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Trans-species transmission of *Brucellae* among ruminants hampering brucellosis control efforts in Egypt

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Abstract

Aims: This study aimed to identify the genotypic fingerprinting of *Brucella melitensis* biovar 3 isolates from ruminants in Kafr El-Sheikh, Egypt, to compare with other peers globally and to highlight the epidemiology and potential causes of brucellosis control failure.

Methods and Results: A multilocus variable-number tandem-repeat analysis (MLVA 16) was carried out on 41 *B. melitensis* bv3 isolates, 31 from the preferential hosts (28 sheep and three goats) and 10 from atypical hosts (nine cattle and one buffalo), identified by bacteriological and molecular techniques. MLVA-16 analysis revealed 19 genotypes with nine as singletons. The most prevalent genotypes were M3_K.E (3,5,3,13,1,1,3,3, 7,43,8,7,6,7,5,3), M13_K.E (3,5,3,13,1,1,3,3,7,43,8,5,8,7,7,3) and M5_K.E (3,5,3,13,1, 1,3,3,7,43,8,4,8,7,11,3) circulating between different animal species. The *B. melitensis* isolation from aborted cows in farms that had never reared small ruminants indicates the likelihood of cow to cow *B. melitensis* transmission. Different genotypes of *B. melitensis* could be isolated from the same animal. The local geographic distribution of genotypes showed a very close genetic relatedness with genotypes reported outside the study area. Worldwide, our genotypes were mostly related to the Western Mediterranean lineage and less likely to the America's clonal lineage.

Conclusion: There is a high genetic similarity of *B. melitensis* bv3 genotypes among different ruminant species, and the same animal could be infected with different genotypes. There is a high probability of spreading of *B. melitensis* among atypical hosts in the absence of the original hosts. The genetic relatedness of *B. melitensis* bv3 genotypes in the study area with other different geographic areas highlighted the national and international ruminants movement role as a potential factor for maintaining *B. melitensis* infection.

Significance and Impact of the Study: Further investigations are required to understand the impact of the presence of more than one genotype of *B. melitensis* in the same animal on the efficacy of brucellosis control strategies.

1 | INTRODUCTION

Brucellosis is a common anthropozoonosis caused by members of the genus *Brucella* and associated with a major economic burden worldwide (Corbel et al., 2006). The infective dose is low (10–100 bacteria) and is easily transmitted to humans by ingestion, direct contact and inhalation causing undulant fever and other severe health problems (Christopher et al., 2010). In ruminants, it is a common cause of contagious abortion, decrease in milk production and infertility (McDermott et al., 2013). Until now, 11 species of *Brucella* have been described, of which *Brucella melitensis* is the most serious and virulent to humans (OIE, 2018). Furtheremore, *B. melitensis* is widely spread among ruminant population especially in Mediterranean region, Latin America and Central and Western Asia (Benkirane, 2006). The high mobility of small ruminants, which are the preference host of *B. melitensis*, facilitates its dissemination between farms and regions (McDermott et al., 2013).

Seropreavlence of brucellosis in different regions of Egypt was investigated using rose bengal plate agglutination test as screening test followed by the complement fixation test as a confirmatory test. In Kafr El-Sheikh and Giza governorates, the seroprevalence of brucellosis in sheep flocks was estimated as 41.3% and 11%, respectively (Abdel-Hamid et al., 2017; Hegazy et al., 2011). *Brucella melitensis* bv3 is the most common and predominant strain isolated from different animal species from almost all Egyptian governorates (Abdel-Hamid et al., 2016; Abdel-Hamid et al., 2020). In Egypt, large ruminants are usually reared either as single species or mixed with small ruminants and equines. There are also different rearing systems, for instance, ruminants graze during the day and are then kept in pens at night, while other farmers rear their ruminants indoors or in mobile herds. The shelterless small ruminant mobile flocks could play a major role in the spread of *B. melitensis*, as they pass across different Egyptian governorates, searching for pasture, and come in to contact with other ruminants (Hegazy et al., 2016). This animal husbandry method is common in the majority of Middle Eastern countries and has resulted in the contact of different species of ruminant for a long period of time.

Limited genetic polymorphisms exist in *Brucella* sp., which show >98% similarity in their nucleotide sequences (Corbel & Brinley-Morgan, 1984; Halling et al., 2005). Genus-specific PCR techniques targeting 16S rRNA or genes coding for *Brucella* membrane proteins have previously been used (OIE, 2018). Other molecular techniques such as the repeat of short nucleotide sequences or the variable number of tandem repeats (VNTR) have being used to differentiate *Brucella* sp. and biotypes depending on the wide variation in the number of these repeats (Christopher et al., 2010).

Multiple locus VNTR (MLVA-16) has been efficiently used in epidemiological studies to identify *Brucella* strains targeting 16 loci and seeking their genetic associations (Maquart et al., 2009). The MLVA-16 includes eight minisatellite loci (repeat size of ≥9 bp) and eight microsatellite (repeat size ≤8 bp) loci termed Panels 1 and 2, respectively; the latter is subdivided into Panels 2A and 2B (Kattar et al., 2008; Vergnaud & Pourcel, 2006). This is a powerful discriminatory tool in subtyping bacteria of high genomic homology regardless of their geographic origin such as *Brucella* (García-Yoldi et al., 2007). However, further studies on large numbers of isolates from different countries are required to improve strain relatedness and to enhance the MLVA database 'http://microbesgenotyping.i2bc.paris-saclay.fr,' a database that contains the VNTRs' metadata of more than 5000 isolates (Kattar et al., 2008). The efficiency of MLVA to detect the genetic divergence between different isolates of *Brucella* is judged by the Hunter–Gaston diversity index (HGDI), which includes the results of each marker of the panels separately as well as in combination (Hunter & Gaston, 1988).

The use of the MLVA technique could play an important role in explaining the geographic distribution of some genotypes and the role of animal movement and animal trade in spreading the infection. The MLVA technique allows for the characterization of species and biovars of *Brucella* and allows for new strains to be traced back to their original source (de Massis et al., 2019; Wareth et al., 2020). Furthermore, the technique could provide more information on the biology of *Brucella* in different animal species, which is essential to undertake effective control measures against brucellosis.

Thus, this study investigated the genetic diversity and strains' relatedness of *B. melitensis* in Kafr El-Sheikh Governorate, Egypt under various husbandry systems. Also, it highlights the epidemiology of brucellosis, potential causes of brucellosis control failure and the genetic relatedness of the local Egyptian *Brucella* genotypes with their peers, based on the MLVA-16 metadata, worldwide.

2 | MATERIALS AND METHODS

2.1 | Ethical approval

The research ethics committee for experimental and clinical studies, Animal Health Research Institute (no. 165567), approved the protocol of this study. This study follows the guidelines of the Egyptian Network of Research Ethics

Committees and the international laws and regulations concerning ethical considerations in research.

2.2 | Study area

Kafr El-Sheikh Governorate is about 134 km north of Cairo, the fourth largest city in the Nile Delta of Lower Egypt, and it consists of 10 districts and 206 villages with a population exceeding three million according to the Central Agency for Public Mobilization and Statistics. It is an agricultural governorate with a high density of livestock and human population. Sheep are raised either in small numbers kept in the household by farmers or in village flocks managed by shepherds (Aidaros, 2005). One shepherd would often keep sheep from many different owners; as a result, animals from different households are part of the same flock for grazing and breeding during most of the year. Some village flocks are sometimes combined to make a large flock managed by more than one shepherd. The flocks are reared in a free-range system, where animals graze freely all over the country as there is no restrictions for animal movement in Egypt (Aidaros, 2005). These flocks could have other ruminant species as cattle, buffaloes or goats. On the other hand, cattle and buffaloes are being raised as household animals, mobile herds just like sheep or in farms. Kafr El-Sheikh Governorate has large animal markets in which livestock owners and traders from different parts of Egypat purchase and sell their animals.

2.3 | Sampling, animals and specimens

According to the national control programme for brucellosis in Egypt, the public health authority has to notify the general organization for veterinary services (GOVS) of any cases of human brucellosis. Hence, GOVS collects blood samples from all animals owned by the notified positive human cases to identify their infection status and cull the positive ones. In the current study, samples were collected from animals reared in various husbandry systems (farms, mobile flocks and households), across different districts of Kafr El-Sheikh governorate, Egypt (Table1). Farms 'A' and 'B' have cattle and buffalo, farms 'F' and 'G' have only cattle, mobile flock 'C' has only sheep, mobile flock 'D' has mixed breeds of (goats, cattle and buffaloes) and mobile flock 'E' has sheep, goats, cattle and buffaloes. The remaining samples were taken from individual household ruminants that could have had contact with other animals. The individual household animals were five ewes and one cow.

Samples were given a code such as G-Cow6 when derived from cow number 6 of farm 'G' or HH-Ewe83 for ewe number 83 from the household.

For the aim of *Brucella* isolation and identification, specimens were collected from seropositive animals in four farms (A, B, F and G), three mobile flocks (C, D and E) and six households. These animals were targeted upon the confirmed seropositivity, abortion at the third trimester and/or the notification of a brucellosis case among the humans in contact with these animals. Farm 'G' is a dairy farm with 119 cows that had no history of abortion, nor *Brucella* spp. infection declaration, no history of introducing animals from outside the farm and had no contact with small ruminants. In 2017, the farm received a replacement cow of unknown brucellosis status for the first time, and 2 months later, abortions had occurred in 20% of the herd.

Full data about total animal numbers, seropositive cases, localities, specimens concerning farms, mobile flocks and household animals under the field of this study were shown in Table 1. Samples for bacterial isolation and identification were collected from live seropositive animals and upon slaughtering the seropositive animals through the national control programme. Supra-mammary ($n = 81$), retropharyngeal lymph nodes ($n = 9$), testicles ($n = 1$), fetal membranes ($n = 52$), spleen ($n = 79$) and milk samples ($n = 40$) were collected from seropositive live and slaughtered animals.

2.4 | Bacterial strains isolation, phenotypic characterization and molecular typing

The phenotypic characterization of *Brucella* isolates was done at the genus level based on colony morphology, urease activity, oxidase and catalase production. Then, species determination was carried out by phage lysis using Tbilisi (Tb), Izatnagar (Iz), Weybridge (Wb) and rough–canis (R/C) phages. Agglutination with monospecific A, M and R antisera besides, CO_2 requirement, H_2S production, growth on thionin and basic fuchsin (20 µg/ml in serum dextrose agar) were performed to identify *Brucella* at the biovar level. Full typing at these three levels was done according to Alton et al. (1988) and OIE (2018). DNA was extracted from bacterial culture harvested in phosphate-buffered saline with PH 7.2 and inactivated at 100℃ for 15 min using QIAamp® DNA Mini Kit (Qiagen). DNA concentrations were measured by NanoDrop™ 2000/2000c Spectrophotometers (Nanodrop Technologies). Molecular typing using AMOS-PCR described by Bricker and Halling (1994) and Bricker et al. (2003) was conducted under the following conditions: initial denaturation at 94℃ for 5 min, followed by 35 cycles of 94℃ for 30 s, 55℃ for 40 s and 72℃ for 45 s, with a final extension of 72℃ for 10 min. The extracted genomic DNA from the *B. melitensis* bv3 reference strain Ether (ATCC 23458) was used for the allele assignment control.

TABLE 1 Details of animal numbers, species, reactors and specimens collected for bacteriological examination under various husbandry systems in Kafr El-Sheikh Governorate

Flock/herd ID	Locality	Animal species/total numbers in the herd	Seropositive animals (RBPT+CFT)	Criteria of notification	Specimen collected for bacteriological examination
Farm A[a]	Sidi Salim	Cattle (52) Buffalo (33)	Cattle (5) Buffalo (0)	Late stage abortion	Spleen, uterus, udder, milk, fetal membranes, suppramammary l.n.
Farm B[a]	Motobas	Cattle (39) Buffalo (18)	Cattle (6) Buffalo (0)	Late stage abortion	Spleen, uterus, udder, milk, suppramammary l.n.
Mobile flock C	Quillin	Sheep (45)	4	Human brucellosis	Spleen, uterus, udder, suppramammary l.n., placenta, fetal fluids
Mobile flock D	Kafr El-Sheikh district	Goat (30) Cattle (12) Buffalo (8)	Goat (12) Cattle (1) Buffalo (1)	Human brucellosis	Fetal membrane, milk, udder, suppramammary l.n., uterus, spleen
Mobile flock E	Quillin	Ewe (47) Ram (1) Goat (6) Cattle (6) Buffalo (4)	Ewe (21) Ram (1) Goat (0) Cattle (1) Buffalo (0)	Human brucellosis	Fetal membrane, milk, udder, suppramammary l.n., uterus, spleen, testicle, testicular l.n.
Farm F	El-Hamool	Cattle (30)	5	Late stage abortion	Udder, suppramammary l.n., uterus, spleen, retropharyngeal l.n.
Farm G	Disuq	Cattle (115)	22	Late stage abortion	Udder, suppramammary l.n., uterus, spleen, fetal membranes
HH56	Biala	Ewe (1)	1	Late stage abortion	Supramammary L.N.
HH58	Biala	Ewe (1)	1	Late stage abortion	Supramammary L.N.
HH83	Biala	Ewe (1)	1	Late stage abortion	Retropharyngeal L.N.
HH87	Biala	Ewe (1)	1	Late stage abortion	Retropharyngeal L.N.
HH88	Biala	Cow (1)	1	Late stage abortion	Retropharyngeal L.N.
HH90	Biala	Ewe (1)	1	Late stage abortion	Retropharyngeal L.N.

Abbreviations: CFT, complement fixation test; HH, household; L.N., lymph node; −ve, negative.

[a]No *Brucella* isolates have been recovered from samples of farm A and B.

2.5 | MLVA-16 analysis

MLVA-16 including Panel 1 (bruce06, bruce08, bruce11, bruce12, bruce42, bruce43, bruce45 and bruce55) and eight microsatellite markers including Panel 2A (bruce18, bruce19 and bruce21) and Panel 2B (bruce04, bruce07, bruce09, bruce16 and bruce30) (Al Dahouk et al., 2007; Le Fleche et al., 2006) were performed for *B. melitensis* bv3 isolates ($n = 41$). For the phylogeny, dendrograms were performed after uploading the VNTRs data and estimating the *Brucella* genotypes online through the MLVA bank for microbe genotyping (http://microbesgenotyping.i2bc.paris-saclay. fr). Dendrograms seeking the genetic similarities among the 41 *Brucella* strains were based on the categorical coefficient with distance calculation and unweighted pair group method with arithmetic mean using BioNumerics version 7.6 (Applied Maths).

Variable number of tandem repeats data of the local *B. melitensis* strains used in this study were compared with 118 *B. melitensis* strains recovered from different animal species and humans from other Egyptian governorates (Sayour et al., 2020). The standard minimum spanning tree (MST), based on categorical coefficient with double locus variance priority rules as well as the dendrogram of the Figure S1, was used to study the genetic similarities between the local strains along with the MLVA-16 global metadata of the *B. melitensis* bv3 strains ($n = 358$) isolated from selected African countries (neighborhood) and worldwide. The genetic diversity of each MLVA-16 loci was estimated using the HGDI with 95% confidence intervals through the V-DICE tool available at the HPA website (http://www. hpa-bioinformatics.org.uk/cgi-bin/DICI/DICI.pl), where it ranged from 0 (identical strains) to 1 (different strains) as reported by Hunter and Gaston (1988). Sola et al. (2003) have classified the allelic diversity (HGDI) as high if the discriminatory power of HGDI is more than 0.6, moderate discrimination if $0.3 \leq HGDI \leq 0.6$ and poor discrimination if HGDI <0.3.

3 | RESULTS

3.1 | Bacteriological examination and identification

A total of 41 *Brucella* isolates were obtained in this study. Phenotypically, all *Brucella* isolates ($n = 41$) proved to be *B. melitensis* bv3 by fitting the identification scheme (Alton et al., 1988; OIE, 2018). AMOS-PCR showed a *B. melitensis*-specific band of 731 bp. Thirty-one isolates were obtained from 28 sheep and three goats (the preferential host), and 10 isolates were obtained from nine cattle and one buffalo (non-preferential hosts) as shown in Figure 1. No *Brucella* isolates

were identified from farms 'A' and 'B' regardless of the se-ropositive cases.

3.2 | MLVA 16 fingerprinting

The genetic diversity among *B. melitensis* isolates ($n = 41$) using HGDI values was estimated for each MLVA-16 locus subsets. Based on the HGDI classification by Sola et al. (2003), the markers of Panel 1 (Table 2) were monomorphic displayed single alleles in all *B. melitensis* isolates ($n = 41$) with no discrimination and HGDI of zero. Similarly, Bruce19 and Bruce21 of Panel 2A displayed the same
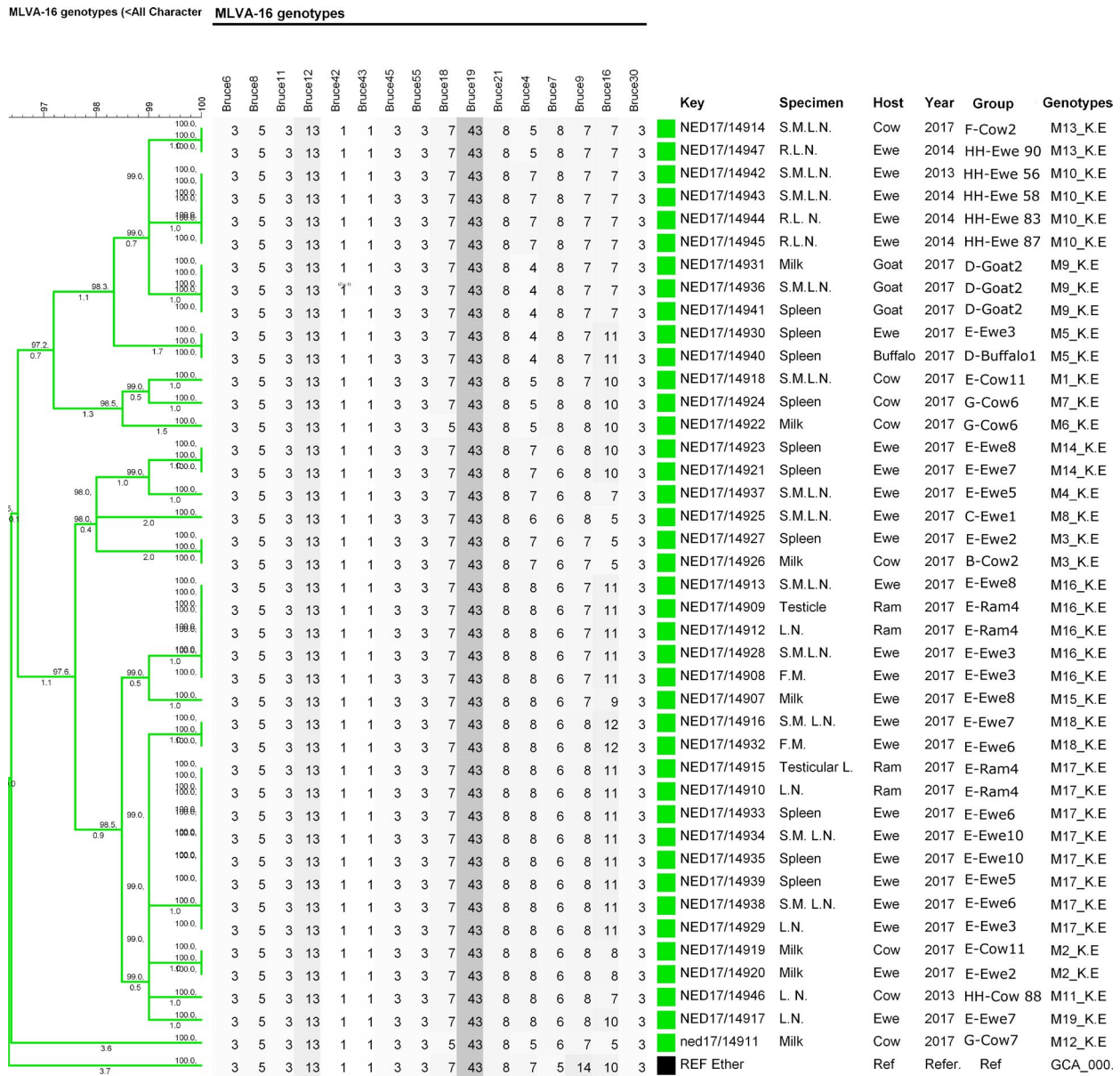

MLVA-16 genotypes

Bruce6	Bruce8	Bruce11	Bruce12	Bruce42	Bruce43	Bruce45	Bruce55	Bruce18	Bruce19	Bruce21	Bruce4	Bruce7	Bruce9	Bruce16	Bruce30	Key	Specimen	Host	Year	Group	Genotypes
3	5	3	13	1	1	3	3	7	43	8	5	8	7	7	3	NED17/14914	S.M.L.N.	Cow	2017	F-Cow2	M13_K.E
3	5	3	13	1	1	3	3	7	43	8	5	8	7	7	3	NED17/14947	R.L.N.	Ewe	2014	HH-Ewe 90	M13_K.E
3	5	3	13	1	1	3	3	7	43	8	7	8	7	7	3	NED17/14942	S.M.L.N.	Ewe	2013	HH-Ewe 56	M10_K.E
3	5	3	13	1	1	3	3	7	43	8	7	8	7	7	3	NED17/14943	S.M.L.N.	Ewe	2014	HH-Ewe 58	M10_K.E
3	5	3	13	1	1	3	3	7	43	8	7	8	7	7	3	NED17/14944	R.L. N.	Ewe	2014	HH-Ewe 83	M10_K.E
3	5	3	13	1	1	3	3	7	43	8	7	8	7	7	3	NED17/14945	R.L.N.	Ewe	2014	HH-Ewe 87	M10_K.E
3	5	3	13	1	1	3	3	7	43	8	4	8	7	7	3	NED17/14931	Milk	Goat	2017	D-Goat2	M9_K.E
3	5	3	13	1	1	3	3	7	43	8	4	8	7	7	3	NED17/14936	S.M.L.N.	Goat	2017	D-Goat2	M9_K.E
3	5	3	13	1	1	3	3	7	43	8	4	8	7	7	3	NED17/14941	Spleen	Goat	2017	D-Goat2	M9_K.E
3	5	3	13	1	1	3	3	7	43	8	4	8	7	11	3	NED17/14930	Spleen	Ewe	2017	E-Ewe3	M5_K.E
3	5	3	13	1	1	3	3	7	43	8	4	8	7	11	3	NED17/14940	Spleen	Buffalo	2017	D-Buffalo1	M5_K.E
3	5	3	13	1	1	3	3	7	43	8	5	8	7	10	3	NED17/14918	S.M.L.N.	Cow	2017	E-Cow11	M1_K.E
3	5	3	13	1	1	3	3	7	43	8	5	8	8	10	3	NED17/14924	Spleen	Cow	2017	G-Cow6	M7_K.E
3	5	3	13	1	1	3	3	5	43	8	5	8	8	10	3	NED17/14922	Milk	Cow	2017	G-Cow6	M6_K.E
3	5	3	13	1	1	3	3	7	43	8	7	6	8	10	3	NED17/14923	Spleen	Ewe	2017	E-Ewe8	M14_K.E
3	5	3	13	1	1	3	3	7	43	8	7	6	8	10	3	NED17/14921	Spleen	Ewe	2017	E-Ewe7	M14_K.E
3	5	3	13	1	1	3	3	7	43	8	7	6	8	7	3	NED17/14937	S.M.L.N.	Ewe	2017	E-Ewe5	M4_K.E
3	5	3	13	1	1	3	3	7	43	8	6	6	8	5	3	NED17/14925	S.M.L.N.	Ewe	2017	C-Ewe1	M8_K.E
3	5	3	13	1	1	3	3	7	43	8	7	6	7	5	3	NED17/14927	Spleen	Ewe	2017	E-Ewe2	M3_K.E
3	5	3	13	1	1	3	3	7	43	8	7	6	7	5	3	NED17/14926	Milk	Cow	2017	B-Cow2	M3_K.E
3	5	3	13	1	1	3	3	7	43	8	8	6	7	11	3	NED17/14913	S.M.L.N.	Ewe	2017	E-Ewe8	M16_K.E
3	5	3	13	1	1	3	3	7	43	8	8	6	7	11	3	NED17/14909	Testicle	Ram	2017	E-Ram4	M16_K.E
3	5	3	13	1	1	3	3	7	43	8	8	6	7	11	3	NED17/14912	L.N.	Ram	2017	E-Ram4	M16_K.E
3	5	3	13	1	1	3	3	7	43	8	8	6	7	11	3	NED17/14928	S.M.L.N.	Ewe	2017	E-Ewe3	M16_K.E
3	5	3	13	1	1	3	3	7	43	8	8	6	7	11	3	NED17/14908	F.M.	Ewe	2017	E-Ewe3	M16_K.E
3	5	3	13	1	1	3	3	7	43	8	8	6	7	9	3	NED17/14907	Milk	Ewe	2017	E-Ewe8	M15_K.E
3	5	3	13	1	1	3	3	7	43	8	8	6	8	12	3	NED17/14916	S.M. L.N.	Ewe	2017	E-Ewe7	M18_K.E
3	5	3	13	1	1	3	3	7	43	8	8	6	8	12	3	NED17/14932	F.M.	Ewe	2017	E-Ewe6	M18_K.E
3	5	3	13	1	1	3	3	7	43	8	8	6	8	11	3	NED17/14915	Testicular L.	Ram	2017	E-Ram4	M17_K.E
3	5	3	13	1	1	3	3	7	43	8	8	6	8	11	3	NED17/14910	L.N.	Ram	2017	E-Ram4	M17_K.E
3	5	3	13	1	1	3	3	7	43	8	8	6	8	11	3	NED17/14933	Spleen	Ewe	2017	E-Ewe6	M17_K.E
3	5	3	13	1	1	3	3	7	43	8	8	6	8	11	3	NED17/14934	S.M. L.N.	Ewe	2017	E-Ewe10	M17_K.E
3	5	3	13	1	1	3	3	7	43	8	8	6	8	11	3	NED17/14935	Spleen	Ewe	2017	E-Ewe10	M17_K.E
3	5	3	13	1	1	3	3	7	43	8	8	6	8	11	3	NED17/14939	Spleen	Ewe	2017	E-Ewe5	M17_K.E
3	5	3	13	1	1	3	3	7	43	8	8	6	8	11	3	NED17/14938	S.M. L.N.	Ewe	2017	E-Ewe6	M17_K.E
3	5	3	13	1	1	3	3	7	43	8	8	6	8	11	3	NED17/14929	L.N.	Ewe	2017	E-Ewe3	M17_K.E
3	5	3	13	1	1	3	3	7	43	8	8	6	8	8	3	NED17/14919	Milk	Cow	2017	E-Cow11	M2_K.E
3	5	3	13	1	1	3	3	7	43	8	8	6	8	8	3	NED17/14920	Milk	Ewe	2017	E-Ewe2	M2_K.E
3	5	3	13	1	1	3	3	7	43	8	8	6	8	7	3	NED17/14946	L. N.	Cow	2013	HH-Cow 88	M11_K.E
3	5	3	13	1	1	3	3	7	43	8	8	6	8	10	3	NED17/14917	L.N.	Ewe	2017	E-Ewe7	M19_K.E
3	5	3	13	1	1	3	3	5	43	8	5	6	7	5	3	ned17/14911	Milk	Cow	2017	G-Cow7	M12_K.E
3	5	3	13	1	1	3	3	7	43	8	7	5	14	10	3	REF Ether		Ref	Refer.	Ref	GCA_000.

FIGURE 1 Categorical coefficient with distance calculation and unweighted pair group method with arithmetic mean (UPGMA)-based dendrogram showing the relative similarity between the 41 *Brucella melitensis* isolates compared with a reference strain

TABLE 2 Copy repeats and HGDI of each locus with 95% confidence interval

Panels	MLVA-16 markers	Copy numbers of the tandem repeats at each locus	HGDI	Lower and upper limits of HGDI at 95% CI	Alleles' numbers
Panel 1	bruce06	3	0.000	0.000–0.069	1
	bruce08	5	0.000	0.000–0.065	1
	bruce11	3	0.000	0.000–0.074	1
	bruce12	13	0.000	0.000–0.062	1
	bruce42	1	0.000	0.000–0.059	1
	bruce43	1	0.000	0.000–0.048	1
	bruce45	3	0.000	0.00–0.062	1
	bruce55	3	0.000	0.000–0.054	1
Panel 2A	bruce18	7,5	0.109	0.098–0.126	2
	bruce19	43	0.000	0.000–0.057	1
	bruce21	8	0.000	0.000–0.053	1
Panel 2B	bruce04	4,5,6,7,8	0.733	0.711–0.748	5
	bruce07	6,8	0.112	0.091–0.125	2
	bruce09	7,8	0.115	0.096–0.129	2
	bruce16	5,7,8,9,10,11,12	0.912	0.906–0.924	7
	bruce30	3	0.000	0.000–0.052	1

Abbreviation: HGDI, Hunter–Gaston diversity index.

patterns as Panel1 except for Bruce18 that showed different copy numbers of the tandem repeats with poor discrimination (HGDI = 0.109). On the contrary, Bruce04 and Bruce16 of Panel 2B were highly discriminatory in *B. melitensis* (HGDI > 0.7), whereas Bruce07 and Bruce09 of Panel 2B were poorly discriminatory. The remaining locus (Bruce30) exhibited only a single allele with no discrimination.

MLVA-16 analysis of the 41 *B. melitensis* isolates (Figure 1) from Kafr El-Sheikh Governorate showed 19 different genotypes with nine singleton (unique) genotypes. MLVA-16 data analysis illustrates highly consistent results among the local *B. melitensis* strains. All these genotypes along with the *B. melitensis* bv3 reference strain (Ether) were clustered together into one cluster with a genetic similarity of approximately 97% (Figure 1). The nine singleton genotypes were M1_K.E (E-Cow11), M4_K.E (E-Ewe5), two genotypes, M6_K.E and M7_K.E, of G-Cow6, M8_K.E (C-Ewe1), M12_K.E (G-Cow7), M19_K.E (E-Ewe7), M15_K.E (E-Ewe8) and M11_K.E (HH-Cow 88). The most common genotype is M17_K.E with eight identical strains, followed by M16_K.E (five identical strains), M10_K.E (four identical strains) and M9_K.E (three identical strains). Complete data of the performed MLVA-16 analysis and *Brucella* genotypes are shown in Figure 1 and are also available on the MLVA database, 'http://microbesgenotyping.i2bc.paris-saclay.fr.'

Interestingly, there were different genotypes isolated from samples of the same animal; three genotypes each were isolated from animals E-Ewe3, E-Ewe7 and E-Ewe8, and two genotypes each were isolated from animals G-Cow6, E-Ewe2, E-Ram4, E-Ewe5, E-Ewe6 and E-Cow 11. Among the shared *B. melitensis* genotypes (*n* = 10), three genotypes were common between two different animal species. Two of these shared genotypes belonged to different herds (M5_K.E and M13_K.E), whereas the remaining one belonged to the same herd (M2_K.E). The shared genotypes were M2_K.E (E-Cow11 and E-Ewe2), M5_K.E (E-Ewe3 and D-Buffalo1) and M13_K.E (F-Cow2 and HH-Ewe90). Furthermore, M3_K.E (3,5,3,13,1,1,3,3,7,43,8,7,6,7,5,3), M13_K.E (3,5, 3,13,1,1,3,3,7,43,8,5,8,7,7,3) and M5_K.E (3,5,3,13,1,1,3,3 ,7,43,8,4,8,7,11,3) genotypes were the most often observed genotypes circulating between different animal species.

3.3 | Genetic similarity of our *Brucella* genotypes with previously reported genotypes outside the study area

Genetic similarities of different genotypes from different districts were identified (Figure 1). The two strains of genotype M13_K.E have 99% genetic similarity with the four strains of the M10_K.E genotype and the three strains of the M9_K.E genotype. The same degree of similarity was also found between M1_K.E and M7_K.E genotypes; M4_K.E and M14_K.E genotypes; M15_K.E and M16_K.E as well as between the M18_K.E and M17_K.E genotypes; and finally between M2_K.E, M11_K.E and M19_K.E genotypes. The relatedness and association of genotypes MLVA-16 data in our study with those previously reported

in Egypt are shown in the dendrogram, Figure 2. Genotypes 61-Egy-Bm3-Sharq and 18-Egy-Bm3-Kshkh isolated from Sharqia and Kafr El-Sheikh governorates (Sayour et al., 2020) are 100% identical with our genotypes M17_K.E and M11_K.E, respectively. Out of the 118 *Brucella* strains, 115 were grouped with the 41 *B. melitensis* strains of this study into one cluster with a similarity coefficient of approximately 96%, Figure 2.
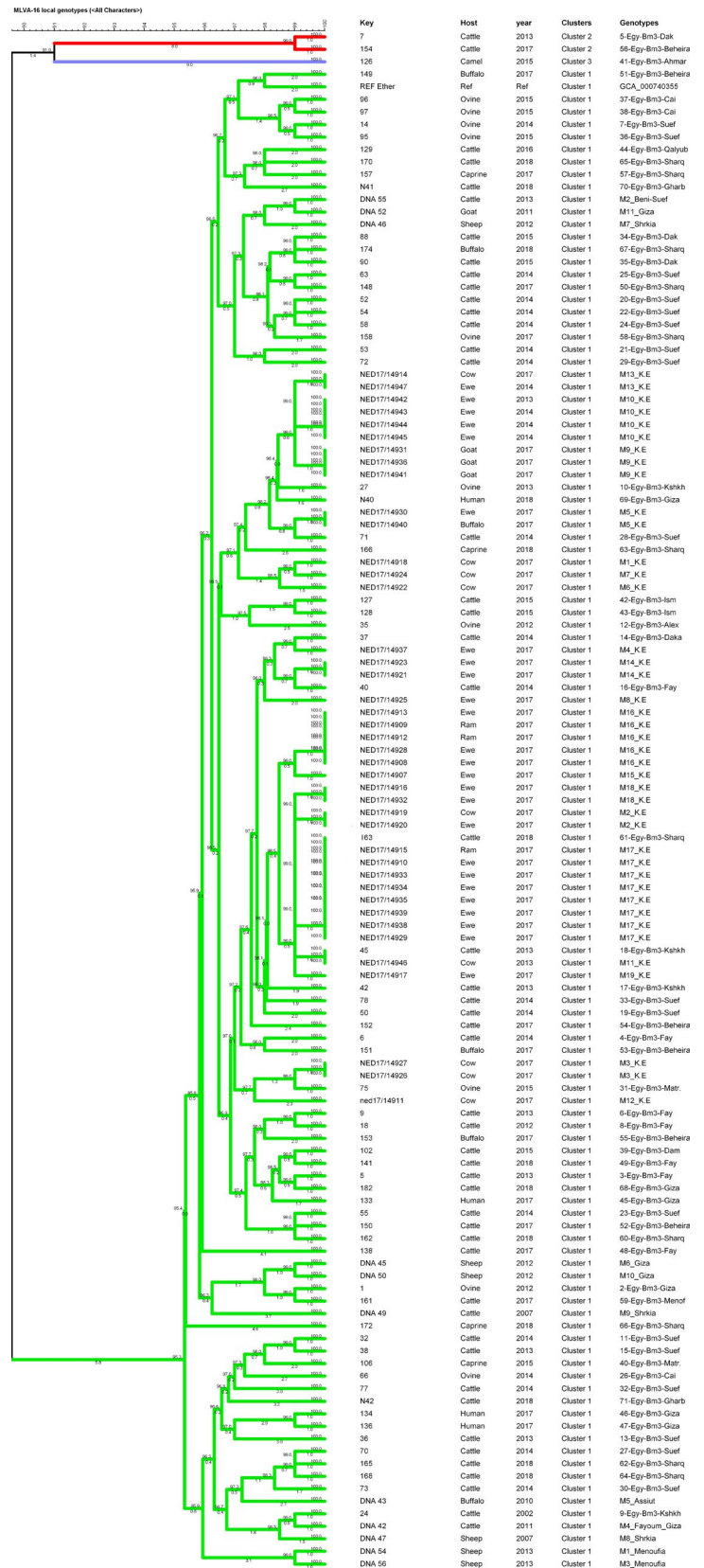

FIGURE 2 Comparative relatedness between the multilocus variable-number tandem-repeat analysis (MLVA-16) data of our genotypes with those previously reported in Egypt

A wider comparison with the worldwide MLVA-16 metadata of 385 *B. melitensis* bv3 (Al Dahouk et al., 2007; Garofolo et al., 2013; Kiliç et al., 2011; Le Fleche et al., 2006; Marianelli et al., 2007; Vergnaud et al., 2018) is illustrated in the dendrogram of Figure S1 as well as the similarity coefficient-based MST, Figure 3.

All genotypes and strains identified in this study belonged mainly to the Western Mediterranean clonal lineage and were less likely related to the America's clonal lineage (Figure S1 and Figure 3). The highest similarity percentages were with neighbourhood Mediterranean and European countries. M12_K.E genotype is of 97% similarity with genotypes 2018Vergnaud#0133 and 2007AlDahouk#013, which were isolated from humans in France (1978) and Tunisia (1992), respectively. M3_K.E genotype is 99% similar to the French genotypes 2018Vergnaud#0531 and 2018Vergnaud#0555, which were isolated in 1983 from cattle (Figure S1). M14_K.E genotype is 99% similar to genotypes 2018Vergnaud#0381 and 2018Vergnaud#0408 isolated from humans in Belgium (1982), which, in turn, have a similarity of 98.5% with M4_K.E genotype (Figure S1).

M13_K.E, M10_K.E, M9_K.E and M5_K.E genotypes are 97.8% similar to 2013Garofolo_6844 and 2018Vergnaud#0928 genotypes, which were isolated from

ovine in Italy (2011) and France (1980), respectively, and represented a similarity of 97.2% with M1_K.E, M7_K.E and M6_K.E genotypes (Figure S1). M8_K.E genotype is 98% similar to 17 genotypes that were isolated from humans and different animals in France and Italy during the period 1978–1999. M16_K.E, M15_K.E, M18_K.E, M17_K.E, M11_K.E, M19_K.E and M2_K.E have 98% similarity with 18 genotypes originated from humans, ovine and cattle in Italy and France during the period 1979–2011 (Figure S1).

4 | DISCUSSION

Brucellosis is a worldwide re-emerging transboundary anthropozoonosis associated with huge economic losses and public health problems (McDermott et al., 2013). *Brucella melitensis* is endemic in ruminants in Middle Eastern countries, and previous studies have shown that current national brucellosis control programmes are not effective in eradicating or reducing disease prevalence in either animals or humans (Abdel-Hamid et al., 2020; Eltholth et al., 2017; Sayour et al., 2020).

In the current study, *B. melitensis* bv3 was the predominant strain isolated from the typical (small ruminants) and atypical hosts (large ruminants). This finding indicates the
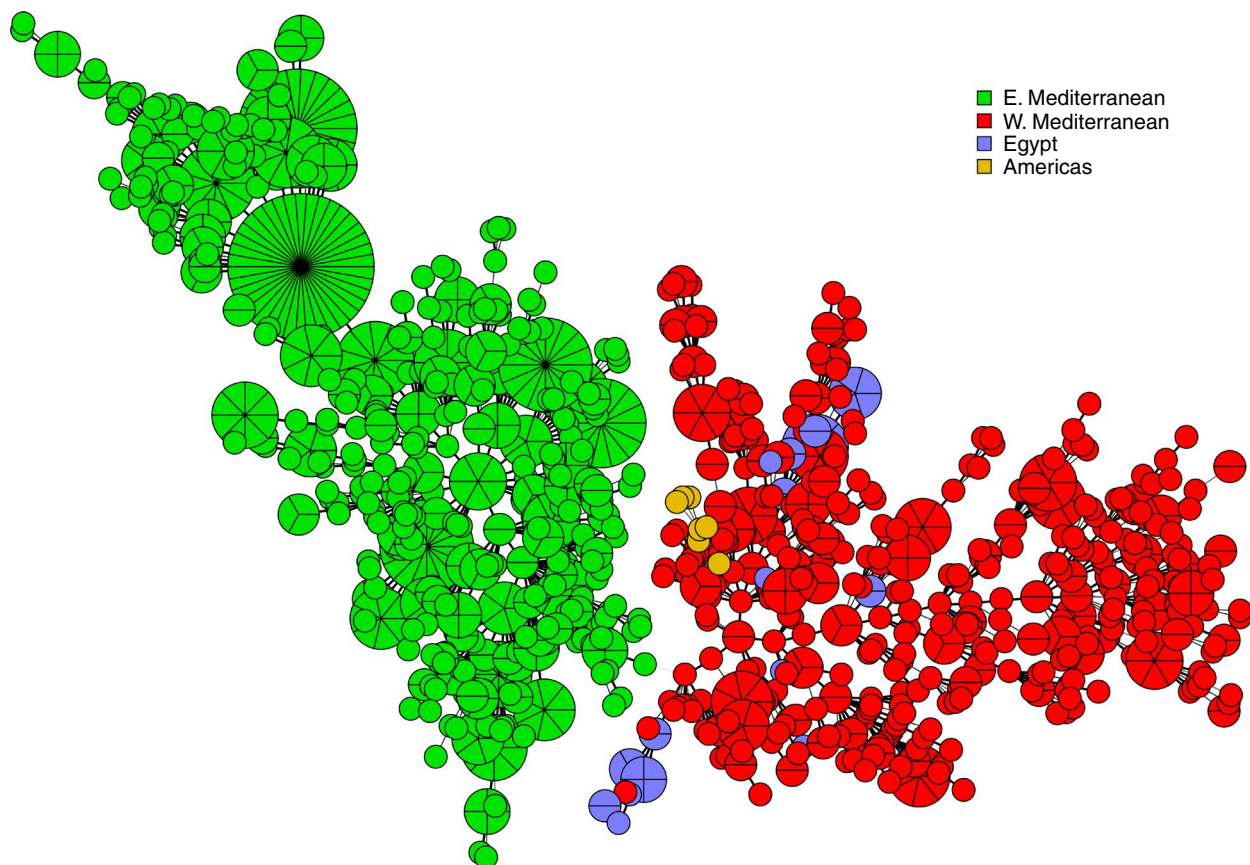
FIGURE 3 Similarity coefficient-based minimum spanning tree of 19 *Brucella melitensis* biovar 3 genotypes of this study with that worldwide

potential cross-species transmission of *B. melitensis* bv3 from the original hosts to large ruminant species in the country, and this may be attributed to the uncontrolled movement of animals in infected areas, as well as the type of animal husbandry practiced (Wareth et al., 2020). In Egypt, part of the national control programme of brucellosis includes the voluntary annual vaccination of heifers with *B. abortus* S19 and kids and lambs with *B. melitensis* Rev 1 vaccine, whereas in some dairy farms, adult naive cows are vaccinated with a commercial *B. abortus* RB51 vaccine (Refai, 2002). The vaccination programme for both small and large ruminants is limited and does not cover all animal population (Eltholth et al., 2017). This may be due to the unsustainability of vaccination programmes due to insufficient budget, uncontrolled animal movement within infected areas and the lack of control on open animal markets (infected animals mixed and in contact with noninfected ones). This was confirmed by the failure of isolation and genotyping of Rev 1 vaccine strain in any specimen (García-Yoldi et al., 2007). These factors may all contribute to the widespread prevalence of *B. melitensis* among small and large ruminants in Egypt.

Our results indicated that *B. melitensis* could be circulated and spread among cattle population, cow to cow transmission, in the absence of sheep and goats (preferential host). These findings are in agreement with previous observations (Godfroid, 2017; Wareth et al., 2020) that cattle could be a reservoir for *B. melitensis* and could transmit it to other cattle in the absence of small ruminants (spill over the infection). Thus, the risk of *B. melitensis* transmission is increased in production systems where ewes, goats and cattle are kept together in the same flock and cannot be isolated during parturition or abortion. Consequently, some *B. melitensis* strains may cross the species barrier and may be sustainably transmitted among cattle, without the persistent influx from the preferential host (Godfroid et al., 2014). This was also concluded by other researchers who isolated *B. melitensis* from cattle, even in the absence of sheep, suggesting a possible role of cattle in maintaining and transmitting this pathogen (Musallam et al., 2016).

The 19 genotypes of *B. melitensis* bv3 based on the MLVA-16 analysis obtained in this study regardless of the high genetic similarities indicate the long-term, widespread prevalence of the disease in Egypt. These results are matching with those reported by (El-Sayed and Awad 2018). Also, the result could be from a small mutation that occurs in the *Brucella* sp. genome (Abdel-Hamid et al., 2020).

Interestingly, in this study, we found more than one genotype from isolates obtained from the same animal, and this was also found by (Mathew et al., 2015) for *B. abortus*. Detection of both *B. abortus* and *B. melitensis* DNA in ovine has been observed in Egypt (Wareth et al., 2015), and this means that depending on the production system, preferential hosts can be infected with two different species or biovars

of *Brucella* at the same time (Martirosyan et al., 2011). The gathering of small ruminants from infected mobile flocks that permits several sources of infection in one place or the introduction of replacement rams purchased from herds with unknown brucellosis status may play a vital role in this trans-species transmission. Another reason for this finding may be the lack of cross protection between genotypes. This may reflect the absence of biosafety and poor hygeinic measures in flocks where different animal species are kept together and in close contact with humans (Hassell et al., 2017). The high seroprevalence and spread of the disease may also stand behind these criteria. The genetic diversity of the *B. melitensis* genotypes identified in this study is in part related to the high discriminatory power of Panel 2B markers.

The geographic distribution of the 19 genotypes obtained in the current study showed the close similarity of some of these genotypes with other genotypes from other governorates. Also, 100% genetic similarity was recorded between two *Brucella* genotypes (61-Egy-Bm3-Sharq and 18-Egy-Bm3-Kshkh) recovered from Sharqia and Kafr El-Sheikh governorates (outside of the study area) with M17_K.E and M11_K.E genotypes found inside the study area. These findings re-ascertain the trans-species transmission of *B. melitensis* across different geographical regions of Egypt. This may be attributed to the lack of control of animal movement in the country. Furthermore, the existence of a large live animal market in close proximity to the study area—Kotor market in Gharbia governorate—may be a risk factor for the distribution of the infection with different genotypes.

Globally, the genotypes identified in the current study are closely related to the Western clonal lineage, with one strain from the African lineage (Tunisia). Egypt does not import animals from African countries for breeding, but animals are imported from European countries, North America and Australia. Our findings suggest that the importation of live animals for breeding from European countries, especially from France and Italy, is a risk factor for the introduction and spread of different *B. melitensis* genotypes in Egypt (Mugizi et al., 2015). In general, the importation of live animals is also a risk factor for the diversity of brucella genotypes (Wareth et al., 2020). These findings could be attributed to the illegal introduction of animals from Libya, a country that suffers from political instability. This allows animals to be smuggled into Egypt through the country's Western borders. Therefore, a risk analysis is required to identify whether the importation and smuggling of live cattle and sheep through country borders for breeding contributes to the existence and spread of the different *Brucella* genotypes obtained.

In conclusion, this study showed a wide diversity of *B. melitensis* bv3 genotypes among different ruminant species in Egypt and the risk of uncontrolled local and international animal movement. It also indicates that this is a

potentially major cause of failure for national control measures for brucellosis. Our novel findings on the biology and epidemiology of *Brucella* spp. are important for reviewing the current strategies for the control of brucellosis in Egypt and other countries with similar production systems. It will also support the selection of the proper vaccinal strain originating from the predominant genotype.

CONFLICT OF INTEREST
The authors declared that they have no competing interests.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.