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Genetic variation of *Brucella* isolates at strain level in Egypt

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Abstract

In this study, Multiple Locus Variable Number Tandem Repeat Analysis (MLVA-16) was performed on 18 *Brucella* isolates identified bacteriologically and molecularly (AMOS-PCR) as *Brucella abortus* (n = 6) and *Brucella melitensis* (n = 12). This was aimed to study the genetic association among some Egyptian *Brucella* genotypes isolated during the period from 2002 to 2013 along with the global genotypes database. MLVA-16 analysis for *B. melitensis* and *B. abortus* strains illustrates a total of 11, and 3 genotypes with 10 and 1 singleton genotypes, respectively. *B. melitensis* strains displayed greater markers diversity by VNTRs analysis of the 16 loci than *B. abortus* and this was attributed mainly to the diverging in panel 2B markers. *B. melitensis* genotype M4_Fayoum_Giza (3,5,3,13,1,1,3,3,8,21,8,7,5,9,5,3) was the only predominated genotype circulating between two different governorates. The most common *B. abortus* genotype, GT A3_Dakahlia (4,5,4,12,2,2,3,3,6,21,8,4,4,3,4,4), was present in three identical isolates. In phylogeny, Egyptian *B. abortus* bv1 genotypes were closely related to East Asian strain (for the first time), Western Mediterranean and Americas clonal lineages. *B. melitensis* local genotypes exhibit a genetic relatedness mostly to Western Mediterranean clonal lineage and one strain of Eastern Mediterranean clonal lineage. In conclusion, the geographic location is not the only factor stands behind the high genetic similarity of the Egyptian *Brucella* genotypes. These low variations may be a result of a stepwise mutational event of the most variable loci from a very limited number of ancestors especially during the transmission through non-preference hosts. The authors encourage the authorities in charge to establish pre-movement testing to reduce the risk of brucellosis spread.

KEYWORDS

Brucella abortus, *Brucella melitensis*, Egypt, markers diversity, MLVA-16, phylogeny

1 | INTRODUCTION

Brucella is Gram-negative bacteria, contains 11 species, which in turn includes many biovars (OIE, 2018). Certain biovars predominate in certain geographical areas, likewise Egypt is mostly enzootic with *B. melitensis* biovar 3 that infect its natural hosts (goats and sheep) and non-specific hosts (cattle and buffaloes) (Abdel-Hamid, Abdel-Mortada, Abd-Elhady, & Farouk,

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2016; Abdel-Hamid, Shell, & Khafagi, 2017a; Afifi, Abdul-Raouf, El-Bayoumy, & Mohamad, 2011; Menshawy et al., 2014; Wareth et al., 2017).

Brucellosis jeopardizes the health of both animals and humans. In animals, it affects animal husbandry economy via abortion, stillbirth, reduction in milk production and slaughtering of serologically positive animals following the Egyptian Brucellosis Control Regulations. In humans, it is an acute febrile illness that may persist and progress to a chronically incapacitating disease with severe complications (Corbel, 2006).

Conventional methods for typing *Brucella* organisms based on biochemical and serological showed incompetence to discern an epidemiological relationship between isolates of the same *Brucella* biovars or to recognize the related isolates derived from a chain of transmission that derived from a single ancestor cell. Subsequently, scholars tried alternatives including antimicrobial susceptibility patterns of the isolated *Brucella* from different animal species and different geographical areas to investigate epidemiological determinants of genus *Brucella*. Besides, there is no methodological adaptation to standardized protocols that can ensure the accurate experimental approach and allow all researchers to compare results (Balouiri, & Ibnsouda, 2016).

Molecular era gives more advantages over traditional bio-typing, serotyping and antimicrobial typing methods but some have limited ability to differentiate Genus *Brucella* at the biovar level. The genus *Brucella* is highly homogeneous (more than 90% DNA/DNA homology (Kattar et al., 2008). Complete genome sequencing of *B.* suis **(**Paulsen et al., 2002**)**, *B. melitensis* **(**DelVecchio et al., 2002**)** and *B. abortus* **(**Halling et al., 2005**)** has revealed remarkable genetic homogeneity.

Variability is the only constant concept in biology even being unapprised until the tools come and bare it (Fraser, 2001). MLVA bounced to solve the mystery *Brucella* DNA homology (highly conserved genome) and it began as HOOF (Hypervariable Octameric Oligonucleotide) print by Bricker, Ewalt, and Halling (2003). The variable number tandem repeats (VNTRs) examine the depth of variability and illustrates the interrelationship among strains. Stable VNTR units at certain loci identify species or even biovar levels in addition to the variable ones discriminates *Brucella* beyond the biovar level (Whatmore et al., 2006).

This study was aimed to investigate the genetic relatedness of local *Brucella* genotypes with the global VNTRs database and to assess the genetic diversity and relationships among the local genotypes through MLVA-16 analysis as a genotyping tool as the data are scarce in this field.

2 | MATERIALS AND METHODS

2.1 | *Brucella* strains' background

Eighteen *B. abortus* (*n* = 6) and *B. melitensis* (*n* = 12) strains were selected for MLVA-16 analysis. During the period from 2002 to 2013, these *Brucella* strains were recovered from 14 districts belonging to seven governorates including Assiut (Dairout) which represents Upper Egypt, Giza (Nahia, Hawamdia and Haram); Fayoum (Dimu) and Beni Suef (Al-Wasta, Beni Suef and Ehnasia) represent Middle Egypt; Sharqia (Kafr Sakr and Minya Al-Qamh), Dakahlia (Mansoora) and Menoufia (Ashmoon and Tookh Tambesha) represent Nile Delta region. The *Brucella* strains were recovered from milk, aborted foeti, fetal fluids, spleen and lymph nodes of live and slaughtered brucellosis serologically positive animals. Isolation and typing of *Brucella* micro-organisms were done at three levels using colony morphology, urease activity, oxidase (Genus level), lysis by phages (Tbilisi, Izatnagar and R/C) and AMOS-PCR (speciation), agglutination with monospecific antisera (A, M and R) dye sensitivity (Thionin and basic fuchsin dyes growth on serum dextrose agar with 20 µg/ml concentration), CO_2 requirement and H_2S production (biovar level) according to Alton, Jones, Angus, and Verger (1988).

2.2 | AMOS-PCR and genotyping of *Brucella* strains using MLVA-16 analysis

Heat inactivation of bacteria was performed at 80°C for 2 hr, then DNA was extracted with the high-pure PCR template preparation kit (Roche Applied Sciences; Mannheim, Germany) according to the manufacturer's instructions. Multiplex (AMOS) PCR for *Brucella* speciation was used to differentiate *Brucella* isolates into species (a step proceeds VNTRs). The AMOS PCR technique was done after Bricker and Halling (1994).

MLVA-16 including eight minisatellite loci (Panel 1) and eight microsatellite loci (Panel 2A and 2B) were performed after (Al Dahouk et al., 2007; Le Fleche et al., 2006) in addition to multicolor capillary electrophoresis (Garfolo, Ancora, & Di Giannatale, 2013a) for 12 isolates of *B. melitensis* bv 3 and six isolates of *B. abortus* bv 1. Genomic DNA of the reference strains *B. abortus* bv 1 strain 544 (ATCC 23448), *B. abortus* bv 1 strain 19 (NCTC 8038), *B. abortus* bv 1 strain 99 (NCTC 11363), *B. abortus* strain RB51 and *B. melitensis* biovar 3 strain Ether (ATCC 23458) was used as a control for alleles assignment. Alleles size (bp) was estimated using **GeneMapper ™** version 5 and converted into the number of repeats by the aid of **BioNumerics** software.

MLVA-16 dendrogram was created based on the categorical similarity coefficient with distance calculation and unweighted pair group method with arithmetic mean (UPGMA) using BioNumerics version 7.6 (Applied Maths, Belgium).

The MLVA-16 data were uploaded to the online MLVA Bank for Microbes Genotyping (http://microbesgenotyping.i2bc.paris-saclay.fr) and genotypes' numbers were generated by the same website. VNTRs data of local *B. abortus* and *B. melitensis* strains were compared with global VNTRs obtained from the previously mentioned website where categorical coefficient with double locus variance priority rules was used to create a standard minimum spanning tree (MST). The genetic diversity of the loci was estimated using the Hunter–Gaston discriminatory index (HGDI) with 95% confidence intervals through V-DICE tool available at the HPA website (http://www.hpa-bioinformatics.org.

TABLE 1 MLVA-16 repeated copy numbers at each locus and Hunter-Gaston Diversity Index (HGDI) with 95% confidence intervals for each locus

Panels	VNTR marker	No. of tandem repeat copies at each locus		HGDI		HGDI lower and upper limits at CI 95%		Number of alleles	
		B. abortus	*B. melitensis*	*B. abortus*	*B. melitensis*	*B. abortus*	*B. melitensis*	*B. abortus*	*B. melitensis*
Panel 1	Bruce06	3,4	3	0.119	0.000	0.098–0.175	0.000–0.069	2	1
	Bruce08	5	5	0.000	0.000	0.000–0.075	0.000–0.065	1	1
	Bruce11	4	3	0.000	0.000	0.000–0.082	0.000–0.074	1	1
	Bruce12	12	13	0.000	0.000	0.000–0.052	0.000–0.062	1	1
	Bruce42	2	1	0.000	0.000	0.000–0.061	0.000–0.059	1	1
	Bruce43	2,3	1	0.201	0.000	0.195–0.237	0.000–0.048	2	1
	Bruce45	3	3	0.000	0.000	0.000–0.051	0.00–0.062	1	1
	Bruce55	3	3	0.000	0.000	0.00–0.052	0.000–0.054	1	1
Panel 2A	Bruce18	6	7,8	0.000	0.205	0.000–0.063	0.198–0.246	1	2
	Bruce19	21	21	0.000	0.000	0.000–0.049	0.000–0.057	1	1
	Bruce21	8	8	0.000	0.000	0.000–0.071	0.000–0.053	1	1
Panel 2B	Bruce04	3,4	5,6,7	0.201	0.335	0.196–0.245	0.312–0.359	2	3
	Bruce07	4,7	5,6,7,8	0.202	0.71	0.196–0.265	0.691–0.762	2	4
	Bruce09	3	5,6,7,8,9,10,11	0.000	0.921	0.000–0.071	0.902–0.972	1	7
	Bruce16	3,4	4,5,6,8,10,11	0.207	0.833	0.191–0.251	0.810–0.869	2	6
	Bruce30	4,5	3	0.204	0.000	0.192–0.248	0.000–0.052	2	1

uk/cgi-bin/DICI/DICI.pl) where it ranged from zero (identical strains) to one (different strains) as shown by Hunter and Gaston (1988). The allelic diversity (HGDI) was classified as high if the discriminatory power of HGDI is more than 0.6, moderately discriminatory if 0.3 ≤ HGDI≤0.6 and poorly discriminatory if HGDI < 0.3 (Sola et al., 2003).

2.3 | The geographic distribution of some Egyptian *Brucella* genotypes

An electronic map of Egypt was obtained from the General Organization of Veterinary Services (GOVS), Egypt. The map was built using Quantum GIS (Quantum GIS Development Team 2017), (http://www.qgis.org.). Then, *B. abortus* and *B. melitensis* genotypes were plotted in the electronic map by using PowerPoint office 2016 and saved as an image.

3 | RESULTS

The provided 18 *Brucella* isolates were fitted to the identification scheme of Alton et al. (1988) and AMOS-PCR (Bricker & Halling, 1994). Of the 18 isolates (Table 2), 12 proved to be *B. melitensis* biovar 3 and six isolates proved to be *B. abortus* biovar 1 phenotypically and genotypically. Molecular typing of the *Brucella* isolates using AMOS-PCR illustrates PCR bands of 731 bp and 498 bp in size-specific for *B. melitensis and B. abortus*, respectively. Most *Brucella* isolates (12/18) in this study were *B. melitensis* bv3. These isolates were recovered from preference host (sheep, n = 6 and goats, n = 1) in addition to occasional host (cattle, n = 4 and buffalo, n = 1) followed by *B. abortus* bv1 (6/18) which isolated only from the preference host (cattle).

To calculate the diversity of *B. abortus*, and *B. melitensis* under the condition of the current research to compare the discriminatory power of MLVA typing approaches (Table 1), HGDI values were estimated for each marker included within MLVA-16 subsets (Table 1). Panel 1 markers were monomorphic displaying single alleles in all *Brucella* species under the field of this study except Bruce06 and Bruce43 in *B. abortus*, where they showed a different tandem repeat copy numbers with low discrimination and HGDI of 0.119 and 0.201, respectively.

In contrast, Bruce07, Bruce16 and Bruce30 from panel 2 B were highly discriminatory in *B. melitensis* (HGDI > 0.7), whereas Bruce18 and Bruce04 displayed low-to-moderate discrimination and the remaining 11 loci exhibited only single allele. Bruce06, Bruce43, Bruce04, Bruce07, Bruce16 and Bruce30 markers displayed low discrimination in *B. abortus* (HGDI > 0.2) and the remaining 10 markers showed no discrimination with HGDI = 0 (single alleles). Markers of Panel 2A were monomorphic in all *Brucella* species except for Bruce18 and 19 markers which showed different copy numbers of the tandem repeats in case of *B. melitensis* with low diversity (HGDI > 0.2).

Table 2 MLVA-16 analysis for the 12 *B. melitensis* and the 6 *B. abortus* isolates showed a total of 11, and 3 genotypes with 10 and 1 singleton genotypes, respectively. Genotype M4_Fayoum_Giza (3 ,5,3,13,1,1,3,3,8,21,8,7,5,9,5,3) was the only genotype circulating

TABLE 2 Details and VNTRs of *B. abortus* and *B. melitensis* genotypes

Key	*Brucella* spp and bv	Governorate	Town/ district	Animal spp.	Specimen	Genotypes	Br06	Br08
DNA 20	*B. abortus bv1*	Beni Suef	Al-Wasta	Cattle	Lymph nodes	A1_Beni Suef	4	5
DNA 44	*B. abortus bv1*	Sharqia	Minya Al-Qamh	Cattle	Abortion fluid	A2_Sharqia	3	5
DNA 26	*B. abortus bv1*	Sharqia	Kafr Sakr	Cattle	Abortion fluid	A2_Sharqia	3	5
DNA 57	*B. abortus bv1*	Dakahlia	Mansoora	Cattle	Spleen	A3_Dakahlia	4	5
DNA 58	*B. abortus bv1*	Dakahlia	Mansoora	Cattle	Aborted fetus	A3_Dakahlia	4	5
DNA 59	*B. abortus bv1*	Dakahlia	Mansoora	Cattle	Aborted fetus	A3_Dakahlia	4	5
DNA 54	*B. melitensis bv3*	Menoufia	Ashmoon	Sheep	Spleen	M1_Menoufia	3	5
DNA 55	*B. melitensis bv3*	Beni Suef	Beni Suef	Cattle	Lymph nodes	M2_Beni Suef	3	5
DNA 56	*B. melitensis bv3*	Menoufia	Ashmoon	Sheep	Lymph nodes	M3_Menoufia	3	5
DNA 42	*B. melitensis bv3*	Fayoum	Dimu	Cattle	Milk	M4_Fayoum_Giza	3	5
DNA 51	*B. melitensis bv3*	Giza	Hawamdia	Cattle	Lymph nodes	M4_Fayoum_Giza	3	5
DNA 43	*B. melitensis bv3*	Assiut	Dairout	Buffalo	Milk	M5_Assiut	3	5
DNA 45	*B. melitensis bv3*	Giza	Nahia	Sheep	Milk	M6_Giza	3	5
DNA 46	*B. melitensis bv3*	Sharqia	Minya Al-Qamh	Sheep	Lymph nodes	M7_Sharqia	3	5
DNA 47	*B. melitensis bv3*	Sharqia	Minya Al-Qamh	Sheep	Lymph nodes	M8_Sharqia	3	5
DNA 49	*B. melitensis bv3*	Sharqia	Minya Al-Qamh	Cattle	Spleen	M9_Sharqia	3	5
DNA 50	*B. melitensis bv3*	Giza	Nahia	Sheep	Milk	M10_Giza	3	5
DNA 52	*B. melitensis bv3*	Giza	Haram	Goat	Milk	M11_Giza	3	5

between two different governorates (Fayoum and Giza). Regardless of genotype M4_Fayoum_Giza, all genotypes are singletons (unique) and circulating in the same governorates where they were recovered (Figure 1). Data regarding, governorates, districts, animal species and specimens of all *Brucella* species and biovars under the field of this study as well as the VNTR profiles of the genotypes are shown in Table 2 and were uploaded in the *Brucella* MLVA database at http://mlva.u-psud.frhttp://mlva.u-psud.fr.

As MLVA-16 genotyping correlated well with epidemiological data (Al Dahouk et al., 2007; Allen et al., 2015), the dendrogram of the genetic variations of all *B. abortus* strains based on MLVA-16 analysis was performed. MLVA-16 data analysis displayed highly consistent results among isolates from the same or nearby governorates and sharing the same MLVA-16 genotypes (Figure 1, Figure 2, and Table 2). Considering a similarity of approximately cutoff value of 95%, *B. abortus* isolates were grouped into two major clusters. Cluster I grouped all the reference strains and the genotype A1_Beni Suef (4,5,4,12,2,3,3,3,6,21,8,3,7,3,3,5) with 96% similarity. Cluster II included the two genotypes (GTs) of the Nile Delta governorates A2_Sharqia (3,5,4,12,2,2,3,3,6,21,8,4,4,3,4,4) which consists of two identical strains (DNA 26 and 44) that belonged to two different districts (Kafr Sakr and Minya Al-Qamh) within Sharqia governorate and A3_Dakahlia (4,5,4,12,2,2,3,3,6,21,8,4,4,3,4,4) which consists of three identical strains (DNA 57, 58 and 59) that belonged to one district Mansoora, Dakahlia, with 99% similarity.

When it comes to the dendrogram of *B. melitensis* genotypes (Figure 3), the high similarity of 95% resulted in the grouping of the genotypes into two clusters. Cluster 1 grouped M1_Menoufia, M2_Beni Suef, M3_Menoufia, M7_Sharqia and M11_Giza genotypes with 97% similarity. Cluster 2 includes M4_Fayoum_Giza, M5_Assiut, M6_Giza, M8_Sharqia, M9_Sharqia and M10_Giza genotypes along with the reference strain with a similarity of 95%.

To get the evolutionary associations between *B. abortus* bv1 lineages (Egyptian genotypes) and the global VNTRs of *B. abortus* bv1 recovered from different localities, dendrogram and MST analysis (Figures 4 and 5) of 41 *B. abortus* bv1 strains along with the reference strains was performed using the MLVA-16 typing data (Figure 4), including those from this study (M1_Menoufia, M2_Beni Suef, M3_Menoufia, M4_Fayoum_Giza, M5_Assiut, M6_Giza, M7_Sharqia, M8_Sharqia, M9_Shrqia, M10_Giza and M11_Giza genotypes) and other published manuscripts (Ferreira et al., 2012; Garofolo, Di Giannatale, et al., 2013b; Her et al., 2009; Jiang et al., 2013; Le Flèche et al., 2006; Minharro et al., 2013; Shevtsov et al., 2015) and available at http://microbesgenotyping.i2bc.paris-saclay.fr/databases/public. All Egyptian genotypes along with strains belonged to Eastern, Western Mediterranean and Americas clonal linages were grouped into one cluster (cluster1) with a global similarity of 95%. Except for those grouped in cluster1, *B. abortus* bv1 strain *B. abortus* 671 and 706 recovered from Kazakhstan and strain 45 and Jr 05 recovered from Brazil were classified into different clusters (cluster 2, 3 and 4, respectively).

Dendrogram and MST of 54 *B. melitensis* bv3 (Figures 6 and 7) including the reference strain (*B. melitensis* bv3 Ether strain) were created seeking the genetic relatedness between the MLVA-16 data analysis of 11 *B. melitensis* genotypes included within 12 *B. melitensis* isolates recovered from different districts and governorates with the global VNTRs data of other published researches (Al Dahouk et al., 2007; Garofolo, Di Giannatale, et al., 2013b; Kilic et al., 2011; Le Fleche

Br11	Br12	Br42	Br43	Br45	Br55	Br18	Br19	Br21	Br04	Br07	Br09	Br16	Br30
4	12	2	3	3	3	6	21	8	3	7	3	3	5
4	12	2	2	3	3	6	21	8	4	4	3	4	4
4	12	2	2	3	3	6	21	8	4	4	3	4	4
4	12	2	2	3	3	6	21	8	4	4	3	4	4
4	12	2	2	3	3	6	21	8	4	4	3	4	4
4	12	2	2	3	3	6	21	8	4	4	3	4	4
3	13	1	1	3	3	8	21	8	5	7	10	10	3
3	13	1	1	3	3	7	21	8	5	7	7	11	3
3	13	1	1	3	3	8	21	8	5	8	10	10	3
3	13	1	1	3	3	8	21	8	7	5	9	5	3
3	13	1	1	3	3	8	21	8	7	5	9	5	3
3	13	1	1	3	3	8	21	8	6	5	6	6	3
3	12	1	1	3	3	7	21	8	6	6	11	6	3
3	13	1	1	3	3	7	21	8	5	7	5	8	3
3	13	1	1	3	3	8	21	8	6	5	9	5	3
3	13	1	1	3	3	7	21	8	6	5	8	4	3
3	13	1	1	3	3	7	21	8	6	6	11	6	3
3	13	1	1	3	3	7	21	8	5	7	9	11	3

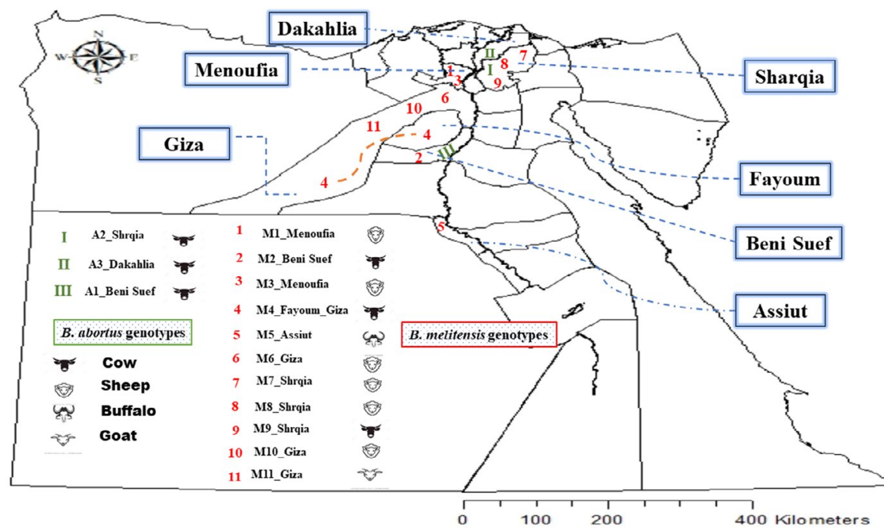

FIGURE 1 Geographic distribution of *B. abortus* and *B. melitensis* genotypes recovered from ruminants in Egypt

et al., 2006; Marianelli et al., 2007; Vergnaud et al., 2018) available at http://microbesgenotyping.i2bc.paris-saclay.fr/databases/public. All the Egyptian genotypes are grouped with all Western and one strain of Eastern Mediterranean clonal lineage into one cluster (cluster1).

4 | DISCUSSION

Multiple studies have been confirmed regarding the usefulness of MLVA in *Brucella* genotyping and identification where the metadata can be used in the epidemiological monitoring and tracking of the source of *Brucella* infection (Ferreira et al., 2012; Garofolo, Di Giannatale, et al., 2013b; Shevtsova et al., 2016).

At this moment, over a five thousand variable number tandem repeats data of different *Brucella* species and biovars are available online through http://microbesgenotyping.i2bc.parissaclay.fr/databases/public, facilitate the epidemiological traceback purpose and seeking the genetic association and relatedness of the Egyptian *Brucella* genotypes with their peers worldwide.

The bovine infection with *B. melitensis* represents a serious problem as a result of a large volume of infected milk produced by infected animals and because of the high environmental contamination induced

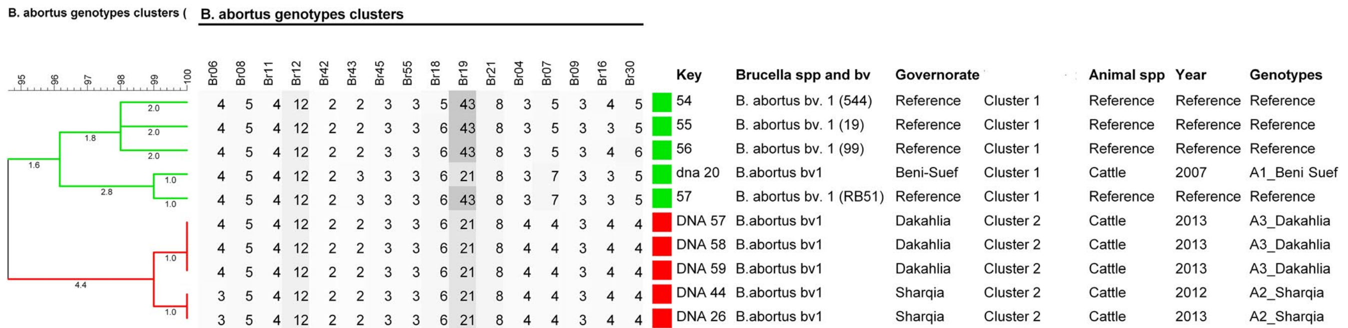

FIGURE 2 MLVA-16 dendrogram showing the genetic relatedness of six *B. abortus* strains (3 genotypes) in comparison with the reference strains

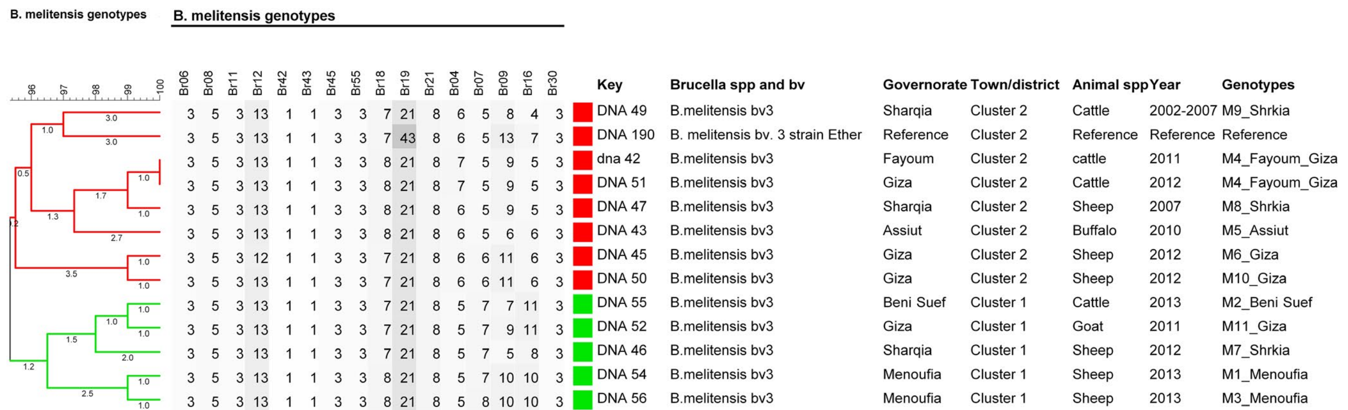

FIGURE 3 MLVA-16 dendrogram showing the genetic relatedness of 12 *B. melitensis* strains (11 genotypes) in a comparison with the reference strain (*B. melitensis* bv3 Ether strain)

as a result of abortions or infected births (Corbel, 2006). At the same time, *B. melitensis* consider the most virulent type among the known brucellae (Corbel, 2006). Both of *B. melitensis* and *B. abortus* are the predominant strains in Egypt with *B. melitensis* being the more prevalent and this finding is matching with the results of other published researches (Abdel-Hamid et al., 2016; Abdel-Hamid, Shell, et al., 2017a; Afifi et al., 2011; Menshawy et al., 2014; Wareth et al., 2017) and this was reflected by the relatively high number of *B. melitensis* isolates (n = 12) compared with *B. abortus* isolates (n = 6) in this study as shown by Table 2. The high prevalence of *B. melitensis* bv 3 throughout the country as a predominating *Brucella* species compromises the whole epidemiological situation necessitates a high discriminatory tool to assess the genetic diversity and relatedness among the local genotypes.

B. melitensis strains displayed a greater markers diversity by VNTRs analysis of the 16 loci than *B. abortus* (Table 1). It seems that the diversity of *B. melitensis* isolates in Egypt is related with the high frequency of infected sheep, goat and cattle and the role of the mobile flocks in this pattern (Abdel-Hamid, Ghobashy, et al., 2017b) and their subsequent movement within or throughout the governorates in addition to the predomination of *B. melitensis* as the most circulating *Brucella* among different animal species.

The most commonly noticeable genotype of *B. abortus* genotypes as shown by Figure 2 and Table 2, GT A3_Dakahlia, was present in three identical isolates recovered from one district. Followed by GT A2_Sharqia which consists of two identical strains isolated from two districts. The low variation or the diversity among clusters is mainly due to diverging in panel 2B markers and Bruce06 and Bruce43 markers of panel 1 and these results are matching the results of Kiliç et al. (2011) and Shevtsova et al. (2016). Unlikely, *B. abortus* can be isolated from small ruminants as this considered rare (Aparicio, 2013) and all the *B. abortus* isolates in this study are recovered from preference host (cattle). Two *B. abortus* genotypes (A2_Sharqia, and A3_Dakahlia) of three (Figure 1) were recovered from Delta region where the high animal populations are located.

MLVA-16 analysis of *B. melitensis* (Figure 3) local strains showed a homogeneity or very low variation among the included genotypes of both clusters 1 and 2. The variation or the diversity among clusters of *B. melitensis* genotypes (genetic polymorphism) is mainly due to diverging in only three markers of panel 2B (Bruce07, Bruce09 and Bruce16) of 16 markers with high diversity (HGDI > 0.7). Besides, Bruce04 of panel 2B (HGDI = 0.335) as well as Bruce18 marker of panel 2A (HGDI = 0.205) which exhibited low-to-moderate diversity.

B. melitensis Genotype M4_Fayoum_Giza circulating between two different governorates (Figure 1 and Figure 3) is, in fact, a reflection of corrupted or improper implementation of the current control program applied over 30 years (test and slaughter of adult serologically positive and vaccination of young replacement) which

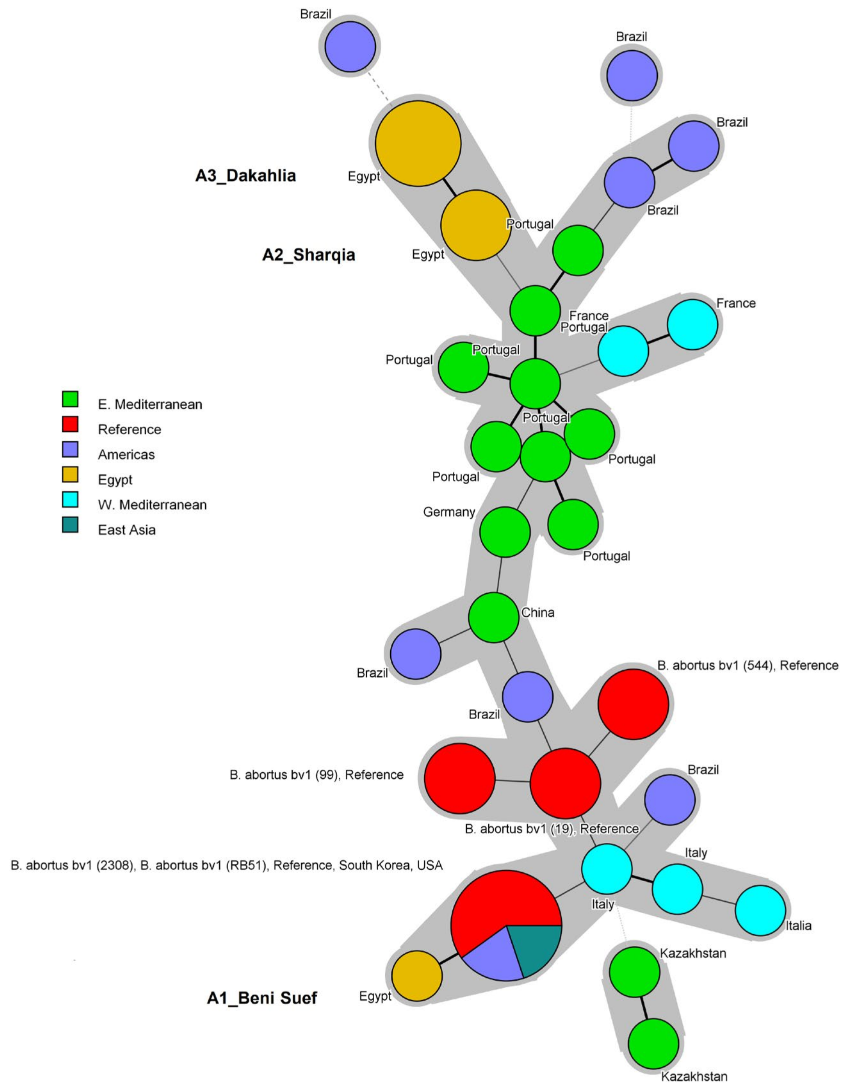

FIGURE 4 Minimum spanning tree based on categorical similarity coefficient of six *B. abortus* bv1 Egyptian genotypes isolated from cattle compared with the global VNTRs database

basically required two steps of paramount importance; 1 – control of animal movement, as it considered one of the most problematic issues facing any veterinary services involved in eradication programs, which are completely lacked as shown in the results (one genotype circulating between two different governorates); 2 – animal identification which is lacked as well. Isolation of *B. melitensis* from infected cattle and buffaloes under the field of this study (M2_Beni Suef; M4_Fayoum_Giza, M5_Assiut, M9_Shrqia genotypes) is a reflection of low biosecurity where different animal species are reared together in the same place as in case of Egypt and Middle East countries (Blasco, 2010) and in close contact to humans. In these environments where mixed breeding is existing, *B. melitensis* high infectious possibilities for cattle may be enhanced (Blasco & Molina-Flores, 2011; Neiderud, 2015; OIE, 2018). As a sequel of this, *B. melitensis* strains may cross the interspecies barrier and may be sustainably transmitted to cattle (reservoir), without the constant influx of *B. melitensis* from small ruminants (spillover infection) as cited by Godfroid, (2017). *B. melitensis* infection in non-preference hosts is characterized by shedding much greater organisms especially if an abortion occurs and thus dangerous to the contact persons because of the high virulence of most *B. melitensis* strains and the large numbers of excreted bacteria by cattle (Corbel, 2006).

None of the 18 *B. melitensis* or *B. abortus* local genotypes have been included in the MLVA Bank for Microbes Genotyping (cumulative excel sheet *Brucella*_4_3).

In phylogeny (Figure 4) and (Figure 5) seeking the genetic association between *B. abortus* local strains with their peers worldwide and by looking deeper inside cluster1, genotype A1_Beni Suef, the singleton GT, exhibit a closer relation to biovar 1 of East Asia (South Korea) clonal lineages (sub-cluster 1F) and *B. abortus* bv1 reference strain (RB51 and 2308) with a global similarity of approximately 99%, while GTs A2_Sharqia and A3_Dakhlia are clustered together in one separate sub-cluster (1B) with a similarity of 99%. GTs A2_Sharqia, and A3_Dakhlia are genetically closer to the E. Mediterranean clonal lineage (Portugal) and Americas clonal lineage (Brazil). In the light of host species associations revealed from the Figure 5 in all clusters (1,2,3,4), the prevailing host species are cattle (70.7%; 29/41) followed by buffalo (4.8%; 2/41). In Egypt, *B. abortus* was recovered mostly from preference host (cattle) as well as non-preference hosts *Viz* buffalos and camels (Abdel-Hamid, Shell, et al., 2017a;

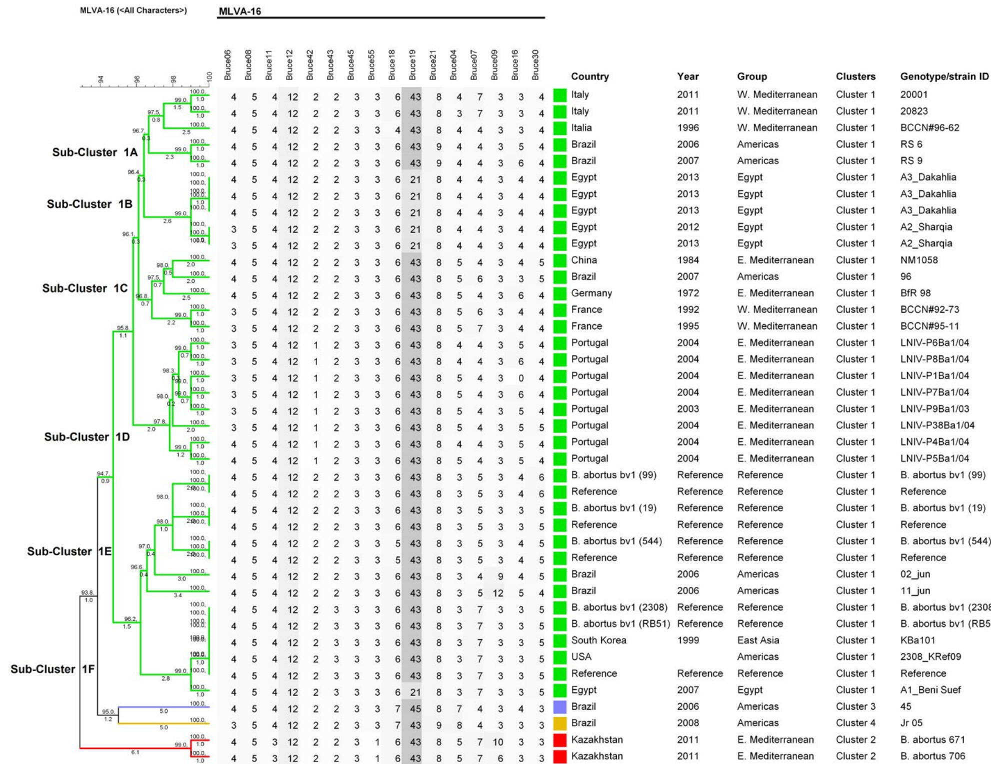

FIGURE 5 Dendrogram based on categorical similarity coefficient of six *B. abortus* bv1 Egyptian genotypes isolated from cattle compared with the global VNTRs downloaded from the MLVA Bank for Microbes Genotyping

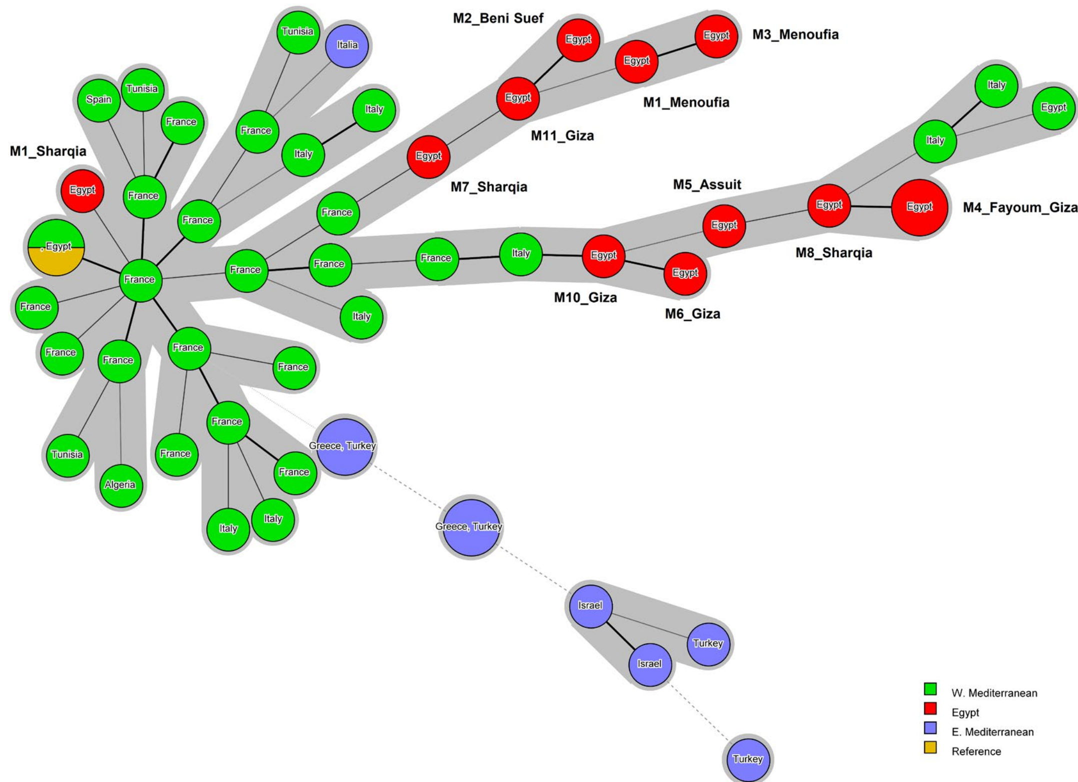

FIGURE 6 Minimum spanning tree based on categorical similarity coefficient of 12 *B. melitensis* bv3 Egyptian genotypes isolated from cattle compared with the global VNTRs database

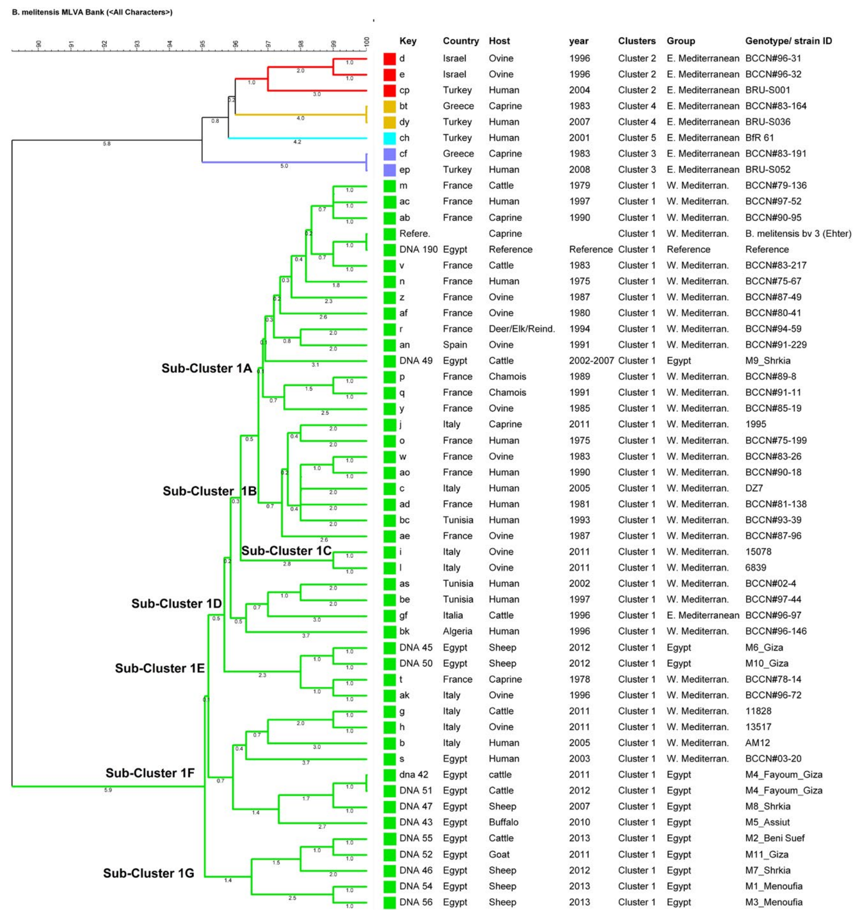

FIGURE 7 Dendrogram based on categorical similarity coefficient of 12 *B. melitensis* bv3 Egyptian genotypes isolated from cattle compared with the global VNTRs downloaded from MLVA Bank for Microbes Genotyping

EL-Seedy, Radwan, & El-Shabrawy, 2000; Menshawy et al., 2014). Amplification of the DNA of *B. abortus* from samples of aborted small ruminants was reported by Wareth, Melzer, Tomaso, Roesler, and Neubauer (2015), but still no definite isolation of *B. abortus* from small ruminants yet.

With a global similarity of 97% (Figures 6 and 7), GT M9_Sharqia was grouped with *B. melitensis* strains recovered from France, Spain (W. Mediterranean clonal lineage) and the reference strain in the sub-cluster 1A. GTs M6_Giza and M10_Giza with two strains of France and Italy (W. Mediterranean clonal lineage) are grouped in sub-cluster 1E with a global similarity of 96%. GTs M5_Assiut, M8_Sharqia and M4_Fayoum_Giza were grouped with a human strain isolated from Egypt (BCCN#03-20) and three *B. melitensis* bv 3 Italian strains (W. Mediterranean clonal lineage) into sub-cluster 1F with a similarity of 95%. GTs M2_Beni Suef, M11_Giza, M7_Sharqia, M1_Menoufia and M3_Menoufia have grouped alone into a separate sub-cluster 1G with a similarity of 95%. Strains recovered from Turkey, Greece and Israel were grouped into different clusters (cluster 2, 3 and 4) with a global similarity of approximately 90% to cluster 1. None of the local *B. melitensis* and *B. abortus* strains is genetically identical to the strains of the African origin, hence MLVA-16 database of the African strains in the MLVA Bank for Microbes Genotyping is scarce especially from neighbourhood countries and this hinders to somewhat the possibility of traceback the source of *Brucella* infection.

When it comes to the host species associations revealed from Figure 7 in all clusters (1,2,3,4), the prevailing host species are small ruminants (44.4%; 24/54) followed by human strains (29.6%; 16/54) and large ruminants (16.6%; 9/54). Few *B. melitensis* isolates have been recorded from Chamois (3.7%; 2/54) and one isolate from reindeer (1.8%) in addition to two reference strains. *B. melitensis* is the most frequent causative agent of small ruminant brucellosis and the main highly virulent pathogen responsible for human brucellosis, followed by *B. suis* and less frequently *B. abortus*. These facts reflecting the second-high percentage of infection in humans after the preference host (small ruminant) as shown by Figure 5, which represents 54 VNTRs data of published researches including our genotypes.

This study is considered the second one that deals with the same topic in Egypt after Menshawy et al., 2014. On the contrary, in this study, MLVA-16 was performed instead of MLVA-15 which is not included the Bruce19 pattern of panel 2A. Discrimination of some strains included in the MLVA Bank was based on Bruce19 pattern only where the other 15 markers were monomorphic and displayed only a single allele like the patterns of strain baboCR6 and baboCR64 cited in Hernández-Mora et al. (2017). Matching the results of Menshawy et al. (2014), *B. melitensis* local strains belonged to Western Mediterranean clonal lineages.

To our knowledge, the present data revealed in this study is the first report cited the similarity of *B. abortus* local strains to field

strain of East Asia group (KBa 101) isolated from South Korea earlier in 1999 with a global similarity of 99%.

High genetic relatedness and similarities between the local *B. abortus* and *B. melitensis* genotypes under the field of this study may reflect micro-evolution through a stepwise mutational event of the most variable loci from a very limited number of ancestors of the indigenous local genotypes (Liu et al., 2017) and that excludes the idea of foreigner strains new infection occurrence from other countries as the variations were located mainly in panel 2B loci.

4.1 | Conclusion and recommendations

From the previous results, authors concluded that the geographic location is not the only factor that stands behind the similarity of the isolated strains of *Brucella* but also some stains seem to be genetically very close, although they were not isolated from the same locality which may indicate that uncontrolled animal movement between different governorates may play a vital role in the transmission of the diseases.

As the restriction of the animal movement is a cornerstone in the current applied eradication program of brucellosis, the authors recommended that authorities in charge shall establish pre-movement testing to reduce the risk of spread of brucellosis between or within governorates which in turn will provide additional assurance of the proper implementation of the control strategy provided that other basic requirements shall be fulfilled.

It seems that panel 2 precisely panel 2B offered high diverting power for genetic relatedness or association of *Brucella* strains in the endemic areas with the disease as in case of this study and others and may be used as an epidemiological tool in this purpose putting in the consideration that this panel cannot be used solely for *Brucella* speciation. However, further investigation on a large scale is required to strengthen this finding along with the whole genome sequencing.

MLVA-16 analysis and DNA sequencing of a larger number of isolates from various geographic areas in Egypt still needed to develop the required insight *Brucella* fingerprint database that can be searched for epidemiological use worldwide.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

AUTHOR CONTRIBUTION

Nour H. Abdel-Hamid: Data curation; Formal analysis; Project administration; Visualization; Writing-original draft; Writing-review & editing. **Essam Mohamed El-bauomy**: Methodology; Project administration; Supervision; Visualization; Writing-review & editing. **Hazem Mohamed Ghobashy**: Data curation; Investigation; Supervision; Writing-original draft; Writing-review & editing. **Abeer Ahmed Shehata**: Data curation; Supervision; Visualization; Writing-review & editing.

ETHICAL STATEMENT

The authors confirm that the ethical policies of the journal, as noted on the journal's author guidelines page, have been adhered to. No ethical approval was required as samples collection from animals have been not gathered.

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