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Original Research Article

Seroprevalence and bacteriological identification of brucellosis in buffaloes in Upper Egypt.

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ABSTRACT

A total of 1317 samples were collected; 1164 serum samples, 122 milk samples, 24 lymph nodes and 7 aborted foeti from buffaloes in 10 Governorates from farms and villages in Upper Egypt. The serological tests used for the diagnosis of brucellosis on blood sera were the Rose Bengal plate (**RBT**) , Buffered acidified plate antigentest (**BABAT)**, EDTA modifiedstandard tube agglutination test (**MSAT**), Revanol test (**RT**). On the other hand, the milk ring test (**MRT**) was performed on buffalo-cow's milk. Suspected colonies were stained with Gram' ^{s} stain and Modified Zeil-Neelson stain. The isolated Brucella organisms on antibiotic free *Brucella* agar medium were subjected to the following tests for biochemical identification tests as CO_2requirement, H_2S production, Urease activity, growth in the presence of dyes,The indirect solid phase **ELISA** technique was carried out according to serum and milk samples.Agar gel immune diffusion test (**AGID**) and **PCR** applied on isolated Brucella strains. The results of the serological tests wereRose Bengal test **34.7%**, BAPA **(37%),** Revanol test **(28.2%)**,modified SAT **(23.7%)**, indirect ELISAwere **(32.3%)** and **AGPT (33.8%)**in this study.*Brucella*organisms from lymph nodes of slaughtered buffaloes by culturing method showed that **3 (13.64%)** isolates(2) of *B. melitensis*biovar 3 and (1)*B. abortusbiovar*1. The isolated strain from aborted foeti was one isolate **(14.29%)** typed as *B.melitensis*biovar 3. isolated only from Beni-Suef.By milk ring test (**MRT**) milk samples were **10 (8.20%)** of *B. melitensis* biotype 3. A multiplex was format that will allow the rapid identification of *Brucella* spp., *B. abortus*, and *B.melitensis* in a single test within 2 to 3 h. *B. melitensis* was identified at **731bp** and *B. abortus* identified at **498bp. Finally,** we made measures of the control program for eradication of brucellosis in buffaloes by a reasonable system of compensation, Veterinarians for field work and state laboratories capable of serological techniques.Also, information technology solutions and further logistic means as animal identification techniques are in many governorates in Egypt.

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Introduction

Bovine brucellosis, a disease of major economic andpublic health importance, is a worldwide problem (**Hussain, *et al.* 2008**). Thedisease is predominantly an occupational illness in farmand livestock workers, veterinarians, slaughterhouse employees, meat inspectors, and laboratory personnel(**Nimri, 2003**). Individuals consuming dairy products in areas of endemic infection and those that handle animals and animal carcasses are at high risk of contracting brucellosis (**Abu-Shaqra**, **2000**). In different areas of Egypt the prevalence of *B.abortus*in bovines ranged between 3.25% and 4.4%.

Buffaloes normally become infected by eating contaminated fodder, drinking contaminated water, or licking the afterbirth or vaginal mucous secretion of an infected buffalo-cow that has aborted or calved. Buffaloes can also become infected by inhaling air borne bacteria when an infected female urinates, or through wounds or the mucous membrane of the eye. Flies can transmit the bacteria by feeding on an after birth and then transmitting it through the mucous membrane of the eye, or an open wound of an animal **(Fosgate, *et al.*2002**).

When a brucellosis-infected buffalo-cow aborts, it excretes bacteria with the aborted foetus, uterine secretion and afterbirth. This infects the environment with *Brucella* bacteria. Such a buffalo-cow can also become temporarily infertile. The infected female also periodically secretes bacteria in her milk during the entire lactation period, and in some buffalo-cows this may persist for the duration of its life. These bacteria are regularly isolated in the secretions of infected non-lactating udders. Some infected buffalo-cows that abort and then calve normally afterwards will also secrete bacteria from the uterus **(Apan, *et al*. 2007)**.

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 Brucellainfection(**Cox.1986**).

ELISA canbeused as a diagnostic test for the screening of antibodies,

it is reported to have a sensitivity of 95%-100% (**Ruppanner, *et al*. 1980**).ELISA and the milk ring test(MRT) used for the detection of *B. abortus* antibodies in dairy farms**(Güllüce and Leloğlu, 1996**). An indirect ELISA was standardized and used to detect*Brucella* antibodies in serum (**WHO, 2012).**

The aim of this study was to investigate the seroprevalence of brucellosis from infertile and aborted buffaloes in Upper Egypt. The comparative

evaluation of *Brucella* infection in serum samples obtained from buffalo based on serological tests as RBPT , SAT and ELISA test used for the detection of *Brucella* antibodies in buffaloes.

Materials and methods:

1-Sampling:

A total of **1317** samples were collected; **1164** serum samples, **122** milk samples, **24** lymph nodes and **7** aborted foeti from buffaloes in **10** Governorates from farms and villages in Upper Egypt(**Table, 1**).

Results:

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

Locality	No. of serum samples	No. of milk samples	No. of Lymph Nods	No. of aborted foeti	Total
Giza	205	35	9	2	251
Beni-Sweif	210	23	7	3	243
Fayoum	200	19	--	--	219
Menia	170	30	5	--	205
Assuit	110	15	3	2	130
Sohag	90	--	--	--	90
Quena	82	--	--	--	82
Red-Sea	22	--	--	--	22
Aswan	45	--	--	--	45
Wadi-Elgadid	30	--	--	--	30
Total	1164	122	24	7	1317

2-Serological identification of brucella antibodies in buffaloes:
The serological tests used for the diagnosis of brucellosis on blood sera were the Rose Bengal plate (**RBT**) , Buffered acidified plate antigentest(**BABAT)** , modified standard tube agglutination test (M**SAT**),Revanol test (**RT**). On the other hand, the milk ring test (**MRT**) was performed on buffalo-cow's milk (**Alton, *et al.* 1988**).

3- Bacteriological identification of Brucella species in buffaloes:
Isolation and identification of Brucella microorganism by culture of milk specimen, lymph nodes and aborted foeti were cultured on Brucella agar medium of 2 plates of selective medium for sample. Plates were incubated aerobically and under 10% CO_2 for 10 days according to **OIE, (1992**).Suspected colonies were stained with Gram' ^{s} stain and Modified Zeil-Neelson stain. The isolated Brucella organisms on antibiotic free *Brucella* agar medium were subjected to the following tests for biochemical identification tests asCO_2requirement, H_2S production,Urease activity, growth in the presence of dyes, and agglutination with monospecificantiseraaccording to **Alton, *et al* (1988).**

4- Rapid detection of Brucella by enzyme-linked immunosorbent assay, using indirect ELISA technique:
The indirect solid phase ELISA technique was carried out according to **Mathison*et al. (1984)* and Mettias*et al. (1996)*.**

5- Agar gel immune diffusion test (AGID): Test was carried out on isolated *Brucella*strains according to **Modolo et al. (2000).**

6- Preparation of genomic DNA from Brucella (Sambrook 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).

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

Primer code	Primer sequences	Product size	Species specificity
BrF **Br R**	5"TGGCTCGGTTGCCAATATCAA3" 5"CGCGCTTGCCTTTCAGGTCTG3" (Baily et al.,1992)	223 bp	all Brucella species
Eri 1 **Eri 2**	5"GCGCCGCGAAGAACTTATCAA3" 5"CGCCATGTTAGCGGCGGTGA3" (Bricker and Halling, 1995)	178 bp	all Brucella spp. except S19
wboA F **wbo A R**	5"GCCAACCAACCCAAATGCTCACAA3" 5"TTAAGCGCTGATGCCATTTCCTTCAC3" (Vemulapalli et al., 1999)	1300 bp	RB51 only
		400 bp	other Brucella
IS711-SP Ba-SP **Bm-SP**	5"TGCCGATCACTTAAGGGCCTTCAT3" 5"GACGAACGGAATTTTTCCAATCCC3" 5"AAATCGCGTCCTTGCTGGTCTGA3" (Bricker and Halling, 1994)	498 bp	B. abortus (biotypes 1, 2 & 4)
		731 bp	B. melitensis

Table (3):Results of serological testsofbrucella antibodies in buffalo samples.

Governorates	N0.of samples	Positive (RBT)	Positive (BAPA)	Positive (MSAT)	Positive (RIVANOL)	Positive (Indirect ELISA)	Positive (AGPT)
Giza.	205	102 (49.7%)	105 (51.2%)	65 (31.7%)	82 (40%)	92 (44.8%)	100 (48.7%)
Beni-Sweif.	210	94 (44.7%)	99 (47.1%)	61 (29%)	74 (35.2%)	83 (39.5%)	90 (42.8%)
EL-Fayoum.	200	81 (40.5%)	90 (45%)	56 (28%)	66 (33%)	75 (37.5%)	80 (40%)
EL-Menia	170	65 (38.2%)	70 (41.1%)	48 (28.2%)	56 (32.9%)	62 (36.4%)	65 (38.2%)
Assuit.	110	34 (30.9%)	37 (33.6%)	27 (24.5%)	29 (26.3%)	29 (26.3%)	32 (29%)

Sohag.	90	17 (18%)	17 (18%)	13 (14%)	13 (14%)	16 (17.7%)	17 (18%)
Qena.	82	14 (17%)	14 (17%)	9 (10.9%)	12 (14.6%)	9 (10.9%)	12 (14.6%)
Red-sea.	22	3 (13.6%)	3 (13.6%)	2 (9%)	2 (9%)	3 (13.6%)	3 (13.6%)
Aswan.	45	7 (15.5%)	8 (17.7%)	4 (8%)	5 (11.1%)	5 (11.1%)	7 (15.5%)
EL-Wadi-Elgadid.	30	3 (10%)	5 (16.6%)	2 (6%)	3 (10%)	2 6%)(	3 (10%)
Total.	1164	420 (34.7%)	448 (37%)	287 (23.7%)	342 (28.2%)	376 (32.3%)	409 (33.8%)

Table (4): Number of biovar of brucella strains isolated from lymph nodes of slaughtered buffaloesand aborted foetiby the direct method.

Locality	No. of Lymph node	No of isolated strains of L.N.	No. of aborted foeti	No of isolated strains of aborted foeti	Types of *brucella*isolated strain
Giza.	9	2 (22.22%)	2	--	*1-B.melitensis biovar3* *1- B.abortusbiovar1*
Beni-Suef.	7	1 (14.29%)	3	1 (33.33%)	*B.melitensis biovar3*
Assuit.	5	-	2	--	-
Sohag	3	-	--	--	-
Total	24	3 (13.64%)	7	1 (14.29%)	3 *B.melitensis biovar3* 1 *B. abortus*

Table (5): *Brucella* isolated from Buffaloes milk by milk ring test (MRT).

Locality	No. of samples	No. of positive samples	Types of *brucella*isolated strain
Giza	35	5 (14.29%)	*B.melitensis biovar3*
Beni-sweif.	23	2 (8.70%)	*B.melitensis biovar3*
EL-Fayoum	19	--	--
Menia	30	3 (10%)	*B.melitensis biovar3*
Assuit.	15	--	--
Total	122	10 (8.20%)	10 *B.melitensis biovar3*

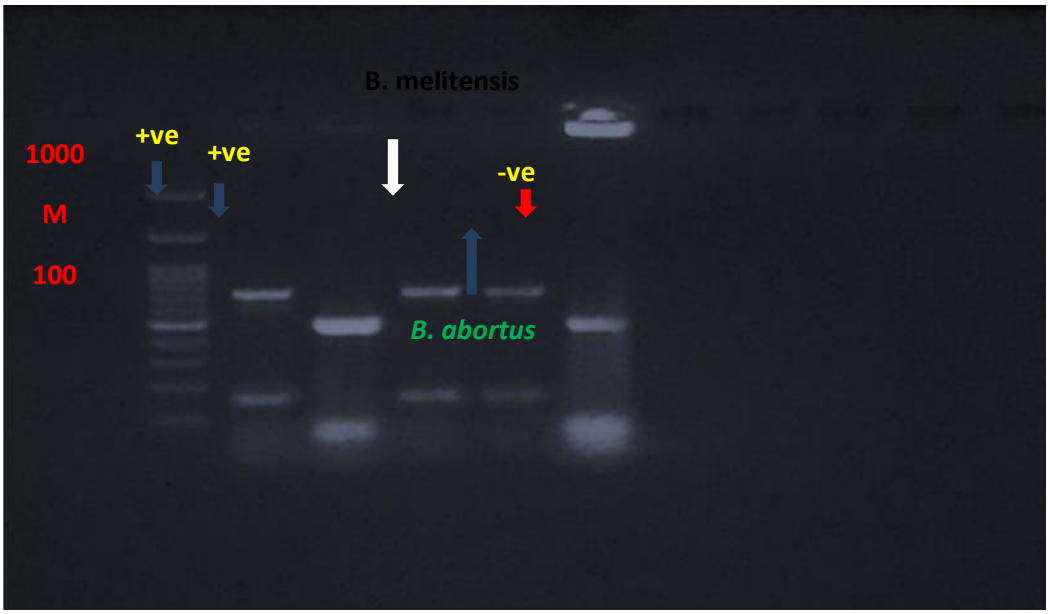

Fig. 1: AMOS PCR on 1.5% agarose gels. +ve= positive -ve= negative. Lane 1: 100 bp – 1000 bp ladder; Lane2,Control+ve *B. melitensis* Lane3,Control+ve *B.*abortusLane: 4, 5*B. melitensis* at 731bp; Lane: 6 B. abortus*at 498 bp.*Lane7,Control–ve.

Discussion

Bovine brucellosis is a disease that causes high economic loses and hazardous to human health worldwide.Accurate diagnosis must include laboratory tests that allowthe direct or indirect demonstration of *Brucella.* Classicalserological tests are routinely used for the diagnosis of brucellosis (Modolo, *et al.*, 2000).

The results of Rose Bengal test cleared 34.7% in this study, the higher incidence of brucellosis in buffaloes were observed in Giza (49.7%) while the lowest incidence of brucellosis in EL-Wadi-Elgadid (10%)(Table, 3). These results were 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. These results agreed with those of *MacMillan et al. (1990) and Garin-Bastuji et al. (1998)* where they reported that, the results of rose Bengal test differ from region to another according to the sensitivity of the serotypes of brucella*(Benkirance, 2006).*

The results of BAPAwere (37%)indicated that, the highest prevalence ofbrucellosis was in Giza (51.2%) and Beni-Suef (47.1%), the lowest prevalence in Aswan (17.7%) and EL-Wadi-Elgadid (16.6%) (Table, 3).These results indicated that, BABA test differ from region to another and had a higher sensitivity to brucella infection than that of Rose Bengal test.

These results may be attributed to the differences in environmental conditions. These results agreed with those of Alton *et al.* (1988) and Mehanna, (1989).(OIE, 2000) reported that, BAPA were performed as a manual of standards for diagnostic tests and vaccines and for accurate control and diagnosis of brucellosis.

The results of Revanoltestwere(28.2%), the highest prevalence was observed inGiza(40%) and Beni-Suef(35.2%) respectively, while minimum level was detected in EL-Wadi-Elgadid (10%)(Table, 3). The test was highly sensitive to brucellosis even at dilution 1/200, characterized by a higher ability for brucellosis detection than rose Bengal and BABA tests (Kim *et al*, 2008*)*.Revanol test detects principally IgG1, and to a lesser extent IgG2, because initial treatment of sera with Revanol removes IgM by precipitation, reduces the reactivity of IgG2, and promotes the reactivity of IgG1. This gives the Revanol test low sensitivity but high specificity *(*MacMillan *et al*., 1990 and Nielsen *et al*., 1984).

Table (3) showed results of modified SAT(23.7%) in agreement with those of *Buxton and fraser (2006)* where they indicated that, SAT when used for detection of chronically infected animals gave +ve reaction later than the other tests. Also, *Bale and Naru (2001)* concluded that SAT may be negative in chronic infections because IgM is no longer present. It is possible that in such cases, the low level of antibodies due to IgG could still be picked up by the RBPT (Hosie*et al*. 1985*)*.

Results of indirect ELISAwere(32.3%)and AGPT(33.8%)both tests gave very sensitive reactions to identify true, positive animals, while the Revanol and SAT test gave the most specific reactions to identify true negative animals Hamdy (1992). Also, Andrea *et al. (*1998)found that indirect ELISA highly specificities 95% than other serological tests.

The isolation of *Brucella*organisms from lymph nodes of slaughtered buffaloes by the direct culturing method showed that3 (13.64%)isolates; 2isolates (22.2%) from Giza(1) *B.melitensis* biotype 3and (1)*B.abortusBiotypes*1; one (14.3 %) from Beni-Suef(Table, 4). These results agreed with *Refai et al. (1990)*incow'sbuffaloes lymph nodes samples. The Brucella isolates recovered from Supra-mammary lymph nodes .

The isolated strain from aborted foeti was one isolate (14.29%)typed as *B. melitensis* biotype *3* isolated only from Beni-Suef(Table, 4). This result agreed with those of Garin-Bastuji *et al.* (2008*).* Whenever possible, brucella species should be isolated by culture using adequate selective media from uterine discharge and aborted fetuses; biovar identification is routinely based on cultural criteria and biochemical tests.

The results of milk samples were 10 (8.20%)by milk ring test (MRT) (Table, 5). Results of isolation of Brucella organisms from milk were

5isolates from Giza (14.2%), Beni-Suef2 isolates (8.6%) and Assuit3 isolates (10%).

These results agreed with those of Kolar*et al (*2004*)* as they observed that, the milk samples may gave a false –ve results for detection of brucellosis and these results attributed to many reasons, include the formation of small dyed clumps under the cream layer, and occasional true rings, different degrees of clearing milk as the dye precipitates. Some antibody-positive milk samples will not have complete clearing but a gradient of light to dark purple from top to bottom. The problem is that all milk samples have some precipitate, so a subjective decision is made of how much whitening constitutes clearing. The tests, therefore, pose substantial problems in standardization and have inadequate sensitivity and specificity. These tests were evaluated only in individual milk samples (Nielsen, 1995).

The recently developed polymerase chain reaction methods (PCR) proved additional means of detection and identification. Results of PCR bymultiplexprimersforbrucella organisms were applied on the isolated strains from L.N, aborted foeti and milk samples. A multiplex was format that will allow the rapid identification of *Brucella* spp., *B. abortus*, and *B.melitensis* in a single testwithin 2 to 3 h. In Figure (1)*B. melitensis*was identified at 731bp and *B. abortus*identified at 498bp.

These methods are becoming very important tools for the identification of Brucella, at the species level and recently also at the biovar level. These techniques can provide results in a very short time. PCR-based methods are more useful and practical than conventional methods used to identify Brucella spp., and new methods for Brucellaspp identification and typing are still being developed (William, *et al.* 2004).

Finally, we concluded that measures of the control program have to be made and a reasonable system of compensation has to be implemented.

The basic tools for a program needveterinariansfor field work and state laboratories capable of serological techniques are available.Information technology solutions and further logistic means such as animal identificationtechniques are in place in many governoratesinEgypt.

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