.8	52	32.5	37.4	<0.001
		No	31	29.2	108	67.5		
	Family history	Yes	33	31.1	11	6.9	27.2	<0.001
		No	73	68.9	149	93.1		
Cattle-contact Practices	Household Cattle breeding	Yes	80	75.5	49	30.6	51.3	<0.001
		No	26	24.5	111	69.4		
	Number of Household Cattle[a]	1	55	51.9	45	28.1	9.5	0.009
		2	15	14.2	3	1.9		
		>2	10	9.4	1	0.6		
	Mixed breeding with small ruminant[a]	Yes	42	39.6	23	14.4	0.4	0.5
		No	38	35.8	26	16.3		
	Exposure to Abortion	Yes	58	54.7	15	9.4	65.8	<0.001
		No	48	45.3	145	90.6		
Dairy-products Consumption	Raw milk	Yes	26	24.5	11	6.9	16.6	<0.001
		No	80	75.5	149	93.1		
	Homemade Cheese	Yes	87	82.1	106	66.3	8.01	0.05
		No	19	17.9	54	33.8		
	Ice-cream	Yes	36	33.9	58	36.3	0.2	0.7
		No	70	66.04	102	63.8		

[a] This variable is limited to cattle breeder.

Table 2
Univariate and multivariate logistic regression analysis for risk factors for human brucellosis.

Variable		Category	Number		Univariate model		Multivariate model	
			Cs	Ct	*P*	Odds (95% CI)	*P*	Odds (95% CI)
Socio-demographic	Education	Illiterate	49	36	<0.001	4.3 (2.2–8.7)	EC	
		Moderate	40	70	0.08	1.8 (0.9–3.5)		
		High	17	54	–	–		
	Occupation with animal contact	Yes	75	52	<0.001	5.03 (2.9–8.6)	0.02	2.1 (1.1–4.1)
		No	31	108	–	–	–	–
	Family history	Yes	33	11	<0.001	6.1 (2.9–12.8)	0.007	3.2 (1.4–7.4)
		No	73	149	–	–	–	–
Cattle-contact Practices	Household Cattle breeding	Yes	80	49	<0.001	6.9 (3.9–12.2)	EC	
		No	26	111	–	–		
	Number of Household Cattle	˃2	10	1	0.05	8.1 (1.01–66.4)	NA	
		2	15	3	0.03	4.1 (1.1–15.02)		
		1	55	45	–	–		
	Exposure to Abortion	Yes	58	15	<0.001	11.7 (6.1–22.5)	<0.001	6.9 (3.4–14.4)
		No	48	145	–	–	–	–
Dairy-products Consumption	Raw milk	Yes	26	11	<0.001	4.4 (2.1–9.4)	NS	
		No	80	149	–	–		
	Homemade Cheese	Yes	87	106	0.005	2.3 (1.3–4.2)	0.03	2.3 (1.1–4.6)
		No	19	54	–	–	–	–

EC: Excluded Confounder; NS: Not significant; NA: Not applicable (because this variable limited to Cattle breeder).

Soft cheese consumption was 2.3 times more likely associated with brucellosis cases than controls in multivariable model (*P* 0.03) (Table 2). Of note, consumption of raw milk habit was more common among individuals who reared cattle at home (22.5% in cattle breeders vs. 5.8% in others; Phi coefficient 0.2, $P < 0.001$). In contrast, soft cheese consumption was not associated with household cattle breeding (77.5% in cattle breeders vs. 67.9% in others; Phi coefficient 0.1, *P* 0.08).

3.3. Risk Factors for Cattle Brucellosis in Study Region

The multivariate regression analysis showed that higher odds for cattle brucellosis were associated with mature cattle ≥2 years old (OR 2.9, *P* 0.01), two or more animals reared in the same household (OR 3.7–6.9, $P \leq 0.001$) and recent abortion (OR 15.2, *P* 0.01) (Table 3). Of note, soft cheese consumption was not associated with household cattle breeding (77.5% in cattle breeders vs. 67.9% in others; Phi coefficient 0.1, *P* 0.08).

3.4. Isolation and Molecular Genotyping of Detected Brucella Pathogens

We recovered 12 *Brucella* isolates from blood samples of eight human cases (7.5%, 8/106) and milk samples of four cattle cases (6.2%, 4/65) (Fig. 3). Three of the bacteriologically positive participants owned bacteriologically positive cattle at home (ID F1–F6, Fig. 3). Another two of these human cases owned cattle that were serologically positive but bacteriologically negative (ID F8 and F10). One human case was bacteriologically negative but owned a bacteriologically positive cow (ID F9). The remaining two bacteriologically positive cases did not keep cattle at home (ID F7 and F12).

We confirmed all detected isolates in humans and cattle (n = 12) as *B. melitensis* bv 3 by phenotypic characterization and molecular (AMOS-PCR) confirmation tests.

Nucleotide sequence analysis and phylogenetic tree construction of *IS711* genes of detected *B. melitensis* isolates showed complete homology (100% identity match) between all of them (Fig. 2). Moreover, the detected isolates matched isolates recovered from humans in Turkey (CP018570BwIM_TUR-30), and in Morocco (CP018502BwIM_MAR_25). However, there were differences between our isolates and other isolates from Afghanistan (CP018478BwIM_AFG_63; 99.9% identity), Europe (LT963348; 99% identity), and China (CP034103BmWS93; 89.9% identity).

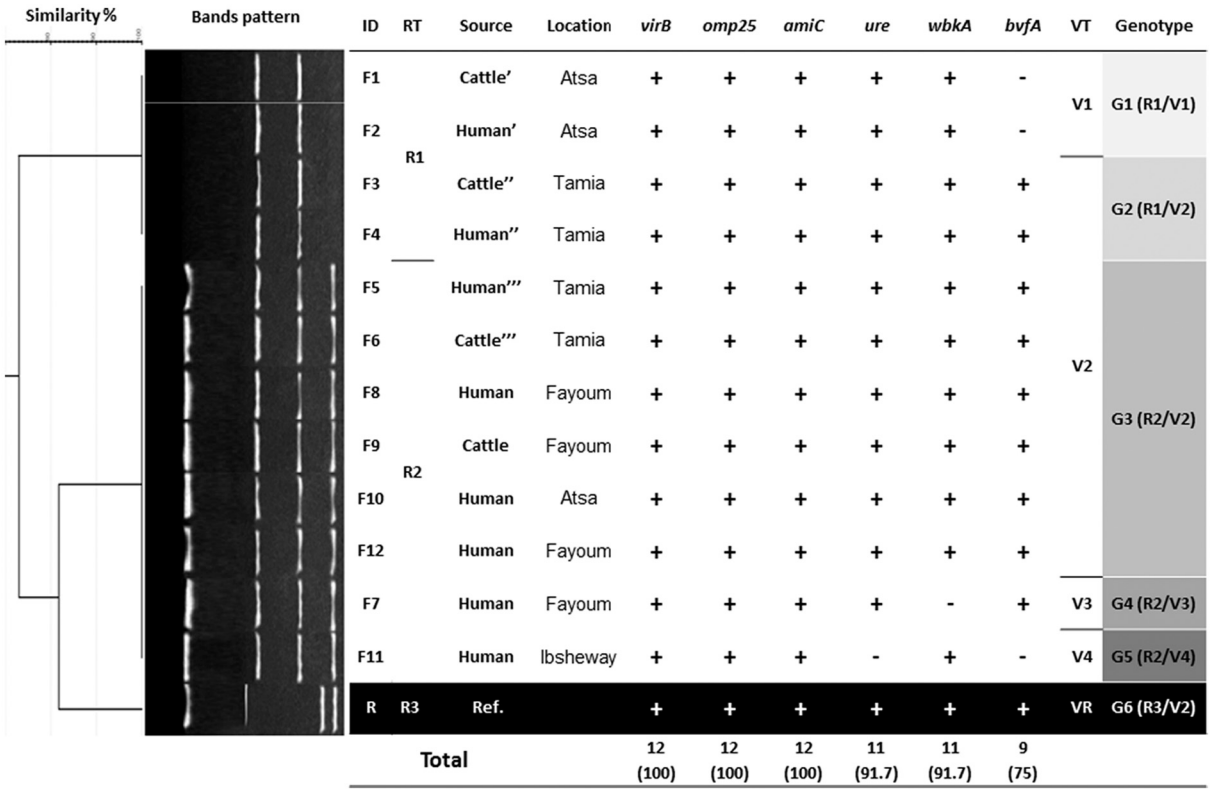
Molecular detection of virulence genes showed that the *virB*, *omp25*, and *amiC* genes were present in all isolates (100%, 12/12). The *ure*, *wbkA*, and *bvfA* genes were detected in 91.7%, 91.7%, and 75% of isolates, respectively (Fig. 3). The detected isolates showed four virulence patterns (V1–V4, Fig. 3); the most prevalent pattern was V2 (8/12, 66.7%), which contained all examined virulence genes (100% per each).

REP-PCR typing of detected isolates revealed two REP types (R1 and R2). Genotyping of detected isolates by combined virulence profiles and REP-PCR types revealed five different genotypes (G1–G5, Fig. 3). These genotypes differed from the reference strain *B. melitensis* Ether bv 3 (G6: R3/V2). Importantly, cattle and human isolates from the same households showed a matched genotype, and these matched cattle/human cases belonged to three genotypes (G1–G3). The most prevalent genotype was G3, which was distributed in cases (humans and cattle) in three out of four positive districts (Fig. 3).

Table 3
Univariate and multivariate logistic regression analysis for risk factors for Cattle brucellosis.

Variable	Category	Number		Univariate model		Multivariate model	
		Cs	Ct	*P*	Odds (95% CI)	*P*	Odds (95% CI)
Age	≥2 y	67	73	0.01	2.7 (1.2–5.7)	0.01	2.9 (1.3–6.8)
	9 m–˂2 y	11	32	–	–	–	–
Household animal number	>2	25	23	0.005	2.8 (1.3–5.6)	0.001	3.7 (1.7–8.1)
	2	25	11	<0.001	5.8 (2.5–13.3)	<0.001	6.9 (2.9–16.6)
	1	28	71	–	–	–	–
Mixed breeding with small ruminant	Yes	45	48	0.1	1.6 (0.9–2.9)	NS	
	No	33	57	–	–		
Abortion	Yes	9	1	0.01	13.6 (1.7–109.5)	0.01	15.2 (1.8–129.6)
	No	69	104	–	–	–	–

m: Months; y: Years; NS: Not significant variable.



ID	RT	Source	Location	*virB*	*omp25*	*amiC*	*ure*	*wbkA*	*bvfA*	VT	Genotype
F1	R1	Cattle'	Atsa	+	+	+	+	+	-	V1	G1 (R1/V1)
F2		Human'	Atsa	+	+	+	+	+	-		
F3		Cattle''	Tamia	+	+	+	+	+	+	V2	G2 (R1/V2)
F4		Human''	Tamia	+	+	+	+	+	+		
F5		Human'''	Tamia	+	+	+	+	+	+		G3 (R2/V2)
F6		Cattle'''	Tamia	+	+	+	+	+	+		
F8	R2	Human	Fayoum	+	+	+	+	+	+		
F9		Cattle	Fayoum	+	+	+	+	+	+		
F10		Human	Atsa	+	+	+	+	+	+		
F12		Human	Fayoum	+	+	+	+	+	+		
F7		Human	Fayoum	+	+	+	+	-	+	V3	G4 (R2/V3)
F11		Human	Ibsheway	+	+	+	-	+	-	V4	G5 (R2/V4)
R	R3	Ref.		+	+	+	+	+	+	VR	G6 (R3/V2)
Total				12 (100)	12 (100)	12 (100)	11 (91.7)	11 (91.7)	9 (75)		

Fig. 3. Genotyping of *B. melitensis* bv3 isolated from human and cattle detected in this study.
',","': highlight isolates from cattle and human in same household; RT: REP type; VT: Virutypes; Ref.: Reference strain *(B. melitensis bv 3 strain* Ether); VR: reference strain virutype.

4. Discussion

4.1. Risk Factors for Human Brucellosis

4.1.1. Risk Factors Associated With Socio-Demographic Variables

The results of univariate regression analysis revealed that socio-demographic predictors of brucellosis were illiteracy, animal-related occupations, and previous infection in other household members within less than six months.

Illiteracy has been shown to be a risk factor for human brucellosis in Yemen [5]. Conversely, a study performed in the northern region of Egypt reported no significant association between education and brucellosis [20]; however the rate of illiteracy among participants (26.7%) in that study was lower than in our study (31.9%). This indicates that differences in socio-demographic variables between the northern and southern regions of Egypt may alter the nature and magnitude of different risk factors. Lack of education may reflect lack of awareness of protective measures against diseases such as brucellosis, thereby exposing these individuals to a high-risk of infection.

Animal-related occupations have been associated with brucellosis in Yemen [5], and Georgia [6], but they have not been found to be a risk factor in Iran [30]. Brucellosis is an occupational disease, and people in animal-related occupations such as farming, butchering or veterinary medicine experience more exposure to brucellosis than do others [31]. There may be regional variation in the nature and frequency of animal-related occupations because of differences in culture, education, market needs, and other factors. This may account for the difference in the effect of this variable between different countries.

Similar to our findings, the presence of another brucellosis case in the same household has been a predictor of brucellosis in previous studies in Egypt [20] and elsewhere [30]. Family history of brucellosis was collinear with several risk practices as cattle breeding, raw milk consumption, and occupation in this study. In Egypt, many members of the same family, especially in rural dwellings, share customs, feeding habits, and practices such as animal husbandry, and sometimes occupation such as farming. These shared high-risk practices justify multiple members' exposure to infection in same household.

4.1.2. Risk Factors Associated With Animal Contact Practices

We have found that keeping cattle at home was significantly associated with brucellosis cases in the univariate regression model. Cattle are important source of zoonotic brucellosis infection for humans in Egypt and worldwide [17,30,32]. In agreement with our findings, keeping cattle either alone or with other animals at home has been shown to be a risk factor for brucellosis in other studies [20,30].

In this study, abortion exposure risk exhibited the highest odds for brucellosis in multivariate analysis. Similar finding was recorded in other studies in Egypt and elsewhere [20,32]. High loads of *Brucella* pathogens are shed in vaginal discharge and afterbirth materials (10^{13}/g of tissue) during abortion [1], and individuals in contact with these materials are at high-risk. All cases that exposed to abortion did not use protective equipment. This agreed with a previous study [32], which recorded bare hand birth-aid as a risk factor for infection.

4.1.3. Risk Factors Associated With Dairy Products Consumption

In this study, raw milk and home-made cheese were the dairy food products associated with brucellosis (OR 2.3–4.4, P 0.005–<0.001). Consuming raw milk has been shown to be a risk factor for brucellosis in several countries [5,30,32]. However, the findings of studies from several governorates in the northern region of Egypt indicate that this practice is negligible because of the high awareness of residents in this region about the risks of consuming raw milk [7,19]. Fayoum is one of the five lowest ranking governorates in terms of the human development index in Egypt, and it has high rates of poverty and illiteracy [22]. The

difference in the levels of development between southern and northern governorates in Egypt contributes to differences in traditional beliefs and customs. Similarly, it was evident that differences in customs and traditions alter the nature and frequencies of brucellosis-associated risk factors between different regions in the same country [6]. Thus, it is important to define regional differences in risk factors for effective and specific design of control plans to be applied nationwide.

Consumption of home-made cheese was a predictor of brucellosis both in this study and in others [6,20]. This practice was not associated with keeping cattle at home (77.5% in cattle breeders vs. 67.9% in others; *P* 0.08) as many participants, especially those in rural dwellings, could buy home-made cheese form informal markets in Egypt.

4.2. Risk Factors for Cattle Brucellosis

The predictors for cattle brucellosis were cattle ≥2 years old, multiple animals reared in the same household, and cattle that had recent abortions. These results are comparable with studies in several countries [32–34]. Abortion is the main source for transmission of infection in animal populations as *Brucella* pathogens are shed in large quantities during abortion, and they can survive for long time in the environment [1,33]. Such contaminated environment may pose risk for reinfection of aborted animals or other animals in same household.

4.3. Isolation and Molecular Genotyping of Detected Brucella Pathogens

In this study, we isolated *B. melitensis* bv 3 from 7.5% and 6.2% of the human cases and cattle cases, respectively. In agreement, *Brucella* was isolated from 7.9% of human cases in Yemen [5]. The *B. melitensis* bv 3 is the most predominate species in Egypt [13,14] and the Middle East [3]. It is also the species most often isolated from cows in Egypt [9,13].

Molecular genotyping of detected isolates was conducted using several techniques. Sequence analysis of the *IS711* gene showed homologies with isolates from the Middle East [8]. Nevertheless, this approach failed to discriminate between the detected isolates themselves. Virutyping is based on the fact that *Brucella* pathogens do not produce plasmids and that their virulence genes are carried on chromosomes [2]. Hence, differences in the existence/absence of these genes not only define virulence traits of the isolates but can also be used as genetic markers for these isolates. The recovered isolates in this study recorded four virulence patterns. Differences in the distribution of virulence genes between different isolates of *B. melitensis* have been reported in Egypt and elsewhere [35,36].

For greater discrimination between isolates, we combined REP-PCR typing and virulence profiles. Combined REP-PCR/virulence genotyping revealed five different genotypes (G1–G5) for the detected isolates and a unique genotype for the reference strain (G6). This finding highlights that using a combination of cost-effective genotyping procedures enhance the discrimination efficiency of these tools and may therefore overcome the limitations of individual use of such procedures as previously described [10].

The G3 genotype predominated among the isolates (50%), and it was distributed in three districts (Tamia, Fayoum, and Atsa). This genotype was also closely related to G4 and G5 genotypes (one or two virulence genes' difference). These results highlight the high degree of similarity of detected isolates that may indicate the circulation of the same or closely related strains of *B. melitensis* bv3 in the study region. Similarly, previous studies from Egypt showed high genetic similarity between *B. melitensis* bv3 isolates in several governorates [9,14]. This similarity of *B. melitensis* genotypes between different districts in Fayoum governorate may be attributed to free movement of animals between different districts. Free animal movement has been reported to contribute to the distribution of genetically similar strains within the same governorate and between close governorates in Egypt [9].

In our study, isolates from cattle and human cases in the same household had matched genotypes which provide a potential genetic evidence of zoonotic transmission of *B. melitensis* from cattle to human within the same household. This is line with the risk analysis data from both this study and elsewhere [30], which showed that rearing cattle at a home is a risk factor for brucellosis.

4.4. Limitations

We were not able to conduct MLVA or WGS-SNP typing due to limited funding resources. These advanced methods would allow comparison with previously published genotypes from other governorates in Egypt. Also, these techniques would help to assess the validity of our suggested combined REP-PCR/virulence genotyping approach in tracing and discrimination of *Brucella* isolates. Future studies will be addressing these limitations.

5. Conclusions

This study recorded widespread risk factors and practices among studied human cases. Some risk factors in study region (e.g. illiteracy and raw milk consumption) were different from other localities in Egypt, which mandate recognition of region-specific risk factors for effective planning of education campaigns and designing comprehensive national-wide prevention strategy. The priority of health education campaigns should be directed toward high-risk regions (as Fayoum governorate) and high-risk population (as workers in animal-related occupations). These campaigns should involve academic, veterinary, and community health extension specialists as well as the target community. Partnership with the community through trained volunteers and sponsors would allow additional resources and increases the reach to stakeholders. The combined REP-PCR/virulence genotyping is based on standard PCR protocols that are affordable and applicable in many developing countries including Egypt. Thus, it is a cost-effective approach that can be used for initial genotyping and tracing of the *Brucella* isolates especially in developing countries that lack lab facilities and resources needed for other advanced typing tools as WGS-SNP or MLVA.

Authors' contributions

Conceptualization: WE, NHA, HMG, EME; **Investigation:** NHA, SFN, SKH; **Methodology:** NHA, HMG, EME, EIB, RII, SFN, WE; **Data analysis:** WE, SKH; **Visualization:** NHA, WE; **Writing - original draft:** WE; **Writing - review & editing:** NHA, HMG, EME, EIB, RII, SFN, SKH, WE.

Declaration of Competing Interest

None.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.onehlt.2021.100281.

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