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Brucellosis Seroprevalence, REP-PCR-Based Genotyping, and Virulence-Associated Genes Distribution Among *Brucella melitensis* Strains Isolated from Ruminants in Kafr Elsheikh Governorate of Egypt

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Brucellosis is a neglected bacterial zoonosis that has a significant impact on public health and the livestock industry. The purpose of this study was to determine the brucellosis seroprevalence, the circulating *Brucella* species, *Brucella* virulence-associated genes distribution, and repetitive extragenic palindromic PCR (REP-PCR) genotyping of *Brucella* isolates among ruminants in the Kafr Elsheikh Governorate. Blood samples were randomly collected from 926 cattle, 422 buffaloes, 301 sheep, and 333 goats in Kafr Elsheikh from January 2021 to December 2022. We determined apparent and true brucellosis prevalence in ruminants in various Kafr Elsheikh locations. Isolation and molecular confirmation (AMOS-PCR), phylogeny based on REP-PCR *Brucella* genotyping was inferred. Virutyping was performed on *B. melitensis* differences in virulence patterns. Brucellosis's true prevalence was calculated to be 13.3%, 11.2%, 15.8%, and 16.8% in cattle, buffaloes, sheep, and goats. *Brucella* isolates (n=8) were recovered from the supramammary lymph nodes and confirmed molecularly (AMOS-PCR) and bacteriologically as *B. melitensis* biovar 3. The *B. melitensis* isolates showed three virulence patterns (V1-V3), with V1 being the most common pattern (62.5%). REP-PCR clearly discriminated between the detected *B. melitensis* bv3 isolates into two clades (C1 and C2) and 7 REP genotypes (G1 – G7). In conclusion, the *B. melitensis* bv3 high diversity genotypes based on REP-PCR patterns highlight the cost-effective discrimination efficiency of the reproducible REP-PCR. *B. melitensis's* consistent isolation at low rates across the year of sampling may suggest various previous outbreaks of established infection rather than a recent introduction of foreign strains in the Governorate.

Keywords: *Brucella*, prevalence, Rep-PCR, virulence genes, virutype.

Introduction

Brucellosis is a bacterial zoonotic disease, transmitted from various animal species to humans and is a worldwide significant public health problem that shows a high incidence rate in developing countries due to the lack of control strategies. This disease is considered the most widespread zoonoses transmitted by animals and remains as a neglected disease in developing countries [1].

In most of the developing world, brucellosis is an endemic zoonotic disease that has severe effects on the cattle industry and small-scale livestock owners.

Clinical symptoms of brucellosis in infected animals include infertility, abortion, stillbirth, arthritis, retained placenta, and a significant drop in milk output. Brucellosis is manifested with a variety of nonspecific signs in humans [1,2]. The respiratory tract, gastrointestinal tract, conjunctiva, broken skin, and any mucous membrane that comes into contact with contaminated fluids or tissues are further entry routes for *Brucella*. In Egypt, *B. melitensis* is the most prevalent *Brucella* species in ruminants [3] followed by *B. abortus* and less frequently by *B. suis* biovar 1 and 2 [4,5]. Recently, *B. canis* DNA was detected in the blood of stray and owned dogs in Egypt [6].

Cross-sectional studies were conducted to finger out the seroprevalence of ruminants' brucellosis in Kafr Elsheikh Governorate, Egypt. Brucellosis seroprevalence was estimated to be 12.2% and 11.3% in sheep and goats [7]. Additionally, 12.2% and 12% of cow and buffalo milk tanks tested positive for *Brucella* spp [7]. In another study conducted by Selim *et al.* [8], the brucellosis incidence rate was estimated to be 12%, 8.4%, 6.4%, and 6.1% in sheep, cattle, goats, and buffaloes in Kafr Elsheikh Governorate. Another study found that 95.5% of villages had at least one brucellosis-positive sheep in the Kafrelsheikh district, where the seroprevalence in sheep was estimated to be 20% [9]. Elmonir *et al.* [10] estimated the prevalence of brucellosis in cattle at 7% in the Kafr Elsheikh district.

All members of the *Brucella* genus exhibit a high degree of homology and are considered to be closely linked immunologically and genetically. However, it has different virulence factors that cause severe pathogenicity [11]. *Brucella* uses several stealth tactics to evade the host immune mechanisms, invade host cells, and multiply inside [12].

Virulence of *Brucella* spp. is mainly due to its ability to escape and modulate the host immune response spread to the predilection site through cellular tropism, and survive intracellularly in host cells [13]. As a result of the host's reticuloendothelial cells being attacked by *Brucella*, an infection occurred. Once *Brucella* spp. invade the host cells, and they are capable with the support of virulence factors to survive within phagocytic cells [14]. The survival and intracellular adaption of *Brucella* species in host cells, as well as their resistance to the body's immunological response, depend on virulence genes [15].

[7]. One shepherd would frequently be comingling sheep belonging to multiple owners, as a result, most of the year, animals from households are brought together for grazing and breeding within the same flock. Local village flocks are sometimes joined to form a large flock managed by several shepherds. Since there are no constraints on how animals can

The REP-PCR method is based on primers created for repeating extragenic palindromic sequences found in bacterial genomes. Extragenic placement and well-preserved repeated reverse sequences are two of its characteristics. This sequence has many copies that are organized into intricate clusters and has high repeatability. This method is straightforward and advantageous as it is not necessary to know the genome's sequence, and DNA can be used in place of a bacterial culture. Additionally, it uses bacterial DNA as a matric and subsequentally lessens the possibility of lab acquired infection [16].

REP-PCR, based on repetitive extragenic palindromic sequences, is used in molecular genotyping of microorganisms such as *Brucella* and for the discrimination of *B. abortus* and *B. melitensis* [17]. This fingerprinting-based PCR technique has the advantages of simplicity, rapidity and stability. Compared with genomic fingerprinting and plasmid profiling, REP-PCR shows better discriminatory power [17,18]. Furthermore, this technique could be a useful tool to seek the genetic diversity among *Brucella* isolates and studying brucellosis epidemiology [19].

This study aims to estimate the ruminant brucellosis seroprevalence in the Kafr Elsheikh Governorate. Determine which *Brucella* species are circulating among ruminants in the study area. Furthermore, to study some virulence-associated genes distribution in different *Brucella* strains among examined animals in the study area. Also, molecular genotyping of recovered isolates to assess their genetic diversity using repetitive extragenic palindromic PCR (REP-PCR).

Material and Methods

A *Study area*

A significant province in northern Egypt, Kafr Elsheikh is located in the Nile Delta along the Nile western bank. It is located at latitude 31.308544 and longitude 30.803947. It shares boundaries with the Mediterranean Sea to the north, the Dakahlia Governorate to the east, and the Gharbia Governorate to the south [20]. There are 11 cities in Kafr el-Sheikh, including the capital city.

Kafr Elsheikh is an agricultural governorate with a substantial animal and human population. Sheep are farmed in either small flocks kept by farmers in their homes or flocks managed by shepherds in villages move, the flocks are raised in a free-range system where the animals can graze anywhere, they like [21]. Other ruminant species, such as cattle, buffaloes, or goats, could be included in these flocks. Cattle and buffaloes, on the other hand, are raised as domestic animals, either on farms or in movable herds comparable to sheep [4,21].

Animals' sampling and estimation of sample size

Using the online Win Epi tool, the minimum sample size was determined to be 386 animals. The sample size calculation required a population size of 292000, a confidence level of 95%, an accepted error of 5%, and an expected prevalence of 20% [22]. Using a design effect (DEFF) of 2 [7], the target sample size became 772 household ruminates.

Increasing the accuracy of the statistical analysis required sample size modification to be 1982 blood samples. Between January 2021 to December 2022, 1982 blood samples were collected randomely from cattle, buffaloes, sheep, and goats in the Kafr Elsheikh district, Bila, El Reyad, Quilline, and Desouqe of the Kafr Elsheikh Governorate. The examined household animals owned by small farmers were explained in Table (1).

TABLE 1. Blood Samples collected from different localities in the Kafr Elsheikh Governorate

| Locality | Animal species | | | | Total |
	Cattles	Buffaloes	Sheep	Goats	
Kafr Elsheikh	180	78	58	65	381
Bila	184	82	61	64	391
El Reyad	181	85	60	66	392
Quilline	196	92	63	70	421
Desouqe	185	85	59	68	397
Total	926	422	301	333	1982

The apparent prevalence (AP) and the true prevalence estimation of ruminants' brucellosis in Kafr Elsheikh

The apparent prevalence (AP) of brucellosis among household ruminants was estimated by dividing the total number of ruminants that tested positive for RBT and CFT by the total number of ruminants investigated [9]. The combined sensitivities (Se) and specificities (Sp) required for true prevalence calculation, were estimated through Win Epi online tool [23]. According to the sensitivities and the specificities recorded by Pfukenyi *et al.* [24] , the in-series combined sensitivities and specificities of both the RBT and CFT were calculated to be 81% and 99%. The true prevalence of brucellosis was determined according to Habibzadeh *et al.* [25].

Blood was obtained from the jugular veins of the studied animals and stored in the refrigerator overnight for serum separation before being centrifuged at 900 × g for 10 minutes. Clear sera were separated, divided into aliquots, and kept at -20^{0}C until used for serological testing.

Serological assessments

According to the lab-applied protocol, we tested serum samples from the studied animals using the buffered acidified plate antigen test (presumptive test), the rose bengal test (RBT) as screening tests, and the complement fixation test (CFT) as a confirmatory test [26]. Serum samples are considered positive if they give positive reactions to both RBT and CFT (series interpretation).

Isolation and bacteriological typing of Brucella species

At the slaughterhouses, supramammary lymph nodes collected from slaughtered seropositive cattle (n=14), buffalos (n=16), sheep (n=1), and goats (n=7) were sent to the Brucellosis Research Department, AHRI for *Brucella* isolation and typing. Also, for *Brucella* isolation, livers and spleens were obtained from 21, 18, 26, and 29 seropositive slaughtered cattle, buffalos, sheep, and goats, respectively. Those samples were transferred immediately to the laboratory for *Brucella* isolation and typing. Typing of *Brucella* isolates at the genus level was performed based on colony morphology, catalase, oxidase and urease. Agglutination with acriflavine was done to test the *Brucella* isolates for the dissociation. Lysis by phages (Tb, Wb, and Iz) were used to identify the species of the *Brucella* isolates. The biovar of the identified *Brucella* species was done based upon the requirement of CO_2, H_2S production, growth in the presence of thionin and fuchsine dyes (20 µg/ml), and Agglutination with monospecific sera (A, M). *Brucella* isolation and typing to the biovar level were done according to [26, 27].

Molecular confirmation of Brucella isolates and Polymerase Chain Reaction (PCR) based virulence-associated genes

For species identification, AMOS PCR [28]. DNA extraction and purification from *Brucella* culture were carried out using oligonucleotide primers as described by Bricker and Halling [28]. The primers sequences used for the virulence-associated genes and the cyclic conditions are given

in Table (2) and were provided by Creative Biogene (USA).

Amplification of PCR Primers was used in a 20-µl reaction that contained 10-µl of MyTaqTM HS Red Mix (Meridian Bioscience, USA), 1 µl of each primer at a concentration of 20 pmol, 3 µl of water, and 5 µl of DNA template. The reaction was carried out in a T3000 thermocycler (Biometra Gmbh, Germany).

The PCR products were separated by electrophoresis on 1% agarose gel (Compact XS/S, Biometra Gmbh, Germany) in 1x TBE buffer at room temperature with gradients of 5V/cm. For the gel analysis, 8 µl of the products were deposited in each gel slot. We used A DNA ladder H3 RTU (Genedirex, Taiwan), a gene ruler 100 bp ladder (Fermentas, Germany), and a gel pilot 100 bp plus DNA ladder (Qiagen, Gmbh, Germany) to measure fragment sizes. The amplified DNA products in agarose gel were seen with a UV transilluminator after gel staining with ethidium bromide dye. The gel documentation system (BioRad's ChemiDoc Imaging System) was employed to photographe the gel. Sterile DNA-free water was employed as a negative control, and *B. melitensis* biovar 1 reference strain 16M (ATCC 23457) was used as a positive control.

TABLE 2.PCR primers sequences, target genes, amplicon sizes and cycling conditions of virulence-associated genes.

Target gene	Sequence	Amplified product	Primary denaturation	Secondary denaturation	Annealing	Extension	No. of cycles	Final extension	Reference
bvfA	ACCCTTCGTCGATGTGCTGA	1282 bp	95℃ 5 min.	95℃ 1 min.	65℃ 1 min.	72℃ 1.3 min	35	72℃	Derakhshandeh *et al.*, 2013
	CCGCGCTGATTTCATCG								
ure	GCTTGCCCTTGAATTCCTTTG	2100 bp	95℃ 5 min.	95℃ 1 min.	65℃ 1 min.	72℃ 1.3 min	35	72℃	
	ATCTGCGAATTTGCCGGACTC								
wbkA	AATGACTTCCGCTGCCATAG	931 bp	94℃ 5 min.	94℃ 30 sec.	60℃ 40 sec.	72℃ 50 sec.	35	72℃ 10 min.	Awwad *et al.*, 2015
	ATGAGCGAGGACATGAGCTT								
omp25	TTT CCG TGT CCA ATT ATG CTA	701 bp	94℃ 5 min.	94℃ 30 sec.	60℃ 40 sec.	72℃ 45 sec.	35	72℃ 10 min.	
	ACCGCGCAAAACGTAATTT								

Virutyping

Based on variations in virulence patterns, four virulence genes—*ure*, *omp25*, *wbkA*, and *bvfA*—were used to identify the *Brucella* recovered isolates.

Molecular genotyping of detected B. melitensis bv3 isolates

The repetitive extragenic palindromic PCR (REP-PCR) was conducted for the genetic discrimination and genotyping of the eight recovered *B. melitensis* isolates as previously described [18]. The REP-PCR dendrogram was created using the Jaccard coefficient and the unweighted pair group method with arithmetic mean with the aid of GelJ software v.2.3 [29]. Simpson's diversity index for discrimination efficiency of REP-PCR genotyping of detected *B. melitensis* isolates was conducted as previously described [30].

Results

Between January 2021 to December 2022, a cross-sectional study was done to investigate the seroprevalence of brucellosis among small and large ruminants in different localities in Kafr Elsheikh governorates. The apparent prevalence was estimated in five localities of the Kafr Elsheikh governorate. These localities are Kafr Elsheikh district, Bila, Quilline, and Desouqe in the southern part of the

governorate and El Reyad in the middle of the governorate. The highest estimated seroprevalence figures (Table 3; Fig. 1) in these localities were recorded in Quilline across all the species tested in this study. The estimated prevalence was 16.3%, 12%, 17.5%, and 18.6% in cattle, buffaloes, sheep, and goats. The estimated prevalence in Desouqe, El Reyad, Bila, and Kafrelsheikh districts was 11.9%, 10.5%, 8.2%, and 11.1% in cattle, 10.6%, 7.1%%, 9.8%, and 10.3% in buffaloes, 13.6%, 13.3%, 11.5%, and 12.1% in sheep, and 14.7%, 13.6%, 10.9% and 13.8% in goats. Remarkably, the seroprevalence of brucellosis was higher in small ruminants than in large ruminants, as demonstrated in Table (3).

TABLE 3. Apparent prevalence estimation brucellosis among ruminants in different localities of Kafr Elsheikh Governorate

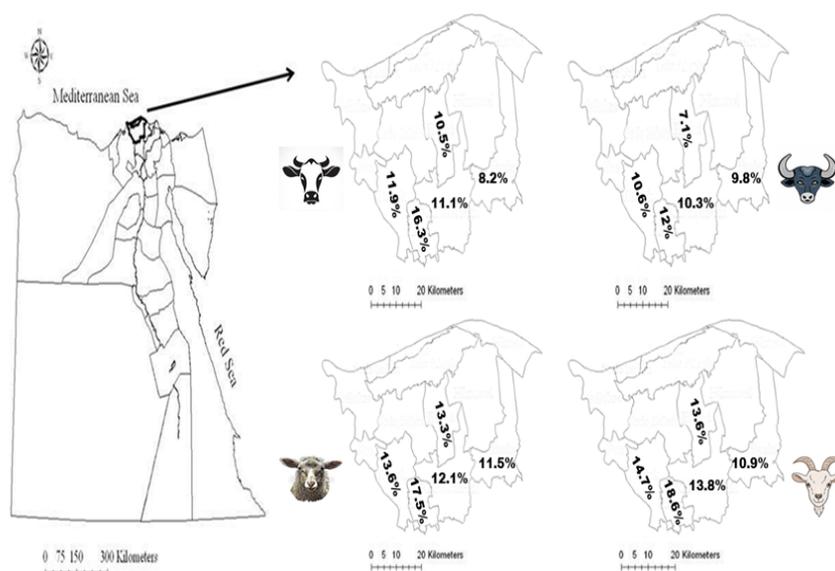| Locality		Animal species			
		Cattles	Buffaloes	Sheep	Goats
Kafrelsheikh district	Apparent prevalence	20/180 (11.1%)	8/78 (10.3%)	7/58 (12.1%)	9/65 (13.8%)
Bila		15/184 (8.2%)	8/82 (9.8%)	7/61 (11.5%)	7/64 (10.9%)
El Reyad		19/181 (10.5%)	6/85 (7.1%)	8/60 (13.3%)	9/66 (13.6%)
Quilline		32/196 (16.3%)	11/92 (12%)	11/63 (17.5%)	13/70 (18.6%)
Desouqe		22/185 (11.9%)	9/85 (10.6%)	8/59 (13.6%)	10/68 (14.7%)

Fig. 1. Brucellosis apparent prevalence estimates among small and large ruminants in five localities of Kafr Elsheikh Governorate.

The apparent prevalence of brucellosis was estimated using both RBT and CFT (Table 4) to be 11.66% (108/926) and 9.95% (42/422), 13.62% (41/301), and 14.41 (48/333) in examined cattle, buffaloes, sheep, and goats respectively. Brucellosis's true prevalence was calculated in the study area after incorporating the error due to inperfect sensitivities and specificities of both tests, to be 13.3%, 11.2%, 15.8%, and 16.8% in cattle, buffaloes, sheep, and goats.

TABLE 4. Bacteriological examination, overall apparent, and true seroprevalence estimation of ruminants' brucellosis in Kafr Elsheikh Governorate

Test Species	Apparent prevalence (AP)		True prevalence %	*Brucella* isolates
	No	%	(AP + Sp-1)/(Se + Sp-1)	
Cattle	108/926	11.66	13.3	3/14
Buffaloes	42/422	9.95	11.2	2/16
Sheep	41/301	13.62	15.8	1/8
Goats	48/333	14.41	16.8	2/7

*All *Brucella* isolates were identified bacteriologically at genus, species, and biovar level as *B. melitensis* bv 3

Eight *Brucella* isolates were recovered from the supramammary lymph nodes of the slaughtered serologically positive cattle (n=3), buffaloes (n=2), sheep (n=1), and goats (n=2). Molecular confirmation using AMOS-PCR that gave specific bands at 731 bp and the bacteriological-full typing of *Brucella* at genus, species, and biovar levels indicated that all isolates are *B. melitensis* biovar 3 (Table 5).

Molecular detection of virulence-associated genes showed that the *wbkA and bvfA* genes existed in all isolates (100%, 8/8). We detected the *ure* and *omp25* genes in 87.5% and 75% of the *Brucella* isolates, respectively (Table 5). Molecular identification of virulence genes in the recovered eight *B. melitensis* isolates revealed three virulence patterns (V1-V3, Table 5), with V1 (5/8, 62.5%) containing all assessed virulence genes.

The REP-PCR genotyping differentiated the *B. melitensis* isolates in two distinct phylogenetic branches (REP-I and REP-II) that discriminated the *B. melitensis* bv3 isolates (REP-I) from *B. melitensis* bv1 Reference strain and *B. melitensis* isolates number 1-3 (REP-II) with less than 70% similarity between the two biovars' branches (Fig. 2). In the *B. melitensis* bv3 (REP-II) branch, the five *B. melitensis* isolates and the reference strain were classified into two clades (C1 and C2) and 6 REP genotypes (G2 – G7) with band numbers ranged from 7-10 (band sizes from 122 to 2946 bp) and a discrimination index of 0.88 (Fig. 2). The isolates belonged to C1 shared a similarity of 84%; these isolates were divided into three genotypes (G2 – G4) that harbored clusters of three isolates with three singleton genotypes (Fig. 2). The reference *Brucella* strain 16M (G7) shared 77% similarity with the *Brucella melitensis* strains ID 1,2,3, and 8 (G5-G7) in C2. Yet showed a lower average similarity rate of 77% compared to other isolates in C1 (Fig. 2).

TABLE 5. Virulance patterns based on the distribution of the virulence associated genes of the eight *B. melitensis* strains recovered from ruminants

Isolates' ID	Investigated virulence associated genes				Virutypes
	bvfA	*ure*	*wbkA*	*omp25*	
1	+	+	+	+	**V1**
2	+	+	+	+	**V1**
3	+	+	+	+	**V1**
4	+	+	+	+	**V1**
5	+	-	+	+	**V2**
6	+	+	+	-	**V3**
7	+	+	+	+	**V1**
8	+	+	+	-	**V3**
Total	**8/8**	**7/8**	**8/8**	**6/8**	

Strain Number	Species	Governorate
7	B. melitensis bv3	Kafr Elsheikh
6	B. melitensis bv3	Kafr Elsheikh
5	B. melitensis bv3	Kafr Elsheikh
4	B. melitensis bv3	Kafr Elsheikh
8	B. melitensis bv3	Kafr Elsheikh
3	B. melitensis bv3	Kafr Elsheikh
2	B. melitensis bv3	Kafr Elsheikh
1	B. melitenis bv3	Kafr Elsheikh
	B. melitensis Ref	Kafr Elsheikh
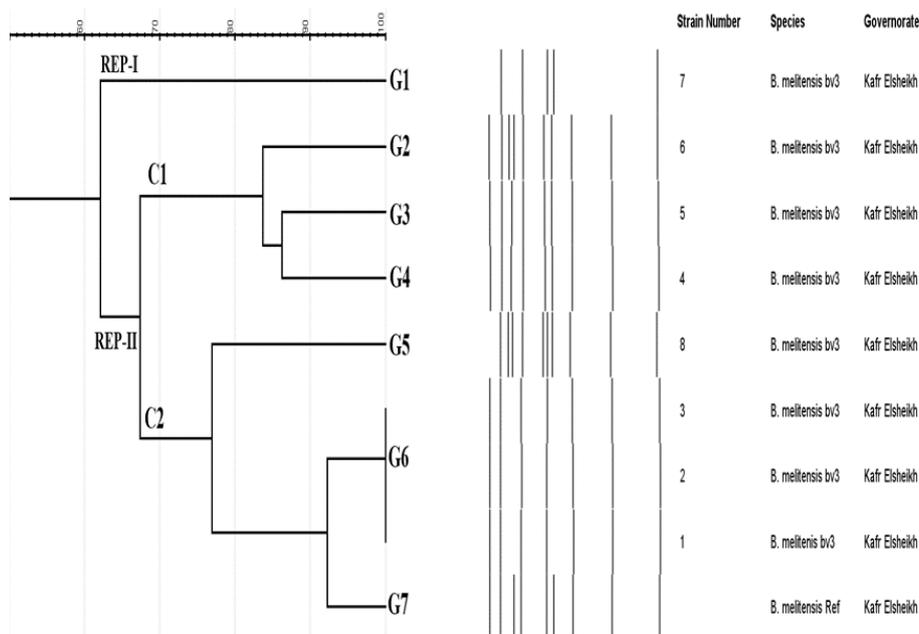
Fig. 2. REP-PCR genotyping of *B. melitensis* bv3 isolates detected in this study. G: REP genotype; REPI-II: REP-branches; C: Clade.

Discussion

Brucellosis Brucellosis is a widespread zoonotic disease that is a major cause of heavy economic losses in the animal industry and presents serious public health hazards [31], as a result, early diagnosis, control, and eradication of infected animals are critical concerns for brucellosis control [32].

In this study, we considered animals positive for brucellosis if they gave positive serological results to both RBT and CFT (series interpretation). Both of RBT and CFT are recommended by WOAH [27] for the surveillance and the estimation of the prevalence of brucellosis in ruminant herds or flocks. Seroprevalence is an important element in either designing or choosing a proper control strategy. Cross-sectional study was conducted in a period between January 2021 to December 2022 in which 926, 422, 301, and 333 blood samples were collected randomely from cattle, buffaloes, sheep, and goats (Table 1). Approximately, the estimated seroprevalence of brucellosis among ruminants in these localities exceeds 10% as shown in Table (3).

Our findings raised concerns about the effectiveness of the applied control mechanisms and revealed that brucellosis is endemic at high levels in all ruminant species in the study area. The figures of the apparent and true prevalence as described in Table (4) match the results of Hegazy *et al.* [7], Selim *et al.* [8], and Elmonir *et al.* [10].

The high prevalence of brucellosis due to *B. melitensis* in this study among different ruminants may be attributed to 1. keeping sheep and goats nearby or inside households, which is the primary risk factor for brucellosis in household cattle, and buffalo tested serologically positive for brucellosis. 2. Dumping abortion-related materials into water canals and housing aborted and brucellosis-infected animals. 3. Household owners are unlikely to report suspected cases to the authorities fearing inappropriate compensation [7]. 4. The location of an open liveanimal market nearby the study area, the Kotor market, may be a risk factor for the spread of *Brucella*. Uncontrolled open live animal markets facilitate contact of *Brucella*-infected animals with healthy ones. 5. no restriction on animal movement between Governorates and infected areas [4]. 6. Lack of biosafety measurements in households and farms. 7. Villagers' lack of awareness, unfavorable attitudes, and abusive behavior can maintain the risk of brucellosis transmission to animals and humans [33]. 8. Yet, ineffective cooperation, coordination, and communication between the veterinary and the public health authorities hamper the applied control strategy of brucellosis, particularly in the Kafr Elsheikh governorate.

Cattle may act as a reservoir for *B. melitensis* in the absence of small ruminants, spreading it to other cattle (spilling over the infection) [4].

The supramammary lymph nodes were collected, for the isolation of the causative agents, from 45

serologically positive animals by CFT (Table 5). In this study, we isolated eight *Brucella* isolates from the supramammary lymph nodes of the slaughtered serologically positive animals. Moreover, molecular confirmation (AMOS-PCR) and the bacteriological-full typing of *Brucella* at genus, species, and biovar levels indicated that all isolates are *B. melitensis* biovar 3. *Brucella melitensis* is the most predominating *Brucella* species circulating among different animal species in Kafr Elsheikh Governorate, Egypt [3,4,21].

Brucella melitensis isolation from several animal species and localities in Kafr Elsheikh Governorate confirmed active brucellosis in the animals tested, was mainly attributable to keeping ewes, goats, and cattle in the same household, farms, mobile flocks and cannot be isolated during parturition or abortion [21]. So, the risk of *B. melitensis* transmission is thus exacerbated, as in the case of our study. Regardless of how many *Brucella* isolates were recovered from various animal species, the isolation of single *Brucella* isolates, either from household animals or a flock/herd is definitive proof to establish the infection status [33] and supports the serological results.

Brucella has adapted to avoid the immune system's response, withstand intracellular trafficking, and tolerate the low oxygen conditions endured inside macrophages [15]. Among the virulence factors of *B. melitensis* are cell envelope proteins, which responsible for the initial survival inside macrophages [34]. DNA was extracted from all eight *B. melitensis* isolates recovered from the animals examined in this study.

The *bvfA, ure*, *wbkA*, and *omp25*, virulence associated genes were investigated among the *B. melitensis* isolates by the PCR assay. PCR produced specific bands of 1282, 2100, 931, and 701 bp, respectively, for these investigated genes.

The survival of *Brucella* in the acidic pH of the macrophage phagosome has been related to the expression of *Brucella* virulence factor A (*bvfA*). The produced protein probably is involved in the establishment of the intracellular niche [35,36]. In this study, all *B. melitensis* isolates (100%) possessed the *bvfA* gene (Table 5), which matched the findings of Lavigne *et al.* [35].

The *wbkA* gene contributes to *Brucellae*'s intracellular survival and intracellular modulatory activity in host cells. Additionally, it has been demonstrated that these genes protect *Brucellae* from the host's defensive system [37].

WbkA is necessary for O-PS biosynthesis, and the excision of *wbkA* could dissociate *Brucella* smooth colonies into rough ones [38]. Our results were consistent with this hypothesis, as all the recovered *Brucella* isolates possessed 100% *wbkA* gene. Other studies done in Egypt or elsewhere detected the *wbkA gene* in 90% of *Brucella* isolates [39,40].

Our findings are consistent with those of another investigation, where the *wbkA* gene was discovered in eight *B. melitensis* isolates recovered from human patients at Babylon Hospital in Iraq [41].

The *ure* gene is encoded for urease, a metal enzyme, that helps *Brucella* to survive the acidic environment through the decomposition of urea into ammonium leading to a pH increase [36].

The *ure* gene is the most virulence factor for *Brucella melitensis* since it controls urease activity, which contributes to the bacteria's resistance to low pH settings and helps it during passage through the stomach most likely when the mode of infection is through the oral route [42].

Our results illustrate the distribution of *ure*-associated gene in seven out of eight *B. melitensis* strains isolated from ruminants (Table 5).

Interestingly, the virulence genes of the *Brucella* pathogens are borne by chromosomes and *Brucella* doesn't produce plasmids, which is the foundation for virutyping [43]. Three virulence patterns were observed in the recovered isolates used in this study. Different isolates of *B. melitensis* exhibit varying distributions of virulence genes, according to reports from Egypt and other countries [22,44] in concordance with our results.

In this study, REP-PCR clearly discriminated between the eight detected *B. melitensis* bv3 isolates. Additionally, *B. melitensis* bv3 isolates were classified into two clades (C1 and C2) and 7 REP genotypes (G1 – G7) by the REP-PCR. This genotyping tool showed acceptable discriminatory power between the isolates (discrimination index= 0.88). Under the field of this study displayed reproducible patterns, the same band s' sizes and band numbers, using the same REP-PCR cyclic conditions. In agreement, the REP-PCR was previously reported to successfully discriminate *Brucella isolates* to genus and species level with occasional intra-species discrimination between individual strains [22,45]. Interestingly, REP-PCR showed more efficient intra-species discrimination for *B. melitensis* bv3 detected in this study compared to *B. melitensis* isolates recovered from Cattle (e.g. 12 field isolates were classified into only two genotypes) in another study in Egypt [22]. This may allow efficient discrimination between individual isolates using genetic typing tools. These findings endorse the utilization of REP-PCR as an affordable and quick tool for traceability of *B.*

melitensis strains especially in developing countries with limited resources. This variety in genotypes detected may suggest introduction of multiple strains to Egypt either simultaneously or over a period of time. The consistent low rates of *B. melitensis* isolation across the year of sampling in the study area, may suggest an established infection from different ancient outbreaks rather than a recent introduction of foreign strains in a country.

Conclusion

We concluded that the most common virulence genes are *bvfA*, *wbkA* and V1 are the most prevalent virutype across isolates and are thought to play a critical role in the pathogenesis of brucellosis in this location. To prevent the further spread of brucellosis, restrictions to animal movement across governorates and appropriate control policies are required. Eductional campaign and application of proper biosafety measures, and a control of live animal marketing are recommended to reduce the prevalence of brucellosis in Kafr Elsheikh livestock population. Variations in the presence or lack of virulence genes can be employed as genetic markers for Egyptian isolates in addition to defining virulence features of the isolates. The high diversity of REP-PCR genotypes among detected *B. melitensis bv3* isolates highlights the cost-effective discrimination efficiency of REP-PCR as a primary tool for trackbacking the sources of infection that require further study to stand upon its limitations as well as its genotyping efficiency as non-sequencing DNA based fingerprinting tool if compared with the well established discriminatory tools (WGS, MLVA-16). The detection of *Brucella melitensis* at consistently low rates across the period of this study suggest an established endemic status rather than a recent introduction of foreign strains in Egypt.

Conflicts of interest

The authors declared no competing interests.

Ethical approval

The protocol for this study, which conformed to the Helsinki Declaration, was approved by the Animal Health Research Institute's Research Ethics Committee for Experimental and Clinical Studies in Egypt (No. 175878).

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الانتشار المصلي لداء البروسيلات، والتنميط الجيني القائم على التنميط الجيني خارج المنشأ المتكرر وتوزيع الجينات المرتبطة بالضراوة بين سلالات البروسيلا ميليتنسيز المعزولة من المجترات في محافظة كفر الشيخ بمصر

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يعد داء البروسيلات من الامراض المشتركة البكتيرية حيوانية المنشأ والمهملة والتي لها تأثير كبير على الصحة العامة وصناعة الثروة الحيوانية. هدفت تلك الدراسة إلى تحديد الانتشار المصلي لداء البروسيلات، وتحديد أنواع البروسيلا المنتشرة بين المجترات، وتوزيع الجينات المرتبطة بضراوة البروسيلا، والتنميط الجيني خارج المنشأ المتكرر لمعزولات البروسيلا (REP-PCR) بين الحيوانات المجترة في محافظة كفر الشيخ. وقد تم جمع عينات دم بشكل عشوائي من 926 رأسًا من الأبقار، و422 جاموسًا، و301 خروفًا، و333 ماعزًا بمحافظة كفر الشيخ في الفترة من يناير 2021 إلى ديسمبر 2022. وقد تم حساب معدل الانتشار المصلي لداء البروسيلات الظاهري والحقيقي في المجترات في مراكز مختلفة بكفر الشيخ. وقد تم اجراء العزل البكتيري والتأكيد الجزيئي (AMOS-PCR) ، ورسم شرة التطور على أساس التنميط الجيني خارج المنشأ المتكرر لمعزولات البروسيلا (REP-PCR)   وقد تم ايضا التمييز بين عترات البروسيلا ميليتنسيز علي اساس الاختلاف في توزيع جينات الضراوة بين تلك العترات(Virutyping) . تم حساب معدل الانتشار الحقيقي لداء البروسيلات ليكون 13.3%، 11.2%، 15.8%، و16.8% في الأبقار والجاموس والأغنام والماعز. تم عزل ميكروب البروسيلا (العدد = 8) من العقد الليمفاوية فوق الثديية وتصنيف تلك العترات جزيئًا (AMOS-PCR) وبكتيريا على أنها بروسيلا ميليتنسيز النوع الحيوي 3. ومن حيث الاختلاف في توزيع جينات الضراوة بين تلك العترات (Virutyping) أظهرت معزولات البروسيلا ميليتنسيز ثلاثة أنماط من الضراوة (V1-V3) ، حيث كان V1 هو النمط الأكثر شيوعاً (62.5%). أستطاع REP-PCR أن يميز بوضوح بين معزولات البروسيلا ميليتنسيز النوع الحيوي 3 المعزولة من المجترات في مجموعتين (C1) و(C2) و7 أنماط وراثية (G1 - G7). في الختام، فإن الأنماط الجينية عالية التنوع للبروسيلا ميليتنسيز النوع الحيوي 3 المستندة إلى أنماط REP-PCR تسلط الضوء على مدى كفاءة التمييز الفعالة من حيث التكلفة لـ REP-PCR القابل للتكرار. إن العزل المستمر لميكروب البروسيلا ميليتنسيزوبمعدلات منخفضة على مدارعام يشير إلى احتمالية حدوث حالات تفشي قديمة مختلفة للعدوى استقرت بين المجترات عن حدوث اصابات جديدة بسلالات أجنبية للميكروب قد تكون دخلت البلد حديثاً.

الكلمات الدالة: البروسيلا ، معدل الأنتشارالمصلي ، التنميط الوراثي ، جينات الضراوة ، كفر الشيخ