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Conventional, Serological and Molecular Characterization of *Brucella* Species Isolated from Different Governorates in Egypt

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ABSTRACT

Brucellosis is endemic in Egypt, so wherever herd problem associated abortion is present, brucellosis should be suspected, and its sero-diagnosis is needed. This study aimed to determine the seroprevalence of brucellosis in Different Governorates in Egypt and isolation and biotyping of *Brucella* isolated from Egypt confirmed by PCR. A total of 1857 samples were collected including 1531 serum, 148 milk, 58 lymph nodes,58 spleen samples, 58 liver samples and 4 aborted foeti from cattle in 7 Governorates in Egypt. Serological tests; Rose Bengal Plate Test (RBPT), Buffered acidified plate antigen (BAPA) test, modified standard tube agglutination (MSTA) and indirect ELISA were applied on positive serum samples for (RBPT). Brucella was isolated and identified from milk, lymph nodes, Spleen and aborted foeti. The results detected 19 isolates from (aborted foeti 1, milk 8, lymph nodes 8 and spleen 2) were detected and identified as *B. melitensis* biovar3. The results of RBPT, BAPA, MSTA and indirect ELISA tests were 21.8%, 23.7%, 80.2%, and 89.8% respectively. MSTA and indirect ELISA applied on positive sera of RBPT. Multiplex PCR was applied as a confirmation and rapid detection of *B. melitensis* isolates. all isolates showed positive results with oligonucleotide primer that amplified a 731bp fragment confirmed as *B. melitensis.* In conclusion, Serology remains the most practicable method for diagnosis of brucellosis, no currently available single serological test can be considered reliable for the detection of brucellosis and the gold standard for diagnosis of brucellosis is the isolation and phenotypic characterization of the organism. A combination of growth characteristics, serological, bacteriological or molecular methods is required for a definitive identification.

Keywords: *Brucella, Isolation, Identification, Serology, PCR.*

INTRODUCTION

Brucellosis is a major economically important disease of livestock and an infection of prime significance in relation to public health. Although a few countries like Northern and Central Europe, Canada, Japan, Australia and New Zealand have achieved success in eradicating this disease, it continues to be endemic in most parts of the world especially the developing countries. (Wareth *et al*. 2014, Abdelbaset *et al.*, 2018). Diagnosis of brucellosis is based on clinical findings, serological tests, and bacteriological isolation and identification. Serological tests may reveal false positive results; therefore, blood and clinical samples suspected of brucellosis should be cultured for confirmatory diagnosis.Alternatively, serological tests are relatively easy to perform and provide a practical advantage in detecting the prevalence of Brucella infection (Cox, 1986,

Kaltungo *et al.,* 2014, Wareth *et al.,* 2014, Abdelbaset *et al.,* 2018). indirect ELISA was standardized and used to detect *Brucella* antibodies in serum (WHO, 2012, Mirjalili and Hesam, 2016). The choice of the testing strategy depends on the prevailing brucellosis epidemiological situation and the aim of testing. The most widely used methods of diagnosis are based on serology, which measures the ability of the serum (antibody) to agglutinate a standard amount of killed *Brucella abortus* (antigen) containing O-side chain. RBPT, BAPA, MSTA and indirect ELISA These tests are most commonly used because they are safe to handle. However, they are prone to false-positive results due to other cross-reacting bacteria, and also, they are not useful in the detection of *Brucella canis* and *Brucella ovis* which lack the O-side chain (Kaltungo, *et al.,* 2014.) The polymerase chain reaction (PCR) has been found to be a useful and more sensitive test (Umesha, *et al.*, 2018). Cultural methods are time-consuming and costly. Molecular methods, on the other hand, have been increasingly applied for the diagnosis of infection in human and in veterinary medicine. In particular, Polymerase Chain Reaction (PCR)-based methods, have been used successfully for this purpose (Daugaliyeva, *et al.,* 2016). When compared to bacteriological isolation those methods are advantageous for its speed, sensitivity and safety (Ahmed, *et al.,* 2016). In fact, molecular methods allow rapid diagnosis and differentiation of various bacterial species, especially slow-growing ones. PCR assay has been shown to be a valuable rapid and sensitive technique in many national and international publications (Amin *et al.,* 2001; Hamdy and Amin, 2002; García-Yoldi D *et al.,* 2006; Gupta *et al.,* 2014a; Leary *et al.,* 2006). According to OIE Terrestrial Manual (OIE, 2016), there is no single test by which a bacterium can be identified unequivocally as *Brucella*. A combination of growth characteristics, serological, bacteriological or molecular methods is required for a definitive identification. this study was planned to determine the sero prevalence of brucellosis in Different Governorates in Egypt and different methods for accurate diagnosis serological, bacteriological and molecular.

MATERIALS AND METHODS

Sampling:

A total of 1857 samples were collected; 1531 serum samples, 148milk samples, 58 lymph nodes,58 Spleen samples,58 Liver samples and 4 aborted foeti from cattle in different farms and villages in 7Governorates in Egypt (Table 1).

Before collection of blood samples, the animals were prepared by leaning the site of puncture with tincture of iodine, then 10 ml of blood were drowned from jugular vein by using of sterile vacuumed tube/ or sterile separate dry needle for each animal. Blood was allowed to flow freely in a sterile dry McCartney bottle, which were placed in an inclined position at room temperature for about one hour to facilitate blood clot before they were transferred to the laboratory. In the laboratory, bottles were kept in refrigerator overnight to help serum separation. The clear serum that oozes from the clotted blood was aspirated by use of a sterile Pasteur pipettes and was transferred to a sterile strew capped tubes, and kept in the deep freezer until tested. Centrifugation at 3000 r.p.m. for 10 minutes was sometimes adapted to obtain clear serum. Milk samples were collected from animals the udder and teats were washed with water and detergent with clean paper towel, disinfected with alcohol and dried. The first two streams of milk were discarded, then about 20 ml of milk (5ml of milk from each teat) were taken directly into a sterile screw capped bottle, and marked with the number of animals, samples were directly taken to the lab. in ice-box and were kept for 24 hours in refrigerator at 4 Cº before being examined. Supra-mammary lymph nodes, liver and spleen were collected from animals suspected to be infected with *Brucella* at the time of slaughter; samples were directly taken to the laboratory in ice-box and kept in refrigerator until tested in the second day. The stomach contents of aborted foeti from cattle were collected according to S**tableforth & Galloway (1959).**

Serological tests for detection of Brucella antibodies:

The serological tests used for the diagnosis of brucellosis

RBPT, BAPAT and MSTAT were applied on the collected sera according to (**Alton, *et al.*1988**). antigen was obtained from the veterinary serum

and vaccine Research and production. Institute, Abbasia. Cairo. Egypt.

Rapid detection of Brucella by indirect enzyme-linked immunosorbent assay technique:

The indirect solid phase ELISA technique was applied on the collected serum samples according to Mathison, *et al., (1984)*. IDEXX *Brucella* antibodies Serum Test kit. France.

Bacterial isolation and identification of Brucella species

Isolation and identification of Brucella by culturing of milk, lymph nodes,Spleen, liver and aborted foeti sampleson 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**.**Suspected colonies were stained with Modified Ziehl-Neelsen stain (MZN). The suspected isolates of Brucella were subjected to the following characteristics for identification; CO_2requirement, H_2Sproduction, urease activity, growth in the presence of dyes, and agglutination with monospecific antisera according to (**Alton, *et al.* 1988**).

Multiplex-PCR for detection and confirmation of brucella species (Sambrooket, et al., 1989):

Different sets of primers were synthesized using MWG oligo synthesis of MWG Biotech according to the sequence reported in the literature and desalted on HPSF-oligo, Genomic Design Service by MWG (Germany). Target genes and their primer sequences are listed in table (2). Multiplex-PCR was applied as confirmatory test on randomly selected brucella isolates which were morphologically and biochemically identified as B. melitensis biovar3.

Table (1): Number of different samples collected from Cattle in different Governorates.

Locality	No. of serum samples	No. of milk samples	No. of Lymph Nodes	No. of Spleen	No. of aborted foeti	No. of Liver	Total
Giza	**425**	**70**	**33**	**33**	**3**	**33**	**597**
Beni-Suef	**370**	**55**	**25**	**25**	**1**	**25**	**501**
El Fayoum	**280**	**23**	**--**	**-**	**-**	**-**	**303**
El-Minia	**185**	**--**	**--**	**-**	**-**	**-**	**185**
Assiut	**141**	**--**	**--**	**-**	**-**	**-**	**141**
Sohag	**82**	**--**	**--**	**-**	**-**	**-**	**82**
Qena	**48**	**--**	**--**		**-**	**-**	**48**
Total	**1531**	**148**	**58**	**58**	**4**	**58**	**1857**

Table 2: Oligonucleotide primers used for *Brucella* DNA amplification.

Species specific primers		Primer Sequence5'-3'	Amplified product	Reference
B. abortus	*F*	*GACGAACGGAATTTTTCCAATCCC*	498bp	**Gupta*et al*. (2014)**
	R	*TGCCGATCACTTAAGGGCCTTCAT*		
B. melitensis	*F*	*AAATCGCGTCCTTGCTGGTCTGA*	731bp	
	R	*TGCCGATCACTTAAGGGCCTTCAT*		

RESULTS

Results of serological tests.

The results of the serological tests showed that BAPA test showed the highest results among the other serological tests(23.7%) and the highest prevalence of brucellosis was in EL-fayom Governorate (27.2%) followed by Giza (25.7%) while the lowest prevalence was observed in Sohag (18.2%), table (3).

Results of RBPT test was (21.8%) and the highest prevalence were observed in Giza Governorate (24.44%) followed by Beni-suef (23.8 %) and finally the lowest prevalence was observed in Sohag (15.8%). table (3).

Table (3): Results of Rose Bengal Plate Test (RBPT) and Buffered acidified plate antigen (BAPA) test.

Governorates	No.of samples	Positive (RBPT)	Positive (BAPAT)
Giza	425	104 (24.47 %)	110 (25.8 %)
Beni-Suef	370	71 (19.2%)	78 (21.11%)
EL-Fayoum	280	67 (23.9 %)	76 (27.14 %)
EL-Minia	185	42 (22.7 %)	46 (24.8 %)
Assiut	141	28 (19.8%)	28 (19.8%)
Sohag	82	13 (15.8%)	15 (18.2%)
Qena	48	9 (18.75%)	11 (22.9%)
Total	1531	334 (21.8 %)	364 (23.7 %)

Results of Modified standard tube agglutination test (MSTAT) was (80.2%) with the highest prevalence in El-Menia (85.7%) followed by El-Fayoum (83.6%), Assiut (82.1%), Giza (77.8%), Qena (77.7%) ,Beni-Suef (77.1%) and the lowest prevalence was in Sohag (76.9%). table (4).

Table (4): Results of Modified standard tube agglutination test (Mod.STAT).

Locality	**Number of samples**	**Modified sat**								**Positive (MSAT)**
		1/10		1/20		1/40		1/80		
		No	%	No	%	No	%	No	%	
Giza.	104	32	31%	20	18.9%	16	15.5%	13	12%	81 (77.8 %)
Beni-Suef.	71	20	28%	15	21%	14	19.2%	6	8.7%	55 (77.1%)
EL-Fayom	67	22	32.6%	18	26.5%	11	16.3%	5	8.1%	56 (83.6%)
EL-Menia	42	15	35.1%	10	24.3%	8	18.9%	3	8.1%	36 (85.7 %)
Assuit.	28	9	31.8%	6	22.7%	5	18.1%	3	9%	23 (82.1 %)
Sohag.	13	5	38.3%	2	15.3%	2	15.3%	1	7.6%	10 (76.9%)
Qena.	9	3	33.3%	2	22.2 %	2	22.2 %	0	0%	7 (77.7%)
Total	334	106	31.7%	73	21.8 %	58	17.3 %	31	9.2 %	198 (80.2%)

Finally Results of indirect ELISA was (89.8%). The highest prevalence was observed in Sohag (100%) followed by Assuit (96.4%), EL-menia (95%), Giza (89.4%), EL-fayom (88%), Beni-suef (85.9%), the lowest percentage observed in Qena (77.7%). table (5).

Table (5): Results of Indirect ELISA**.**

Governorates	No. of samples	Positive (Indirect ELISA)
Giza	104	93 (89.4 %)
Beni-Suef	71	61 (85.9 %)
EL-Fayoum	67	59 (88%)
EL-Minia	42	40 (95%)
Assiut	28	27 (96.4%)
Sohag	13	13 (100%)
Qena	9	7 (77.7%)
Total	334	300 (89.8%)

Results of isolation of Brucella from lymph nodes, Spleen, Liver sample, aborted foeti and milk samples.
Table (6) indicated that, there is 19 isolates of brucella (5) five of them from the supra mammary lymph node from Giza Governorate samples and (3) isolates from supra mammary lymph nodes from Beni-suef, (2) isolates of Spleen samples from Giza and no isolates from Liver samples, one isolateonly isolated from stomach contents of aborted foeti from Giza Governorate and (8) Brucella isolates were isolated from milk samples. (4) isolates from Giza, (3) Beni-suef and (1) from El Fayoum milk samples.

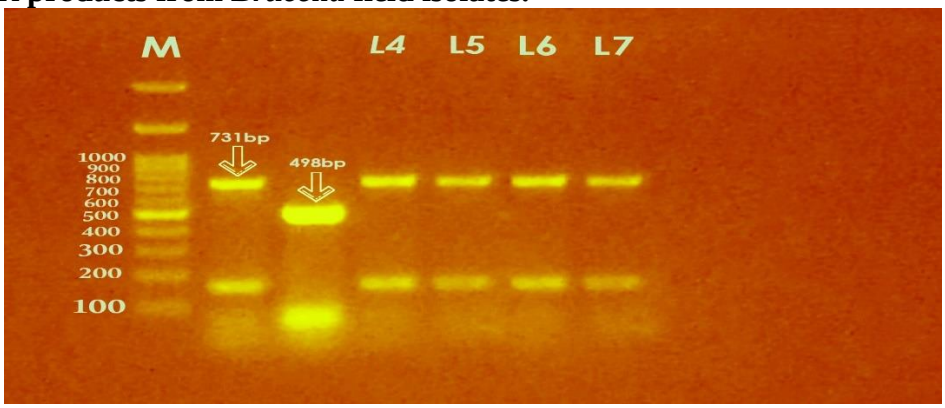
Table (6): Number of Brucella strains isolated from lymph nodes, Spleen, Liver sample, aborted foeti and milk samples.

	Lymph nodes		Spleen		liver		A aborted foeti		Milk samples		
Locality	No of Samples	Number of isolated	No of Samples	No of isolated strains	No of Samples	No of isolated strains	No of Samples	No of isolated strains	No of Samples	Number of isolated	Types of strain of Brucella isolated
Giza	33	5	33	2	33	0	3	1	70	4	Brucella melitense s biovar3
Beni-Suef	25	3	25	-	25	0	1	0	55	3	Brucella melitense s biovar3
El Fayoum	-	-	-	-	-	-	-	-	23	1	Brucella melitense s biovar3
Total	58	8	58	2	58	0	4	1	148	8	Brucella melitense s biovar3

Results of PCR .

Multiplex-PCR was applied as confirmatory test on *Brucella* isolates. morphologically and biochemically identified as *B. melitensis* biovar3. The isolates showed positive results with oligonucleotide primer that amplified a 731bp fragment **(Fig.1)**.

Multiplex- PCR products from *Brucella* field isolates.



(Fig.1) Lane 1: molecular DNA size marker. Lane2: positive control for *Br. melitensis*. Lane3: positive control for *Br. abortus*. Lane 4,5,6 and 7: Brucella field isolates identified as *Br. melitensis* (DNA product at 731 bp).

DISCUSSION

Bovine brucellosis is a disease with a significant economic and public health importance due to losses incurred as a result of infertility in animals and extensive chronic morbidity in humans (Gwida *et al.*, 2016). Brucellosis remains an important zoonotic disease in animals and humans. It is mainly caused by *B. abortus* (cattle and buffaloes), *B. melitensis* (sheep and goats), and *B. suis* (pigs) (Bhat *et al.*, 2012). Brucellosis is endemic among ruminants and humans in

Egypt despite the presence of control programs (Hosein *et al.* 2018). The annual incidence of human brucellosis is estimated to be 5 to 12.5 million cases in Egypt (Hull and Schumaker 2018). The seroprevalence study of brucellosis in cattle revealed that the overall seroprevalence and seroprevalence at herd level was 2.4 and 45.9%, respectively. World Health Organization (WHO) has reported in its fact sheet that around millions of cases of brucellosis are accounted every year but actual rate of incidence is still 10–25 times more than the stated number of cases. One important reason behind this condition is lack of distinct guidelines for diagnosis of brucellosis cases. In this study The sero prevalence of bovine brucellosis in **7**Governorates in Egypt using RBPT, BAPAT was 21.8 % and 23.7 % respectively table (3) This result higher than recorded by Hegazy *et al.,* 2011 was 0.79% by RBPAT , Salem *et al.,* 2014 (6.5% and 6% by using BAPAT, RBPAT), Selim *et al.,* 2015 (RBPT 8.4 % and BAPAT 7.5 %), AL-Habaty *et al.,* 2015 (RB 10.23 % and BAP 9.76 %) Ahmed *et al.,* 2016 (BAPAT 8.9% and, RBPT 8.9%) and Khalafallah *et al.* 2020 (RBPT3.65 %, BAPAT 3.7%) . but lower than H.I. Hosein *et al.* (2017) Serological examination using BPAT and RBT 141 cows revealed 109 (77. 3) and 105 (74.47) respectively and Mahmoud *et al.* (2019) RBPT. 54% and BAPA 60%. the results in these study mainly showed increase the prevalence of brucellosis than the other studies .also there is difference from region to other for example The results of Rose Bengal Plate test (RBPT) cleared that, the higher prevalence of brucellosis was observed in Giza governorate (24.4%) while the lowest incidence of brucellosis in Sohag (15.8%). The results of BAPA indicated that, **t**he highest prevalence of brucellosis was in EL- fayom Governorate (27.2%). and the lowest prevalence in Sohag (18.2%). This result was attributed to the differences in hygienic conditions and the difference in control programs of brucella from region to another and from farm to another farm and also the results indicated there is a difference in sensitivity of rose Bengal from region to another according to the serotypes of brucella. (Benkirance, 2006). It can't depend on one type of serological test to diagnose of tested samples because many types of bacteria have antigen similar to *Brucella* as *Yersinia* and *E-Coli,* and that would give false positive results (Garin-Bastuji *et al*., 2006). Although RBPT is a highly sensitive screening test for diagnosis of animal and human brucellosis, it should be followed by a quantitative test for further confirmation Kumar A *et al.* (2016). Accordingly, we employed RBPT for brucellosis screening and then confirmed the results by STAT; this combination was used to minimize measurement errors of false positives (Franco MP *et al.* 2007, Tumwine G *et al.* 2015). Result of MSTAT in our study is 80.2% table (4) higher than recorded by (Ahmed *et al.,* 2016) (8.9 %), (Khalafallah *et al.* 2020) (3.47 %) and lower than recorded by AL-Habaty *et al.,* 2015 (SAT 90.9 %). Serological methods, most commonly STA, are frequently used in the diagnosis of brucellosis (Gul HC *et al.* 2016). Total brucella antibodies (IgG, IgM, and IgA) are determined by STA (Araj GF *et al.* 2010). However, false-negative results may be observed with STA due to several causes, including the prozone phenomenon. our result in indirect ELISA is 89.8% table (5).the result higher than (Saadat *et al.* 2017)(82.6%), (Rhaman, *et al.,* 2020) (37.24%) and (Mahmoud *et al.* 2020 (60%) and lower than AL-Habaty *et al.,* 2015 (95.4%) .There are many serological tests uses for the determination of brucellosis, but we prefer RBPT, which has considerably high sensitivity while I-ELISA used for the specificity of the brucellosis diagnosis. The I-ELISA has been regarded as a gold standard by many researchers to compare the results for brucellosis diagnosis (Neha, Kumar, *et al.* 2017, Zakaria, *et al.* 2018 and Abd Rhaman, *et al.* 2020). The classical microbiological identification of brucellae strains is based on colonial morphology, microscopic appearance and biochemical properties, such as CO2 requirement, H2S production, urea hydrolysis, sensitivity to basic fuchsin and thionin, and also agglutination with monospecific sera, and phage typing (Alton *et al.,* 1988). Results of culturing of tissue samples from lymph nodes, spleen, liver, aborted foeti and milk were 13.7 %, 0.034 %, 0%, 25 % and 5.4% respectively table (6). These findings come in accordance with Aman et al *et al.* (2020) 5 out of 200 (with an incidence rate of 2.5%) milk samples were positive for Brucella and grow on Brucella specific media On the other hand, a higher rate of isolation of *Brucella* organism reported by Khalafallah *et al.* (2020) Results of

culturing of tissue samples from lymph nodes, spleen and liver were 61.54%, 40.38% and 36.54% respectively H.I. Hosein *et al.* (2017) 104 cows and 46 milk samples of sero-positive cows revealed isolation of 64 (61.5%) and 28 (60.9) brucella isolates respectively that were identified as *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. Results of PCR by multiplex primers for Brucella organisms were applied on the isolated strains from lymph nodes, aborted foeti, Spleen and milk samples. A multiplex was designed that will allow the rapid identification of *Brucella* species, *B. abortus*, and *B. melitensis* in a single test within 2 to 3 hrs. In the current study the results of application of (PCR) assay showed that all isolates are reacted positively with *Br. melitensis biovar* (3) specific DNA products with a molecular size of 731 bp, indicative of *Br. melitensis DNA*. The obtained results were agreed with that reported previously by Ilhan *et al.* (2008) and El- Shymaa (2014)., Wareth *et al.* (2015)**,** who reported that PCR must be considered an alternative to the traditional culturing methods for *Brucella* diagnosis as screening and confirmatory diagnostic tool for saving cost and time, Also these results were similar to that obtained by Lobna M.A.*et al.*, 2016, Khalafallah *et al.* (2020).

CONCLUSION

Serology remains the main method for diagnosis of brucellosis, no single test is adequate for diagnosis of brucella so we need several serological tests, BPAT and RBT are strongly recommended for screening purposes and followed by a confirmatory test like indirect-ELISA has great advantage of sensitivity, specificity with rapid results. Also, molecular diagnosis and applied PCR assay is recommended. We need wide comprehensive monitoring, surveillance programs all governments in Egypt. *Brucella melitensis* biovar3 remains the prevalent brucella type among cattle in Egypt. A control program for brucellosis should be based on routine testing and slaughter of infected animals, vaccination, numbering and restriction of animal movement should be applied.

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