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16S RNA gene based Molecular characterization of Brucella isolates from bovine brucellosis in Egypt

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A total of 227 specimens (3 aborted foeti, 125 milk samples, 33 lymph nodes, 33 livers and 33 spleen) from cattle and buffalo were collected and examined for isolation and identification of *Brucella* microorganism. The study revealed 9 isolates (aborted foeti 1, milk 2, lymph nodes 4 and spleen 2) and these were identified as *Br. melitensis* biovar3. The 16S rRNA gene of one of the *Br. melitensis* isolate was sequenced and found identical to *B. melitensis* bv.3 strain (Gene Bank accession No.MT 378400). On sequencing, the Nucleotide sequence alignment of obtained sequences with other *Brucella* strain indicated isolates with high identity with *Br. melitensis* biovar 3.

Keywords: Egypt, Brucellosis, Cattle, 16S rRNA and PCR.

INTRODUCTION

Brucellosis is an infectious and zoonotic disease widely distributed all over the world; caused serious problems on the animal and human level. Brucella infection is endemic in Mediterranean countries including Egypt (Sayour et al. 1970; Hamdy, 1992 and Franc et al. 2018). After infection for the first time among herd of animals a storm of abortion during last third of gestation period happened followed by series of reproductive problems such as retained placenta, stillbirth and individual cases of abortion in female animals; in male animals orchitis and epididymitis usually observed (Morgan and Mackinnon, 1979 and Coletha 2017). Direct contact of human with infected materials such as placenta or aborted fetuses as well as consumption of contaminated milk or dairy product resulted in the disease in man. Human infection associated with fever, fatigue and headache with joint pain, bone abscess formation and endocarditis (Dean et al. 2012). Brucella consider as member of alpha-Proteobacteria and characterized by its appearance as Gram-negative, coccobacilli and non-motile. Brucella characterized by high homology of its genetic material so that in 1980s it was supposed that Brucella is a mono-specific genus (*Brucella melitensis*) which has 6 biovars differentiated according to their host preference. The advancement in bioinformatics clears that *B. melitensis*, *B. abortus*, *B. ovis* and *B. neotomae* consider as 4 related clones of single organism while *B. suis* (including *B. suis biovar* 5) found in a separate cluster from them (Amr El-Sayed and Walid Awad 2018).

Infection by *Brucella spp*. can be confirmed by using several diagnostic tools such as; isolation, PCR or serological tests. Recently most laboratories used PCR instead of conventional bacteriological techniques because it characterized by safety, high sensitivity, high specificity and more safe than ordinary bacteriological methods (Ahmed et al. 2016). PCR assay has been shown to be a valuable rapid and sensitive technique in many national and international publications (Amin et al. 1995; 2001; Hamdy and Amin, 2002; Richtzenhain et al. 2002; Tantillo et al. 2003; El- Faki et al. 2005b; Garin-Bastuji et al. 2006; Gupta et al. 2006a; Leary et al. 2006). On the other hand the sequencing of PCR products allowed rapid identification and typing of the isolates (Tan et al. 2015). Large number of PCR based assays have been developed and adjusted for the identification of *Brucella* species ranged from genus identification (Genus-Specific PCR assay), that is designed to amplify a single unique genetic locus that was highly conserved in *Brucella* (e.g. 43 KDaomp, BCSP31, IS6501/711 or 16SrRNA genes) (Fekete et al. 1990; Baily et al. 1992; Herman and DeRidder, 1992; Halling et al. 1993; Romero et al. 1995a; Da Costa et al. 1996; Casanas et al. 200***1*** and Rejoice et al. 2020); to species identification (differential PCR-based assay), that depends on strain locus specific multiplexing (e.g. AMOS-PCR based on IS711, PCRRFLP or RAPD-PCR), (Bricker and Halling, 1994; Bricker and Halling, 1995; Sifuentes et al. 1997; Tchernevaetal., 2000; Adone et al. 2001; Redkar et al. 2001; Probert et al. 2004; Ocampo-Sosa et al. 2005). Differential PCR based assays are particularly useful for epidemiological trace back or for species-specific eradication programs (Bricker, 2002). Recently diagnosis depending on 16S rRNA gene for rapid and simple identification had been applied; the 16S rRNA component of 30S small subunit of prokaryotic ribosomes contains hyper-variable regions that provide specific sequences useful for bacterial identification, so it was used for confirmatory identification of Brucella (Weisburg et al. 1991 and Ramya et al. 2015). Molecular identification of *Brucella spp.* depending on16S rRNA gene showed excellent results for its identification (Weisburg et al. 1991; Bricker and Halling., 1994; Casa˜nas et al. 2001; Kumar et al. 2011; Singh et al. 2013). Low variability in the 16S rRNA locus has been noted as an impediment in using 16S rRNA gene sequencing to discriminate at the species level (Vizcaino et al. 2000). Mustafa et al. (2017) finds 90-99% sequence identity with *B. melitensis.* In Egypt several studies on molecular diagnosis of brucellosis had been applied such as; Hosein et al. 2018; used universal PCR targeting immune dominant antigen, gene *bp26* and Bruce ladder multiplex PCR for detection of Brucella DNA directly from tissues of seropositive animals as well as on DNA extracted from bacterial culture. The assay has amplified the target gene (immune dominant antigen, gene *bp26)* with fragment size of 450 bp. Bruce ladder multiplex PCR proved the presence of genetic materials of *Brucella melitensis* in culture DNA extracts as it amplified three fragments (587, 1071 1682 bp in length). Also same techniques had been used by Yosry et al. 2019 during their epidemiological study about bovine brucellosis at Menoufia province in Egypt. While only one paper used the newly PCR assay depending on 16S RNA gene in diagnosis of Brucella infection among sheep (Elsayed et al. 2013). This study considers the first used of newly PCR technique depending on 16S RNA gene for diagnosis of Brucella infection among cattle in Egypt.

MATERIALS AND METHODS

This study had been done during period from March 2018 to March 2020. Different types of samples had been collected from Giza and Beniseuif provinces for isolation of *Brucella spp.* as shown in table (1). 125 milk samples (Twenty ml of milk were collected from udder of reactors animal into a sterile vacationer tube); The stomach contents of 3 aborted foeti from cattle were collected according to Stableforth & Galloway (1959); 33 lymph nodes (supramammary, internal iliac and retropharyngeal lymph nodes); 33 liver samples & 33 spleen samples had been collected and transported to the laboratory under cooling condition for bacteriological examination. All samples had been collected after serological testing of suspected animals by local authorities and confirmed as positive for *Brucella* infection and tissue samples had been collected after emergency slaughtering of serologically positive cases.

The recommendations of the FAO/WHO Expert Committee on Brucellosis (Alton et al. 1988) for isolation and identification of *Brucella* was performed. Milk, lymph nodes, spleen, liver and aborted foeti samples had been cultured on Brucella selective medium consisting of Brucella Medium Base (Oxoid®) supplemented with Brucella Selective Supplement (Oxoid®) and incubated aerobically under 10% CO_2 for 10 days and examined daily for the presence of colonies. Culture procedures were performed in a Biohazard safety cabinet class II type A/B3 (model no. NU-425-400E, Nuaire ™, Plymouth, MN 55447, USA) had been used for culturing and handling procedures. Cultured plates were incubated in a CO_2 incubator (model no. 322-11, NAPCO®, National Appliance Co., Oregon, and USA). Suspected colonies were stained with Gram`s stain and Modified Ziehl-Neelsen stain (MZN) and tested for biochemical characters such as ; CO2 requirement, H_2S production, urease activity, growth in the presence of dyes, and agglutination with mono specific antisera according to (Alton, et al. 1988). For molecular identification and sequencing; firstly the viable bacteria were killed by the addition of mixture of methanol 67% with 33% saline to viable bacteria. The killed bacteria were washed once with distilled water to remove the remnant of killing solution and finally the bacterial colonies were suspended in distilled water at concentration of approximately 10^9 cells per ml. (Salem et al. 2016). Then DNA had been extracted according to manufacturer protocol of used extraction kit {gSync^{TM} DNA extraction kit, Geneaid (New Taipei City, 22180 Taiwan, Cat. No.GS 100)}. For confirmation of the microbiological results; PCR had been carried out targeting 16SrRNA gene according to (Bricker, and Halling, 1994). The reaction carried out in 50-µl reaction volume; reaction mixture consists of PCR buffer, 1.5 mM MgCl2, 20 pmol of primer pairs {(16srRNAF1: 5' AGAGTTTGATCCTGGCTCAG 3' and 16srRNAR1: 5' AATCTTGCGACCGTAGTCCC 3')}. , 0.2 mM each of dNTP mixture, 1.5 U of Taq DNA polymerase and 5 µl of extracted DNA. Control positive sample containing DNA isolated from Rev.1 strain of *Br. Melitensis*and control negative as DNA free mixture were included during PCR. The amplification was performed in Labnet® Multigen Gradient thermal cycler, Catalog TC9600-G- 230V. (Labnet international, Inc. Edison, NJ, USA); started with 3 min of denaturation at 95 °C followed by 35 cycles each consisting of 1 min of denaturation at 95°C, 1.5 min of annealing at 56°C and 1.5 min of extension at 72°C. The final extension was allowed to continue for 7 min. The amplification products were electrophoresed in 1.5% agarose gels and visualized with ethidium bromide (Unver et al. 2006). Direct sequencing was done using ABI PRISM dye terminator cycle sequencing kit with AmpliTaq DNA polymerase and an Applied Biosystems 377 DNA sequencer (Perkin-Elmer, Foster City, Calif.). To find the nearest available database sequences, the obtained sequence was analyzed using the BLAST software (National Centre for Biotechnology Information).The Clustal W software was used to align selected sequences.

RESULTS

The results of bacteriological examination of collected samples illustrated in table (2); (1 from aborted foeti, 2 from milk, 2 from spleen and 4 from lymph nodes) the percentage of total isolation was (3.9%).The grown colonies were smooth elevated, transparent, and convex, with intact borders, brilliant surface and have a honey color under transmitted light. In regarding to collection location 8 strains had been isolated from samples collected from Giza province and 1 from Benisuef province. The percentage of isolation from cattle milk one strain from 95 samples is (1.05%) while percentage of isolation from buffaloes milk one strain from 30 samples is (3.3%) and all isolates from aborted feti is from cattle samples (3.03%), 2 isolates from Spleen of Cattle samples (7.4%) and 1 isolates from L.N. samples of buffaloes (16.6%) and 3 isolates from L.N. Samples of Cattle (11.1%). All 9 *Brucella* isolates were examined biochemically for typing. All strains were identified as *Brucella melitensis biovar3*; as shown in table (3).

The DNA of isolated bacteria (the isolate isolated from cattle lymph node) had been extracted and molecular confirmation of these isolates had been done by using PCR assay targeting 16S ribosomal RNA gene. One clear band had been extracted from the gel and sequenced and the generated sequence was 623bp in length and by using nucleotide blast our sequence was highly similar to *Brucella melitensis* 16S ribosomal RNA gene, and registered in the gene bank under accession number MT378400. Our generated sequence showed 99.36% and 98.38% identity with *Brucella melitensis* EF187230 and CP044986 respectively from India and 98.38% identity with *Brucella melitensis* from Greece, China and Norway. As well as our generated sequence showed 99.20% from Assiut, Egypt as shown in fig (1).

Table 1: types and number of samples collected for *Brucella spp*. isolation:

Gov.	L.N.	Milk	Liver	Spleen	Aborted feti	Total
Giza	33	7o	33	33	2	171
BeniSeuif	-	55	-	-	1	56
Total	33	125	33	33	3	227

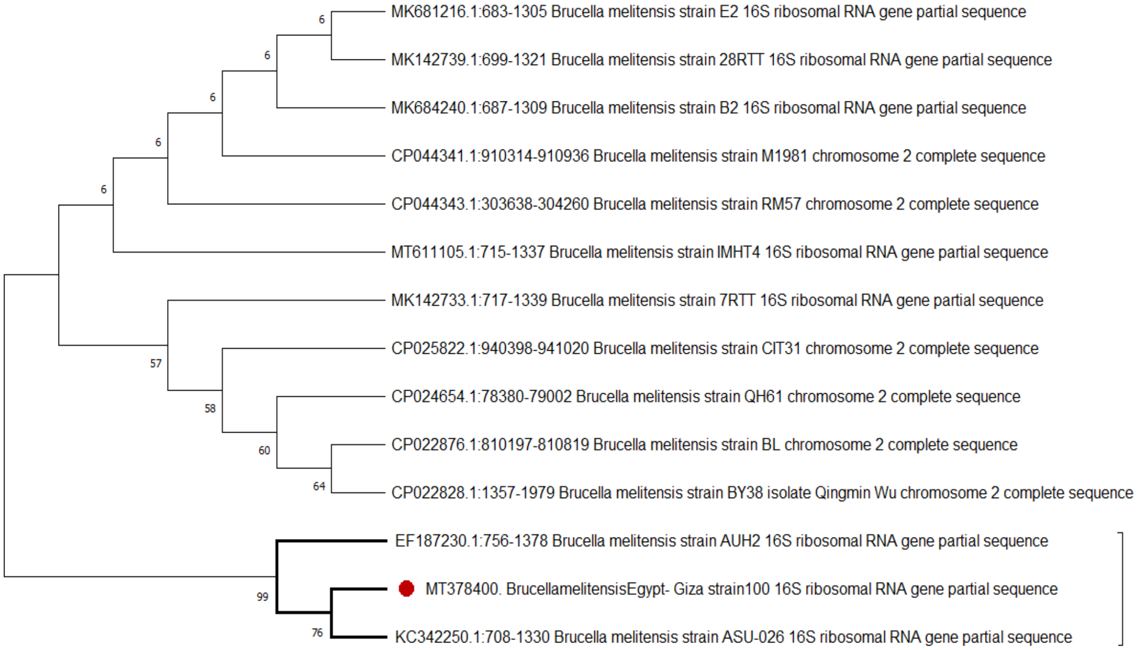

Figure 1: Phylogenetic tree showed that the generated sequence showed 99.36% and 98.38% identity with *Brucella melitensis* EF187230 and CP044986 respectively from India and 98.38% identity with *Brucella melitensis* from Greece, China and Norway. As well as our generated sequence showed 99.20% from Assiut, Egypt.

Table 2: The number and percentage of Brucella isolation from collected samples

province	**L.N. 33**		**Milk 125**		**Liver 33**		**Spleen 33**		**Aborted feti 3**		**Total 227**	
	NO	%	NO	%	NO	%	NO	%	NO	%	NO	%
Giza (171)	4	(12.12%)	1	(1.4%)	-	-	2	(6.06%)	1	(3.03%)	8	(4.6%)
BeniSuef (56)	-		1	(1.8%)	-	-	-		-		1	(1.7%)
Total (227)	4	(12.12%)	2	(1.6%)	-	-	2	(6.06%)	1	(3.03%)	9	(3.9%)

Table 3: Results of biochemical tests.

Bacterial strain	Species	No. of isolates	Biochemical tests								Agglutination in sera		
			CO$_2$ Requirements	H$_2$S production	Growth on days					Urease activity	Monospecific Antisera		Bio variety Metabolic pattern
					Thionin			Fuchsin					
					a	b	c	a	b		A	M	
***Br. melitensis* Field isolates**	Cattle	7	-ve	-ve	-ve	+ve	+ve	+ve	+ve	Variable	+ve	+ve	***Br. melitensis* bv.3**
	Buffaloe	2	-ve	-ve	-ve	+ve	+ve	+ve	+ve	Variable	+ve	+ve	
Reference strains	***Br.melitensis16M***		-ve	-ve	+ve	+ve	+ve	+ve	+ve	Variable	-ve	+ve	
	Br. abortus 544		+ve	+ve	-ve	-ve	+ve	+ve	+ve	1-2 h	+ve	-ve	
	Br. suis133		-ve	-ve	-ve	+ve	+ve	-ve	-ve	0-30 min	+ve	-ve	

DISCUSSION:

Brucella spp. infection in cattle and buffalo caused a serious disease with serious economic effect such as abortion and infertility and zoonotic importance (Gwida et al. 2016).

Brucellosis caused by species specific bacteria; in cattle and buffalo it caused by *B. abortus,* in ovine and caprine it caused by *B. melitensis* while in pigs it caused by *B. suis* (pigs) (Bhat et al. 2012). This illness has a significant detrimental influence on socioeconomic elements in Mediterranean nations, Central Asian countries, and, in particular, rural parts of developing countries, where livestock husbandry and the production of dairy products and by-products are critical for family income (Nicoletti, 2010 and Ali et al. 2017).

Accurate diagnosis of *Brucellosis* is important for the design of control plans of the disease in animals and in human also. Primary diagnosis is depends on the history of reproductive problems in livestock, but it is a tentative diagnosis (Poester et al. 2010) that must be confirmed by confirmatory methods (Poester et al. 2010 and Nielsen 2002) Bacterial isolation is the “gold standard” method for confirmation of the diagnosis of brucellosis but the isolation from clinical specimens requires long incubation periods (4 to 7 days up to 40 days) and often the low recovery rates (Rich et al. 2000). Because of the significant danger of laboratory acquired infections, regular identification and classification of brucellosis suspected specimens, based on culture isolation and phenotypic characterization, necessitates biosafety level-3 (BSL-3) methods (Boschiroli et al. 2001), To solve these challenges, molecular techniques have been investigated.

Furthermore, as compared to the conventional microbiological assay for brucellosis diagnosis, polymerase chain reaction (PCR)-based assays have showed a greater sensitivity (Hoover 1997 and Geresu & Kassa 2016). Bacteriological isolation of brucella species from the specimens had been done successfully as shown in table (2). Also the result of isolation and typing was in agreement with previous trials of isolation from done by Sherif, 2008; they try to isolate and identify of brucella strains from field specimens. All isolates were *Brucella melitensis* biovar 3. The low isolation rate of *Brucella* organism from tissue samples in this study agreed with Seleem et al. 2010 and de Jong &Tsolis 2012 who reported that *Brucella* isolation is challenging. *Brucella spp*. is a fastidious bacterium and requires rich media for primary cultures. Furthermore, its isolation requires a large number of viable bacteria in clinical samples, proper storage and quick delivery to the diagnostic laboratory.

Several advantages have been reported for the PCR assay over the current conventional methods used to identify *Brucella* species. The major advantage being the time taken compared to conventional methods which require several days to isolate and identify the organism **(**Fekete et al. 1992; Ouahrani-Bettache et al. 1996; Bricker and Halling 1994; Ewalt and Bricker 2000). The other advantage is the need for minimal sample preparation because isolation of living organisms is not necessary. The assay is also not affected by contamination with other microbes that might be present in tissue samples used for isolation.

Molecular diagnosis of *Brucella spp.* based on detection of 16S rRNA by PCR followed by sequencing had been used by many researchers to confirm the microbiological identification of *Brucella spp* (Dash et al. 2012). Results mentioned by Gee et al. showed that the generated sequences of 65 strain of *Brucella* isolated from 6 species had 100% identity with *Brucella* 16S rRNA gene sequences registered in GenBank (Gee et al. 2004); these findings confirm that 16S rRNA gene sequencing is a reliable tool for rapidgenus-level identification of *Brucella spp*. and their differentiation from closely related organisms. Results obtained in this study agreed with those previously reported; high density clear band appeared with 623 bp in length and showed showed 99.36% and 98.38% identity with *Brucella melitensis* EF187230 and CP044986 respectively from India and 98.38% identity with *Brucella melitensis* from Greece, China and Norway. As well as our generated sequence showed 99.20% from Assiut, Egypt. More comparative phylogenetic studies should be done on more strains of *Brucella* species isolated from Egypt.

CONCLUSION

Our findings ensure that *Br. melitensis biovar 3* considers as the most prevalent cause of abortion among bovine population in Egypt. As well as the use of PCR technique depending on detection of 16 S RNA gene provides rapid and accurate methods for identification of Brucella spp. So that we recommended the use of this easy, non-expensive methods together with sequencing for direct detection of Brucella infection directly from biological samples; which will save time and efforts and minimize the risk of laboratory infection of laboratory staff. Local veterinary authorities in Egypt should take many strict measures to combat and eradicate brucellosis from animals in Egypt.

CONFLICT OF INTEREST

The authors declared that present study was performed in absence of any conflict of interest.

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