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Bacteriological and genotypic characterization of Brucella isolates from dairy cows and its public health impact

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Abstract

The present study was carried out on a total number of 66 blood and milk samples which were collected from dairy cows (23 suffered from abortion, 16 stillbirths and 27 apparently healthy cows) as well as 65 human blood samples collected from the same rural areas where animal samples were collected. The obtained sera were examined for detection of *Brucella*-antibody titer using serological tests. In this investigation overall reactors of brucellosis in cows was recorded as 21.2% in RBPT, 24.2% in TAT, 18.2 % in Riv T and 18.2 % in CFT. *Brucella* microorganism was isolated from 6 (26.1%) out of 23 milk samples in cases of abortion and from 4(25%) out of 16 of still birth cases. Nine *Brucella* strains were identified as *B. melitensis* biovar 3 and only one strain was identified as *B. abortus*. Reactors of human brucellosis that were occupationally linked with handling of cows using 65 serum samples, when tested by various tests, revealed 13.8% by RBPT, 12.3% by TAT, and 15.4% by iELISA. The human reactors were found to be highest by i-ELISA (33.3%),and (27.8%) by both RBPT and TAT among slaughterhouse workers involved in cows slaughtering as compared to other occupational groups, associated staff, veterinarians and para-veterinary staffs in cows rearing system. However, among 22 blood samples collected randomly from the hospital, all samples were found negative by RBPT and i-ELISA while 1(4.5%) was positive by TAT. Out of 10 DNA extracts of *Brucella* strains isolated from milk samples in the present study, 9 samples demonstrated species *B. melitensis* explicit amplicon of 731 bp, upon PCR utilizing IS711 primer , and only one sample showed species *B. abortus* explicit amplicon of 498 bp, upon PCR utilizing IS711 primer. Also out of 10 DNA extracts of *Brucella* strains isolated from serum samples from humans 8 samples showed species *B. melitensis* exact amplicon of 731 bp .The analyzed gene sequences revealed that *Brucella melitensis* cow isolates were genetically closely related to *Brucella melitensis* isolates in case of human infection.

With respect to the phylogenetic relationship of *Brucella abortus* cow isolate, is compared with other *Brucella melitensis* isolates.

***Keywords**: Bacteriological, genotypic characterization, Brucella, IS711 gene, public health impact.*

Introduction

Bovine brucellosis comprises a financial implication in livestock production in Egypt due to abortion, infertility, premature birth and reduced milk production in females, as well as orchitis and epididymitis in males. In spite of efforts of Egyptian veterinary services to control brucellosis, the disease prevails in dairy cattle and humans and poses a public health hazard in Egypt **(Hosein *et al*., 2018)**. *Brucella melitensis* is the most predominant strain isolated from animals and humans **(Hosein *et al*., 2017)**. In Egypt, brucellosis is a highly contagious, zoonotic and endemic disease in farm animals and humans, where it has been recorded in cattle, buffaloes, sheep, goats and camels and has been recovered from dogs as carrier hosts **(Wareth *et al*., 2017)**. Brucellosis is caused by Gram-negative coccobacilli of genus *Brucella*. *Brucella* is transmitted from the infected animal to human via ingestion of contaminated dairy products **(Kaynak-Onurdag *et al*., 2016)**. The prevalence of animal brucellosis in Egypt in the past 20 years had a range of 0.33% to 1.32 % according to the official report of the General organization for veterinary services **(Wareth *et al*., 2014)**. Brucellosis has been identified by fundamental Organizations as a standout amongst the most significant neglected zoonotic diseases in the world **(Franc *et al*., 2018)**.

Brucella melitensis is considered the most virulent species for human (**Whatmore 2009).** Long term clinical signs of human brucellosis comprise sweats, polyarthralgia, undulant fever, back pain, abdominal pains, headaches, myalgia, and personality changes, other brucellosis symptoms like arthritis, leukopenia, anemia, endocarditis, and meningitis **(Buzgan *et al*., 2010)**. The diagnosis of brucellosis is the corner stone for its perfect eradication and control **(Alton *et al*., 1988)**. Diagnosis of brucellosis is frequently carried out by bacteriological and immunological tests **(Refai 2003)**. The immunological testing for brucellosis in cattle is usually carried out as a component of the disease eradication and surveillance program. In USA, two primary methods for testing of brucellosis in livestock are: the Brucella ring test (detect antibody in the pooled milk samples from dairy farms) and the market cattle identification blood tests (to test serum antibodies in blood samples) as reported by **(Glynn and Lynn 2008)**. The helpful tests for diagnosis of human brucellosis include the Rose Bengal test (RBT), counter immunoelectrophoresis, immunocapture agglutination test and the indirect enzyme-linked immunosorbent assay **(Acha and Szyfres 2003)**.

Bacteriological isolation poses high specificity but is time-consuming and need an appropriate level of biosafety. Polymerase chain reaction (PCR) has been used as an effective assay for rapid detection and confirmation of Brucella infections as well as differentiating Brucella species. Serological assays are used more quick and less expensive diagnostic tools. However, serological tests have many limitations regarding the specificity and sensitivity, particularly in animals **(Kaltungo *et al*., 2014)**. In addition, *Brucella* organism could be identified by PCR in the blood, abomasal contents of aborted fetuses and, when compared to the culture methods, PCR proves more sensitivity **(Diaz-Aparicio *et al*., 1994; Leal-Klevezas *et al*., 1995)**. PCR is considered as a hopeful alternative for bacteriological techniques due to higher speed, more safe and high sensitivity. Brucella species has Insertion Sequence (IS) that is also called as IS6501. *B. melitensis* carries seven complete IS 711 copies (**Ocampo-Sosa and Garcia-Lobo 2008).** *B. abortus* contains six complete and one truncated IS711 copies **(Halling *et al*., 2005)**. Based on stability of IS 711, the polymorphism at *alkB* locus is utilized to differentiate *B. melitensis* from *B. abortus* **(Marianelli *et al*., 2003)**. The present study aimed to detect smooth Brucella antibodies through serological screening of the serum and milk of dairy cows as well as the serum of occupational workers and Veterinarians in Sharkia Province, Egypt. Secondly achieve bacteriological isolation and molecular identification of Brucella species besides studying

the phylogenetic relationship of incriminated Brucella species isolated from different sources based on sequencing of IS711 gene.

Materials and Methods

Sampling

Collection of animal samples:

Blood and milk samples were collected from 66 dairy cows at different rural areas belonging to El-Sharkia Governorate during the period of May, 2017 to March, 2018. The animals had no history of vaccination against Brucella. The dairy cows included (n=23) suffered from abortion, (n=16) stillbirth and (n=27) apparently healthy females. Blood samples were collected from Jugular vein after disinfecting site of injection with ethyl alcohol 70%. The blood samples (10 ml) were collected from each dairy cow in a vacotainer tube. The sixty six blood samples were coded, and then transferred to the laboratory without delay in an insulated box. The sera of dairy cows were separated by centrifugation, then kept at-20°C until serological examination. Also, before sampling of milk, teats were carefully washed, dried then wiped in clean cotton immersed in ethyl alcohol 70%. Afterwards, two ml of the fore milk were collected in sterile vials from each of the functional quarters of the udder to perform Milk Ring Test (MRT); while another 10 ml of milk samples from each quarter were collected in a sterile tightly closed container then kept at 4ºC in a refrigerator until bacteriological examination.

Collection of human blood serum samples:

Sixty five sera samples of humans were selected from the same rural areas from which animal examined in this study. The blood human samples were collected from the respective hospitals. The age of patients was ranged from 12 to 80 years and included males (n=42) and females (n=23). An informed and written consent was obtained from patients after several meetings and explaining the protocol of the current study. The participation was voluntary and the withdrawal from the study was permitted at any time. The medical ethics was according to the Helsinki declaration. The patients were suffered from swinging pyrexia of unknown origin, arthralgia and a history of livestock holdings (symptoms suspected Malta fever caused by brucella). The sera samples were included the followings: randomly collected from hospitals (n=22), associated staff working in the cow breeding farm (n=13), slaughterhouse workers (n=18) and Veterinarians and para-Vets (n=12). Under aseptic precautions, patient's blood was collected in a BD™ (Becton Dickinson) vacutainer by vein puncture and then transported to the laboratory for sera separation to perform serological examination and PCR technique.

Serological assessment for animals:

The sera samples of dairy cows were subjected to serological assessments using tube agglutination test (TAT), Rose Bengal plate test (RBPT). Rivanol test (Riv.T) and complement fixation test (CFT) as previously explained **Alton *et al*. (1988)** and **OIE (2016)**.

Milk Ring Test:

The test (MRT) was carried out according to **OIE (2016)**.

Serological assessment for humans:

The sera of humans were tested by tube agglutination test (TAT), Rose Bengal plate test (RBPT) and *Brucell*a IgG and IgM ELISA according to the standard protocol listed by **WHO (2006)** and **Kazemi *et al.* (2008)**.

Isolation and identification of *Brucella* species from milk samples:

Brucella species was isolated from milk samples of dairy cows according to **Tantillo *et al*. (2003)**. The milk samples were centrifuged at 3000 rpm for 10 minutes for obtaining the sediment-cream mixture. This mixture was cultured on Brucella medium base supplemented with 5% horse serum (Oxoid, CM 0169) and also on the Farrell′s selective medium .The inoculated plates from milk samples were incubated at 37ºC in presence of 10% CO_2 for two weeks. After incubation, the suspected colonies of *Brucella* were morphologically examined. *Brucella* colonies were round, glistening, pinpoint and honey drop like-appearance. The colonies were examined for both Gram stain

and Modified Ziehl-Nelsen stain. The biochemical identification and biotyping of Brucella isolates was performed using oxidase, catalase, urease mono specific sera (A&M) and dyes sensitivity as previously explained **Alton *et al*. (1988)**.

Molecular detection of *IS711* gene in *Brucella* isolates:

DNA extraction:

Genomic DNA was extracted from ten serologically positive human sera samples and from ten bovine milk Brucella cultures that were grown in Brucella selective broth at 37°C overnight using the QIAamp DNA Mini kit (Qiagen, Germany, GmbH) from the manufacturer's recommendations.

Primers and PCR amplification:

The oligonucleotide primers and the program of duplex PCR reactions to amplify IS711 gene for identification of *Brucella* to the species level were carried out according to **Bricker and Halling (1994)**.

Table (1). Primers sequences, target gene, amplicon sizes and cycling conditions for PCR

Target gene	Target agent	Primers sequences	Amplified segment (bp)	Primary denaturation	Amplification (35 cycles)			References
					Secondary denaturation	Annealing (Opticson)	Extension	
1S711	*Brucella* genus	**IR1** GGC-GTG-TCT-GCA-TTC-AAC-G	839	94˚C 5 min.	94˚C 15 sec.	55˚C 30 sec.	72˚C 45 sec.	**Bricker and Halling, (1994)**
		IR2 GGC-TTG-TCT-GCA-TTC-AAG-G						
	B. abortus	**1S711-specificPrimer** TGC-CGA-TCA-CTT-AAG-GGC-CTT-CAT	498	94˚C 5 min.	94˚C 15 sec.	55˚C 30 sec.	72˚C 30 sec.	
		B. *abortus*-specific Primer GAC-GAA-CGG-AAT-TTT-TCC-AAT-CCC						
	B. melitensis	**1S711-specificPrimer** TGC-CGA-TCA-CTT-AAG-GGC-CTT-CAT	731	94˚C 5 min.	94˚C 15 sec.	55˚C 30 sec.	72˚C 45 sec.	
		B. *melitensis*-specific Primer AAA-TCG-CGT-CCT-TGC-TGG-TCT-GA						

Sequencing and phylogenetic analysis of *IS711* gene of *Brucella* isolates:

Partial sequencing of *IS711* gene:

PCR products were purified using QIAquick PCR Product extraction kit (Qiagen, Valencia). Sequencing was carried out in Elim Biopharmaceuticals USA. Bigdye Terminator V3.1 cycle sequencing kit (Perkin-Elmer) was used for the sequence reaction and then it was purified using Centrisep spin column. Partial sequencing was done for IS711 gene of five Brucella isolates including: one *Brucella abortus* isolate from milk of aborted cow; two isolates *B. melitensis* from milk of aborted cows and two *B. melitensis* detected in human blood samples by PCR. The sequences of IS 711 genes were submitted to GenBank under accessions MK490913, MK490914, MK490915, MK490916 and MK490917. Pubmed sequences were obtained by Applied Biosystems 3130 genetic analyzer (HITACHI, Japan), a BLAST® analysis (Basic Local Alignment

Search Tool) was initially performed to establish sequence identity to our GenBank accessions **(Altschul *et al.*, 1997).**

Phylogenetic analysis of I*S711* gene:

The nucleotide sequences of *IS711* genes of *B. abortus* and *B. melitensis* were analyzed and comparative analysis of sequences was performed using CLUSTAL W multiple sequence alignment program, version 1.83 of MegAlign module of Laser gene DNAStar software pair wise **(Thompson *et al*., 1994).** The phylogenetic analysis was carried using maximum likelihood, neighbor joining and maximum parsimony in MEGA6 **(Tamura *et al*., 2013).**

Results

The results are illustrated in tables 2-5, figures 1,2 and 3.

Table (2). Detection of Brucella specific antibodies response in the sera of examined cows by serological tests.

Examined animals History & clinical signs	Serological tests							
	RBPT		TAT		Riv T		CFT	
	No.	%	No.	%	No.	%	No.	%
Abortion (N= 23)	7	30.4	8	34.8	6	26.1	6	26.1
Stillbirth (N= 16)	5	31.3	5	31.3	5	31.1	5	31.1
Apparently normal female (N= 27)	2	7.4	3	11.1	1	3.7	1	3.7
Total (N=66)	14	21.2	16	24.2	12	18.2	12	18.2

*% calculated according to No. of examined samples in each item.

Table (3). Results of Milk Ring test and culture on Brucella selective medium.

Examined animals History & clinical signs	Serological and bacteriological examination			
	MRT		Bacterial isolation	
	No.	%	No.	%
Abortion (N= 23)	9	39.1	6	26.1
Stillbirth (N= 16)	6	37.5	4	25
Apparently normal female (N= 27)	2	7.4	0	0
Total (N=66)	17	25.8	10*	15.2

*=Nine *Brucella* strains were identified as *B. melitensis* biovar 3 and only one *Brucella* strain was identified as *B. abortus.*

Table (4). Detection of Brucella specific antibodies in the sera of examined humans.

Examined humans with clinical signs (65)	Serological tests					
	RBPT		TAT		IElisa	
	No.	%	No.	%	No.	%
Randomly from hospital (22)	0	0	1	4.5	0	0
Associated staff working in cow Breeding (13)	3	23.1	2	15.4	3	23.1
Slaughter House Workers (18)	5	27.8	5	27.8	6	33.3
Veterinarians and para-vet (12)	1	8.3	0	0	1	8.3
Total (n=65)	9	13.8	8	12.3	10	15.4

Table (5). The overall results of molecular PCR detection for *B. abortus* and *B. melitensis* from examined human and animals.

Sample source and type	Results		
	***Brucella* genus**	***B. abortus***	***B. melitensis***
Human serum (10)	8	-	8
Bovine milk culture (10)	10	1	9

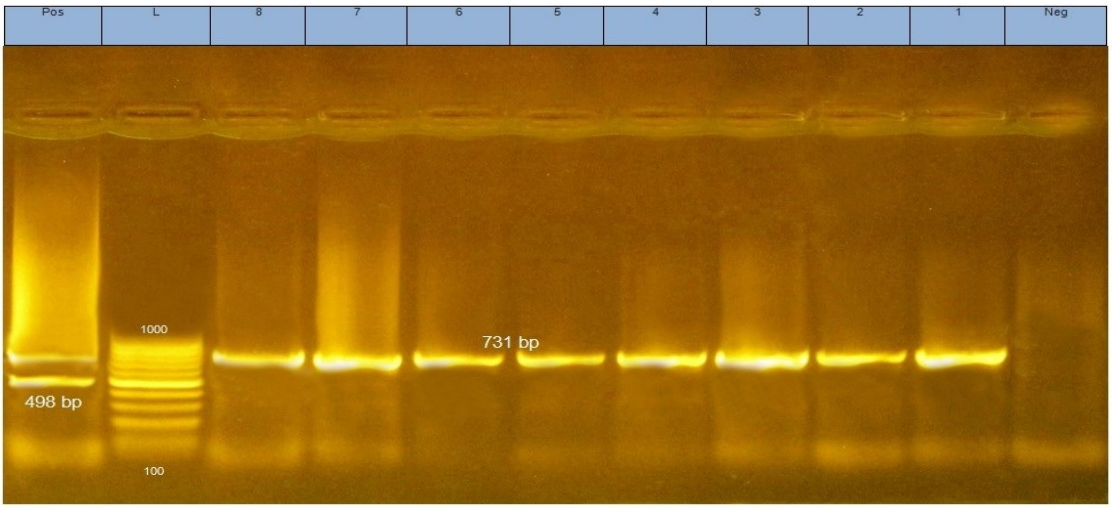

Figure (1). Amplified PCR products of different Brucella spp from seropositive human sera. Agarose gel electrophoresis of PCR products. Lane L: Marker, Lane 1-8: *Brucella melitensis* 731bp. negative and positive controls (*Br. melitensis* 731bp and *Br. abortus* 498bp) were included.

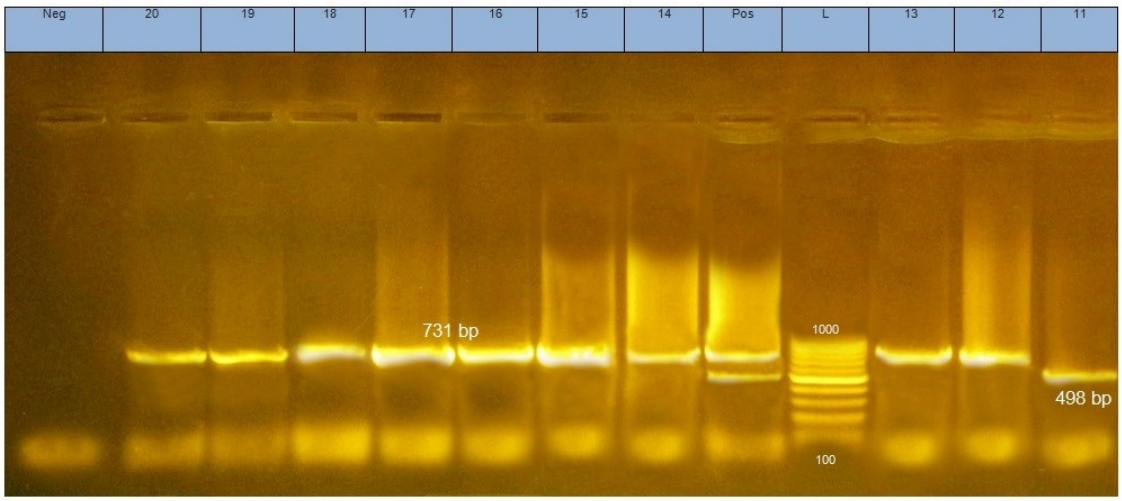

Figure (2). Amplified PCR products of different *Brucella* spp from strain from cow's milk. Agarose gel electrophoresis of PCR products. Lane L: Marker, Lane 11: *Brucella abortus* 498 bp., Lane 12-20: *Brucella melitensis* 731 bp, negative and positive (*Br. melitensis* 731bp and *Br. abortus* 498bp) controls were included.

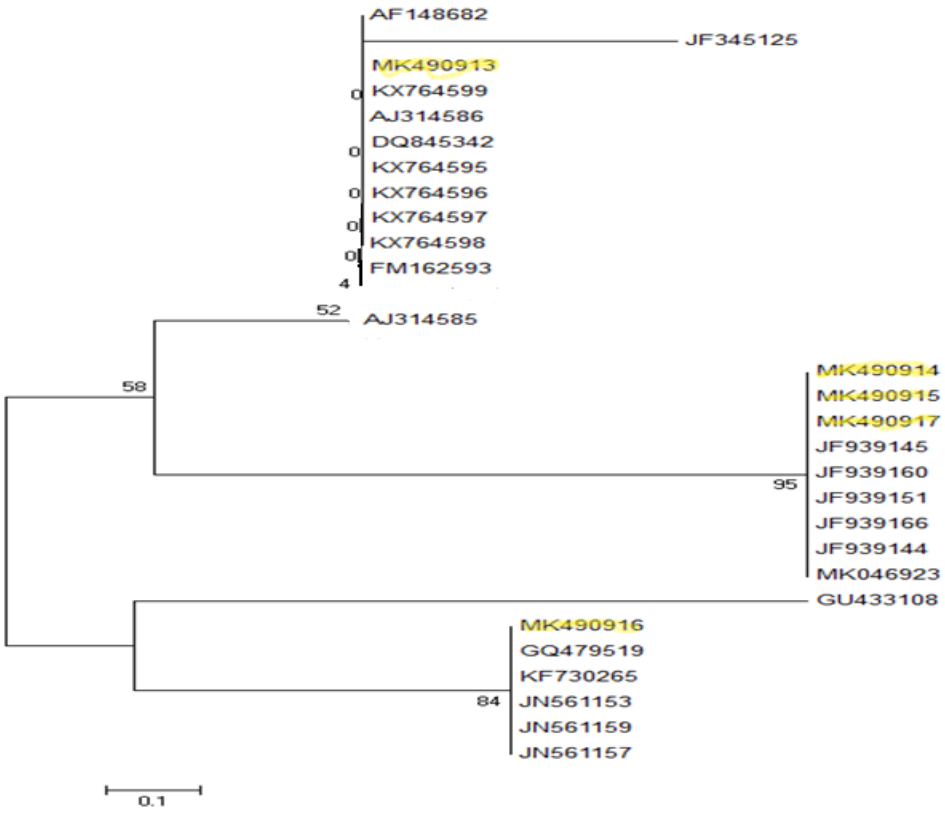

Figure (3). Phylogenetic tree of IS 711 gene in *Brucella abortus and Brucella melitensis* designed by Mega 6 in comparing with sequences of IS 711 gene.
MK490913: >*Brucella abortus* isolate Egy 19 AlkB (alkB) gene, partial cds; and insertion sequence IS711, partial sequence (from milk of aborted cow)
MK490914: *Brucella melitensis* isolate EGY20 insertion sequence IS711, partial sequence (from milk of a cow)
MK490915: >*Brucella melitensis* isolate EGY21 insertion sequence IS711, partial sequence from milk of a cow with still birth fetus
MK490916: *Brucella melitensis* isolate EGY13insertion sequence IS711, partial sequence from blood of human
MK490917: *Brucella melitensis* isolate EGY14 insertion sequence IS711, partial sequence from blood of human

Discussion

Bovine brucellosis is one of the transmissible diseases causing challenges for veterinary authorities that are confronted, not only with animal but also with major public health implications.

The serological outcomes of brucellosis differs from district to district all through the country anyway this infection has the capability to cross the borders especially in developing Mediterranean and Middle Eastern countries **(Kardjadj *et al.*, 2016).**

In this investigation there was variation for serological results between cows with different reproductive problems using RBT, TAT, Riv T and CFT (Table 2). The overall percent of brucellosis in cows was recorded as 21.2% in RBPT, 24.2% in TAT, 18.2% in Riv T and 18.2 % in CFT.

Immunoserological tests, Rose Bengal test (RBT), Tube agglutination test (TAT) Rivanol test (Riv. T.) and complement fixation test (CFT) in particular, are usually used for detecting brucellosis in cattle and sheep, in like way that they perceive as frequently as likely antibodies to antigenic determinants present in the O chain of the S-LPS. The discrepancies between the outcomes of these tests based on sensitivities and specificities with employed tests were in like manner detailed by **Shalaby *et al.* (2003).**

From the got outcomes table (2) it is seen that

higher number of tests responded emphatically with TAT (24.2%) followed by RBPT (21.2%) then Riv. T (18.2%) and CFT (18.2%). This might be credited to the nearness of some Gram-negative microorganisms which respond decidedly with these tests used in determination of brucellosis causing deficiencies in the understanding of the outcomes. It is gave the idea that the TAT among all tests utilized in this study gave the most astounding rate of positive animals compared with other traditional serological tests. This might be clarified by that the test has a high sensitivity to all classes and subclasses of immunoglobulins as stated by **Alton *et al.* (1988).**

The lower positive incidence of Riv.T than RBPT may be due to that the main goal of this test is based on disposing of some non-specific responses by precipitation of high molecular weight serum glycoprotein from serum solutions; which is essentially IgM, leaving generally IgG in the serum **(Poiester *et al.*, 2010).**

CFT is considered as best quality level serological test utilized for recognition of brucellosis as it identify just IgG explicit for *Brucella* infection so it defeat cross response with other comparative Gram- negative micro- organisms thus no false outcomes distinguished **(Abernethy *et al.,* 2012).**

Milk Ring Test had detected, 25.8%, positive cases. The milk ring test is sensitive and considered as an economical test for the surveillance of dairy herds for brucellosis since milk samples can be obtained effectively and they have been generally utilized for testing herds or individual animals. Conversely, the MRT has likewise been accounted for unmistakable hindrances incorporating false positive outcomes in milk samples gathered not long after parturition, close end of lactation, mastitic cows or vaccinated animals **(Kaltungo *et al.,* 2013).**

Isolation of *Brucella* is still the gold standard for diagnosis, but it is prolonged, potentially dangerous, and requires well skilled staff **(Wareth *et al.,* 2014).**

In this study, bacteriological examination revealed that *Brucella* microorganisms were isolated from six (26.1%) out of 23 milk samples examined in cases of abortion and from four (25%) out of 16 of still birth cases (table 3). Nine *Brucella* strains were identified as *B. melitensis* biovar 3 and only one strain was identified as *B. abortus* biovar 1. This finding suggests that *B. melitensis* biovar 3 is the predominant strain isolated from the milk of cow with reproductive disorders. This finding is in harmony with the results of **Hosein *et al.* (2017)** and **Mitrov *et al.* (2010**) who suggested that the high incidence of *Brucella melitensis* may be ascribed to that cattle are set aside in close involvement with sheep and goats and the *Brucella* is usually transmitted from animal to animal by contact following abortion.

In the present study the reactors of human brucellosis (occupationally linked with handling of cows) using 65 serum samples, when tested by various tests, found to be 13.8% by RBPT, 12.3% by TAT, and 15.4% by i-ELISA. The seropositive rates were found to be the highest by i-ELISA (33.3%), then by RBPT and TAT (27.8%) each among slaughterhouse workers involved in cows slaughter as compared to other occupational groups, associated staff, veterinarians and para-veterinary staffs in cows rearing system (Table 4). However, among 22 serum samples collected randomly from hospitalized in patients suffering from fever, polyarthritis and sweating, all samples were found negative by RBPT and i-ELISA while 1(4.5%) was positive by TAT. This finding verifies well with the way that most infections are found among housekeeper, veterinarians and animal farmers as reported by **Kazemi *et al.* (2008).** All over again, this case that was reactor in TAT could be attributed to the fact that this test is detecting all classes of immunoglobulins and that case may be affected by other related Gram negative bacteria leading to false positive results **(OIE 2016)**.

Our results agree with **Pappas and Memish, (2007)** who establish that ingestion, coordinate contact through breaks in the skin and airborne disease (research facilities and abattoirs), fundamentally influencing consumers of crude

milk, subordinates, ranchers, butchers and veterinarians**,** unpasteurized dairy products, uncooked meat and carcasses are sources of infection for employees in the meat-packing industry and general population. Veterinarians and para-veterinarians may acquire brucellosis from aiding parturition in infected livestock, just as through close contact and fortuitous vaccination **(McDermott *et al*., 2013)**. The ELISA test has the upside of giving obvious outcomes with anti complementary and hemolyzed sera. It likewise analyzes quantitative estimation of antibody concentration from a single dilution of serum. The investigation additionally thought about an assortment of routinely used serological tests, RBPT, TAT, and i-ELISA in the diagnosis of brucellosis. However, no single test is in itself fully adequate, and each test was associated with certain disadvantages **(Nielsen 2002).**

The diagnosis of brucellosis requires culturing of Brucellae and sero –conversion that are time consuming **(Al Dahouk and Nockler 2011).** Thus, molecular detection based on PCR is promising options for the conclusion of infectious diseases caused by fastidious microorganisms such as Brucellae. In this investigation, we employed a PCR assess for detection of Brucella spp in human serum samples.

In the present study, out of ten (10) DNA extracts of Brucella strains isolated from milk samples, nine samples demonstrated species *B. melitensis* explicit amplicon of 731 bp, upon PCR utilizing IS711 primer , and only one extract showed species *B. abortus* explicit amplicon of 498 bp, upon PCR utilizing IS711 primer. Also out of ten serologically positive sera samples from human eight samples showed species *B.melitensis* exact amplicon of 731 bp (Figure 1&2).

The *Brucella* genome additionally contains an insertion sequence (IS) element called IS711, explicit to the genus. Information from sequence analysis is used in several fields of biology. It gives information on the association between entity organisms, or between groups of organisms. It shows how closely related they are. Anyway the duplicate number of IS711 varies in the genome of the different Brucella species **(Halling *et al.,* 2005)**. Our sequences had Gene Accessions (MK490913, MK490914, MK490915, MK490916, and MK490917).

Our outcomes showed that the species-explicit PCR assay with primers IS711 recognized higher numbers of *B. melitensis* DNA nine out of ten isolates from cow's milk and eight out of ten iElISA- positive human serum samples (table 5). These findings are in harmony with that reported by **Khosravi (2006)** and **Elfaki (2005)**. Regardless of our satisfying outcomes, a few patients with positive iELISA showed up as negative by PCR this result concurs with **Romero *et al.* (1995**) who reported that these false-negatives could be the result of the presence of either a number of organisms below the detection limit as far as possible or corruption of target DNA in the serum tests.

Phylogeny analysis can give increasingly accurate reports in order to make up for the inability of automated systems to distinguish between closely related bacterial strains. As far back as early microbiological work by **(Wilson 1933)** researchers have been developing gradually refined strategies for classifying Brucella species. However, in spite of technical advances in genotyping, most strategies have possessed the capacity to generally produce the equivalent transformative connections found in entire genome phylogenies.

Analysis on phylogenetic study of the existing available isolates was done for molecular characterization of *Brucella melitensis* and *Brucella abortus* in each case to determine the probable beginning of the organism and identify the resemblance of the organism from dairy cows with the organism from human in the same rural areas

In this study, partial sequencing of IS711 gene of 5 Brucella isolates including *B. abortus* strain from milk of aborted cow (MK490913); two isolates of *B. melitensis* from milk of dairy cows (MK490914 &MK490915) and two strains of *B. melitensis* from human blood (MK490916&MK490917) was carried out,

and the phylogenetic tree was constructed by MEGA6 using the Neighbor Joining method. In the first clade, the *B. abortus* strain Egy19 IS711 from milk of aborted cow (MK490913) exhibited strongest identity with *B. abortus* strains (AJ314586&AJ314585) from Italy **(Marianelli *et al*., 2003)**; from USA (AF148682), from Iran (DQ845342), from piglets in India (FM162593) and from soil in Pakistan (KX764599, KX764595, KX764596 & KX764598). In the second clade, our two sequences of *B. melitensis* strains Egy 20 IS711 from milk of cow (MK490914) and Egy21 from milk of cow with stillbirth fetus (MK490915) displayed the highest homology with *B. melitensis* strain Egy14 IS711 from human blood (MK490917). The present finding confirmed the zoonotic transmission of *B. melitensis* of milk origin to in-contact humans (slaughterhouse workers and veterinarians). Moreover, our three sequences of *B. melitensis* strains from milk of cows (MK490914 & MK490915) and human blood (MK490917) showed maximum identity with other *B. melitensis* isolates from blood of human in India (JF939145, JF939160, JF939151, JF939166 &JF939144) and in China (MK046923). In the third clade, our *B. melitensis* strain Egy13 IS711 from human blood (MK490916) displayed highest similarity with *B. melitensis* strains (JN561153, JN561159 & JN561157) from seropositive goats in Malaysia **(Bamaiyi *et al*., 2012)** and with other *B. melitensis* strains from vaginal swabs of goats in Malaysia (KF730265) and from Singaporeans in Saudia Arabia (GQ479519). This finding indicated that there was an evidence of interspecies and zoonotic transmission of *B. melitensis* strains from goats to humans figure (3).

With respect to the phylogenetic relationship of *Brucella* abortus (MK490913) cow isolate, it is weakly related to other *Brucella melitensis* isolates.

Our results are in harmony with **Arboleda *et al.* (2017)** who established that *B. abortus* and *B. melitensis* as sister species, every one of them monophyletic, when they carried out phylogenetic analysis, through the investigation of housekeeping genes including *O. anthropi as an external group.*

Conclusion we concluded that bacteriological isolation confirmed that *B. melitensis* is the predominant strain isolated from the milk of cow with reproductive disorders. The analyzed gene sequences revealed that *Brucella melitensis* isolates obtained from cows were genetically closely related to *Brucella melitensis* isolate in cases of human infection, the phylogenetic tree showed high resemblance between the two strains, ought to be considered beginning from a similar origin or closely related strains. While, the phylogenetic relationship of *Brucella abortus,* cow isolate, weakly related to other *Brucella melitensis* isolates. This finding confirmed the zoonotic transmission of *B. melitensis* from milk to slaughterhouse workers and veterinarians, and posing a public health hazard.

Recommendation: Further investigation on phylogenetic study of overall isolates in Egypt is necessary for molecular characterization of *Brucella abortus* and *Brucella melitensis* in deciding the believable starting point of the organism and to identify the similarity or diversity with the other Brucella species. Strict hygienic measures should be applied with periodical application of test and slaughter policy in dairy farms associated with suggested vaccination program.

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