ISSN NO. 2320-5407

Journal homepage: http://www.journalijar.com

INTERNATIONAL JOURNAL OF ADVANCED RESEARCH

RESEARCH ARTICLE

Molecular and Serological detection of the most common *Brucella species* infecting bovine and human in Egypt

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Manuscript Info

Manuscript History:

Received: 12 May 2014
Final Accepted: 23 June 2014
Published Online: July 2014

Key words:
PCR; SAT; Multiplex PCR ; ELISA ; IgG;IgM; *B.abortus; B. melitensis*; human

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Abstract

Brucellosis is one of the most important diseases affecting human and animals in most of the developing countries including Egypt. Rapid and accurate diagnosis is fundamental for control and eradication of brucellosis. The present work aimed to determine of the most common *brucella species* biotypes infecting human and animals by using serological and multiplex PCR . A total of 170 samples (90 serum samples from male and 80 serum samples from female as well as the same number from male and female whole blood samples with (EDTA anticoagulant) at different age and 190 animals sera and the same number of samples whole blood with EDTA anticoagulant collected from (135 cow & 55 buffaloes) through the period between (February 2011 to March 2013) in Egypt . Their sera were tested by ELISA and SAT. DNA was extracted and examined by multiplex PCR (MPCR) involving specific primers for *Brucella species (B.SPP), Brucella melitensis (B. melitensis)* and *Brucella abortus (B. abortus)* based on IS711 in the brucella chromosome. Brucellosis was confirmed in patient by ELISA IgM was (23.3%) in male and (17.5%) in female, brucella IgG in male was (37.7%) and in female was (26.25%) and by SAT was (25.5%) in male and (22.5%) in female at El Fayoum Governorate respectively. Comparing serological tests with MPCR in human (male and female) serum and blood samples gave total positive of ELISA IgM (20.58 %) , ELISA IgG, (32.32), SAT (24.11%) and MPCR (32.9 %). Also ELISA (18.5 %), SAT (14.8) and MPCR (21.48%) in cow and ELISA (14.5%), SAT (12.7%) and MPCR (5.5%) in buffaloes in the same region. The present study proved that multiplex PCR assay is a rapid , sensitive and accurate technique for diagnosis of *B.SPP biotype* compared to ELSA IgM, IgG and SAT. However ELISA was simple, accurate, rapid, does not require specialized training or equipment and economical for the detection of *Brucella* antibody...

INTRODUCTION

Brucellosis is a zoonotic disease caused by Gram negative coccobacilli bacteria of the *genus Brucella*, which affecting human and animals. It is socio-economic and public health importance in many developing countries including Egypt (Tsolia *et al.*, 2002 : Pappas *et al.*, 2006 ; Franco *et al.*, 2007 and Mantur *et al.*, 2008 b). *Brucella* organisms are transmitted to man from infected animals by ingestion of unpasteurized milk and milk products or/ by contact with infected animals discharges & inhalation of aerosols containing *Brucella* organisms (Franco *et al*., 2007). Some specific occupational groups including farm workers, veterinarians, ranchers and meat-packing employees are considered at higher risk (CDC, 2008 and Padilla *et al.*, 2010). Serological diagnosis still remains the corner stone of identification of positive cases. Despite the wide range of available serological techniques

they are still suffering from different limitations which may result in development of either false positive or false negative results leading to misdiagnosis of infected and non infected animals (Hussein and Awad,2007). Serological tests including agglutination tests slide agglutination test (SAT) , Enzyme Linked Immunosorbent Assay (ELISA) IgG and IgM ,the timely and accurate diagnosis of human brucellosis continues to challenge clinicians because of its non-specific clinical features, slow growth rate in blood cultures, and the complexity of its serodiagnosis (George and Araj, 2010) However, these methods can lead to false positive results when animals are infected with bacteria possessing O-antigen of similar structure such as *Yersinia enterocolitica O: 9* (Nielsen *et al* ., 2004). Recently PCR-based assays such as nested PCR, quantitative real-time PCR and multiplex PCR for diagnosis of animals and human brucellosis (Mayer-Scholl *et al* .,2010). Developing a multiplex PCR for the identification of all *B. abortus* biotypes, *B. melitensis*, *B. ovis*, *B. canis*, *B. neotomae* and *B. suis* (Lopez-Goni *et al.,* 2008). The present study aimed to investigate multiplex PCR for diagnosis human and animals brucellosis comparing with serological tests in Egypt .

Material and Methods

Blood samples

A total of 170 human blood samples were collected from febrile illness individuals (male and females at different ages) in different localities at El-Fayoum Governorate in Egypt, during February 2011 to March 2013. Animals blood samples were collected from 190 bovine (135 cow & 55 buffaloes) in two plain vacutainer tubes with EDTA and free clotting factor. Allowing blood to clot and sera were separated by centrifugation at 3000 rpm / 15 min then divided into 2ml eppindroff tubes and stored at – 70^{o}C until used .

ELISA Assay

ELISA IgM and IgG : The collected serum samples from human were tested for the presence of the specific IgM and IgG antibodies by using ELISA IgM and IgG kits (Nova Tec brucella IgG- ELISA). ELISA kits are designed for the qualitative and quantitative detection of Brucella IgM and IgG antibodies in human serum.

ELISA in Ruminants : ELISA was carried out according to (Nielsen *et al*., 1996) . *B. abortus* S-19 SLP antigen was kindly obtained from (Veterinary Sera and Vaccine Research Institute (VSVRI) Abassia, Cairo, Egypt . The optimal antigen concentration, antibody and conjugate dilutions were chosen after preliminary checker board titration. The optimum concentration were 10 µg/ml coating antigen , 1:100 serum dilutions for cows & buffaloes sera. 1:1000 Horse radish peroxidase-labeled anti-bovine (Sigma Co.) as conjugate. The absorbance of the colored reaction was read within 20 min at 450 nm using a titertek multiskan ELISA reader. All incubation steps were carried out at 37°C in a moist chamber. The positive threshold value was determined to be two-fold of negative sera.

PCR

DNA Extraction :

Nucleic acid extracts prepared from reference strain according to (Probert *et al.*, 2004).The DNA pellet was dissolved in 50µl of TE (pH 8) and stored at -20°C till used as positive control.

Extraction of Genomic DNA from the Collected Blood Samples:

The genomic DNA from whole blood samples collected from human (170) and animals (190) were extracted with DNA Mini-Prep Biospin Blood Genomic DNA Mini-Prep Kit (BioFlux Cat BSJ040100001S80) according to the manufacturer's instructions (Qiageen USA). Measuring DNA concentration and purity according to (Probert *et al.*, 2004) . One µl of DNA was diluted with 49 µl distilled water and the optical density (OD) was measured in a quarter cuvette at 260 and 280 nm. DNA concentration was calculated using the following formula , DNA concentration (µg/ml) = 50 X A_{260} x dilution factor (50). DNA purity was calculated from the following equation :DNA purity = A_{280} / A_{260}. For ds DNA an absorbance ratio of 1.8 is concerned acceptable.

Multiplex PCR amplification:

According to the methods described by (Sambrook *et al.,* 2001). In a 0.5 ml PCR tubes the following reaction mixture was added was included and the PCR assay adjusted to the following parameters: a 25 µl reaction mixture containing 12.5 µl 2×Qiagen Multiplex PCR Mastermix, Cocktail Primers were performed according to (Bricker and Haling 1994).Cocktail Primers[*B. SPP* 5- TGC-CGA-TCA-CTT-AAG-GGC-CTT-CAT-3, *B. abortus* , 5-GAC-GAA-CGG-AAT-TTT-TCC-AAT- CCC-3 and *B. melitensis* 5-AAA-TCG-CGT-CCT-TGC-TGG-TCT-GA-3].

Briefly mixture was spine and placed in the thermal cycler , which was programmed as follow:- Initial denaturing. 95 °C for 15 min - Cycles (25 cycles) denaturing 94^{o}C 30 minute annealing 55.5^{o}C/120 seconds extension 72^{o}C/120 sec- Final extension 72^{o}C/5 minutes After the last cycle, the reaction mixtures were incubated for an additional 5 min at 72 °C before they were stored at 4 °C.

Agarose Gel Electrophoresis :

According to (Garcia-Yoldi *et al.,* 2006) agarose was heated but not boiled in a microwave till dissolving, left to cool to 60^{o}Cand then 5ul of EBr solution was added. The solution was poured in the casting tray of electrophoresis apparatus and left to solidify. 8µl of PCR products were loaded with 2µl of DNA loading buffer. DNA ladder 100 bp ,(sigma Aldrich Germany) was used and 3µl in 1µl loading buffer as DNA marker. DNA was electrophoresed at 80 v/15 minutes and ultraviolet transilluminator and photographed.

Statistical analysis:

Statistical analysis was performed according to (Perrie and Watson 1999). We used SPSS software version 16. Data were analyzed significant between groups' small and large at $P < 0.05$ and highly significant at $P < 0.01$ by using T test and ANOVA test.

Results

Serological and molecular technique were used to detect and determine different type of *B.SPP* in human (male and female) as well as in bovine (cow & buffaloes) at El Fayoum Governorate in Egypt . When comparing the reliability of the most widely used serological techniques ELISA IgM , IgG & SAT for diagnosis of *brucella SPP* with multiplex PCR . The results obtained showed that the highest positive percent of ELISA IgM in human against *brucella SPP* were (31.8% and 21.7%) in male followed by female (22.2% and 20%) and the total positive percent of brucella ELISA IgM was (23.3%) in male and (17.5%) in female.

The antibody titer determined by ELISA(ODs ± SD) of positive ELISA IgM against *B. species* in human (male and female) were (0.2056± 0.066 and 0.3702 ± 0.112) respectively. The highest positive percent brucella IgG in male were (40.8 % and 39.18%) and female were (38.8 % and 30%) at Province 2 and 4, this area known as rural area at El Fayoum Governorate. The total positive percent brucella IgG in male was (37.7%) and in female was (26.25%) at El fayoum Governorate respectively. The positive ELISA IgG (ODs ± SD) of *B.species* in human (male and female) at large districts were (0.63870±0.119 and 0.657±0.064) at El-Fayoum Governorate respectively.

When comparing serological tests (ELISA IgM , ELISA IgG and SAT) in male , it was found that positive percentage (23.33% ,37.77% & 23.7 %) and in female positive percent were (17.5% ,26.25% & 22. 5 %) at El fayoum Governorate.

Table (1) : Detection of human brucellosis by ELISA IgM, IgG and SAT in human at El-Fayoum Governorate in Egypt

M: Male F: Female

Locations	No. human sera	Serum samples		ELISA IgM				ELISA IgG				SAT				Means ODs ± SD			
				Male		Female		Male		Female		Male		Female		ELISA IgM		ELISA IgG	
		M	F	P	%	P	%	P	%	P	%	P	%	P	%	M	F	M	F
Province 1	45	23	22	5	21.7	3	13.63	8	34.7	4	18.18	5	21.73	3	13.6	0.166±.0.046	0.294±0 .176	0.738 ±.0.153	0.735 ±.056
Province 2	42	22	20	7	31. 8	4	20	9	40.8	6	30	6	27.28	4	20	0.232.±0.085	0.657 ± .048	0.645 ± 0.076	0.657 ±0.065
Province 3	42	22	20	4	18.18	3	15	8	36.36	4	20	6	27.28	5	25	0.209.±0.044	0.308.±0.155	0.678 ±0.120	0.573 ± 0.06
Province 4	41	23	18	5	21.7	4	22.2	9	39.18	7	38.8	7	30.4	5	27.7	0.205.±0.035	0.22.±0.0395	0.674 ±0.128	0.664 ± 0.072
Total	170			23.33%		17.5%		37.77%		26.25%		25.5%		22.5%		0.2056± 0.066	0.370± 0.110	0.638 ± 0.119	0.657 ± 0.064
				35		20.58 %		55		32.35%		24.11%				0.2273± 0.05		0.670 ± .0523	

Figure (1): Prevalence of human brucellosis by ELISA and SAT in human at El Fayoum Governorates

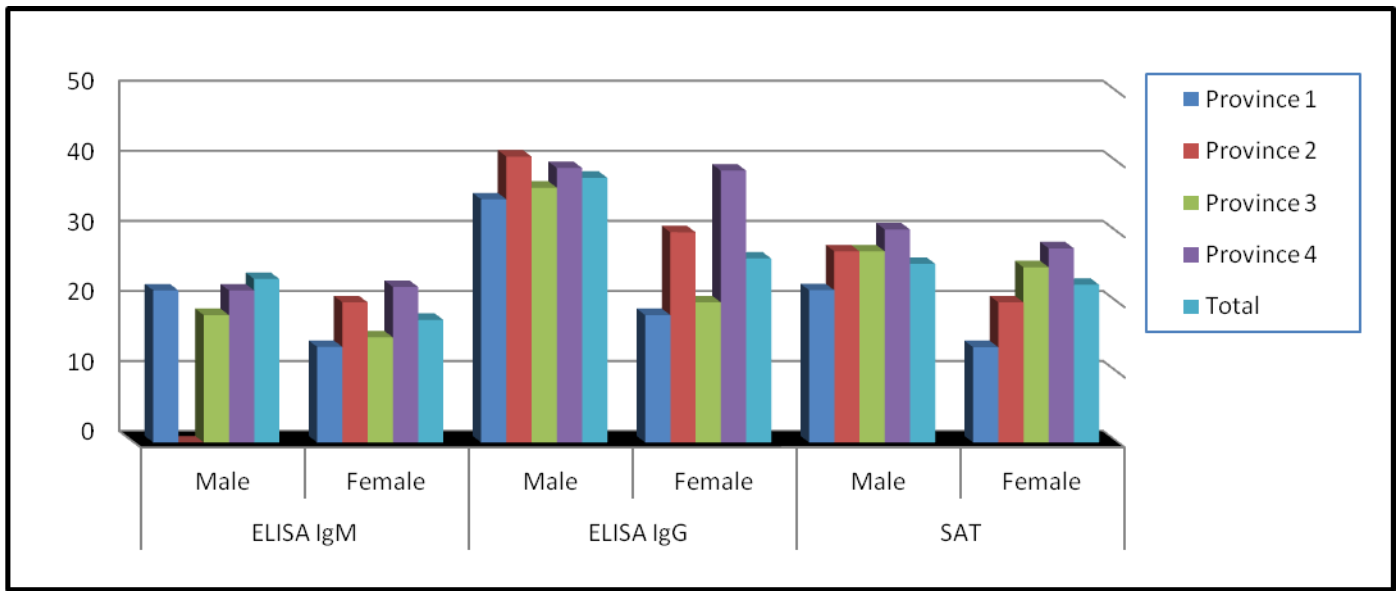


Table (2): Examination of bovine sera against *brucella species* by ELISA and SAT in bovine at El-Fayoum Governorate .

Location	Sera		ELISA				SAT				ELISA Mean ODs ± SD	
			Cows		Buffaloes		Cows		Buffaloes		Cows	Buffaloes
	C	B	PCS	% CS	BS	% BS	P CS	% CS	PBS	% BS	CS	BS
Province 1	30	10	5	16.66	1	10	5	13.33	2	20	0.538±.0.153	0.435±.056
Province 2	35	15	6	17.14	2	13.33	6	14.14	2	13.33	0.645 ±0.076	0.657 ±0.065
Province 3	40	20	7	17.5	3	15	5	15	1	10	0.678±0.120	0.573± 0.060
Province 4	30	10	7	23.33	2	20	8	16.66	7	10	0.474±0.128	0.564±0.072
Total	135	55	25	18.51	8	14.5	24	14.8	12	12.7	0.538± 0.119	0.657± 0.064

CS= Cow BS= Buffalo PCS= Positive cow sera PBS= Positive buffalo sera

The results in table 2, showed that the total positive percent of brucella species in buffaloes (14.51%) and cows (18.5) tested by ELISA at El Fayoum Governorate . Also, lowest number of reactors among buffaloes was 5 % may be due to buffaloes have gene resistance of infections. Means ODs ± SD of ELISA in cow was 0.678 ±0.12 and Buffaloes was 0.656 ±0.065. When comparing serological tests (ELISA, RBT and SAT) in cow , we found positive percent were (28.5% ,14.8 %, 26.7%), in buffaloes positive percent were (14.5% ,12.7 %, 12.7%)

Figure (2): Positive percentage of bovine brucellosis in bovine at El Fayoum Governorate

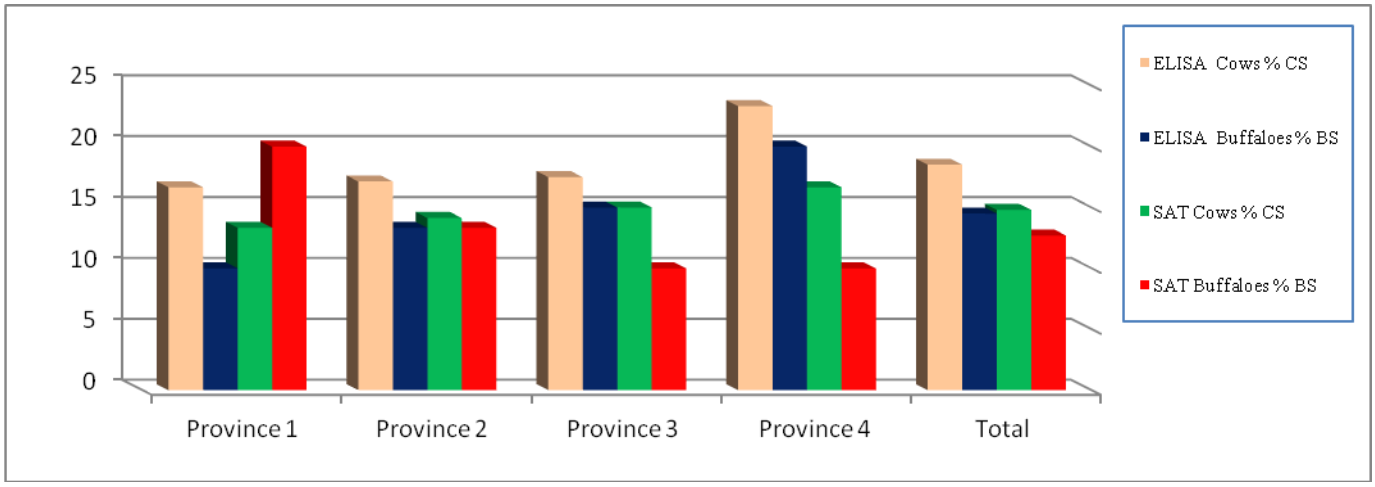


Table (3): Determination of *Brucella spp biotypes* by multiplex PCR in human at El-Fayoum Governorate.

Localities	No WB	No. of W. blood samples		Multiplex PCR									
				Positive			% of Positive			*Br. ab*	Br. M	*Br. ab%*	Br. M%
		M	F	M	F	T	M	F	T				
Province 1	45	23	22	6	5	11	26.08%	22.27%	24.44%	3	8	6.66%	17.7 %
Province 2	42	22	20	8	7	15	36.36%	35 %	35.7%	4	11	9.5 %	26 %
Province 3	42	22	20	9	8	17	40.9%	40 %	40.4%	3	14	7.14%	33.3%
Province 4	41	23	18	7	6	13	30.4 %	33.3 %	31.7%	4	9	9.7 %	21.9 %
Total	170	90	80	30	26	56	33.3%	32.5%	32.9%	14	42	8.23%	24.79%
	170			56			32.9%			8.23%		24.79%	

M: Male F: Female

Figure (3): Determination of *Brucella spp by multiplex PCR* in human at El-Fayoum Governorate.

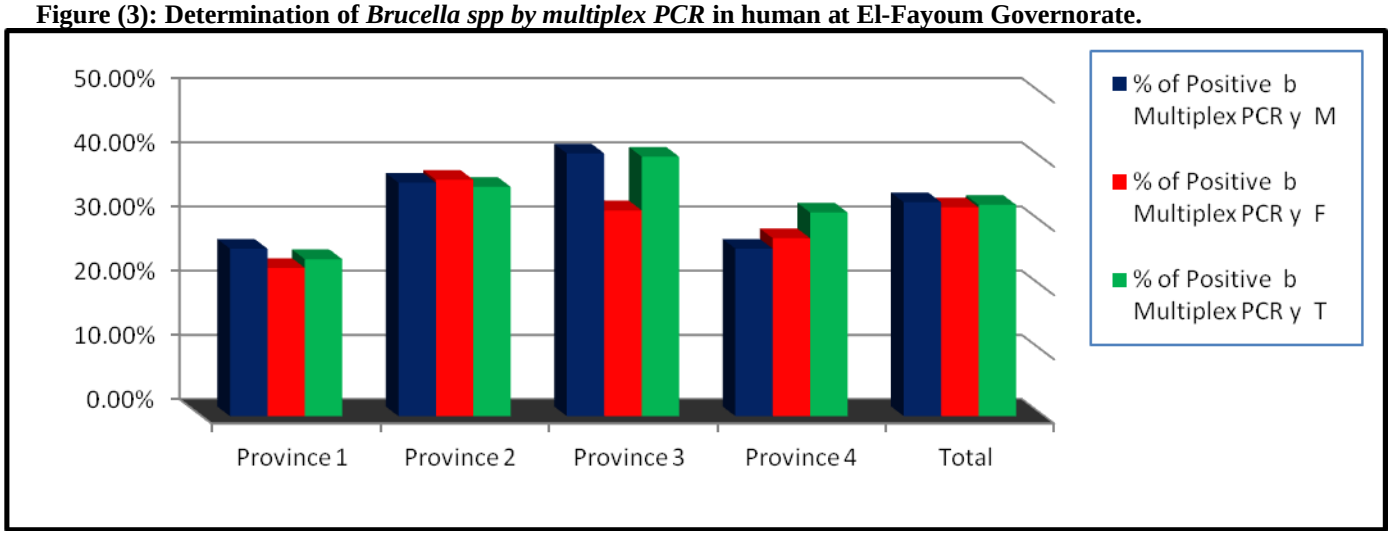


Figure (4):Multiplex PCR for identification of *Brucella species* from human and animals blood samples

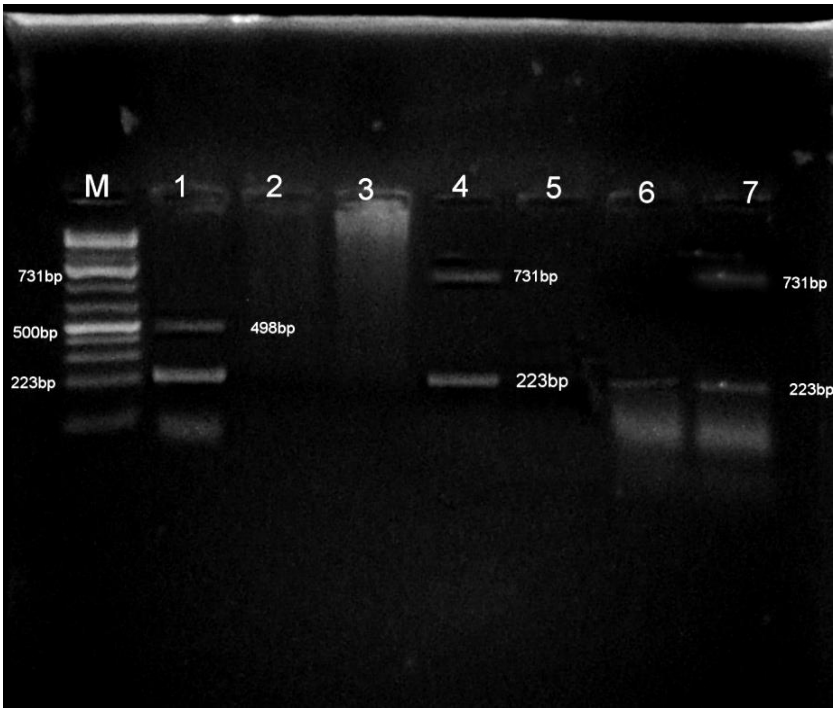


Figure 1 : Multiplex PCR for detection of *Brucella genus* (*B. species* , *B. abortus & B.melitensis*) at 223 , 498 and 731 bp; lane 1 positive *B.abortus* at 223 and 498 bp ; lane 2,3 and 5 : negative samples ; lane 7 : *B. species* positive sample at 223; lane 7 : *B. melitensis* positive sample at 223 and 731 bp and 498 bp for *B.abortus* were considered positive.

Figure (5) Differentiation of *Brucella species* by Multiplex PCR from human blood samples

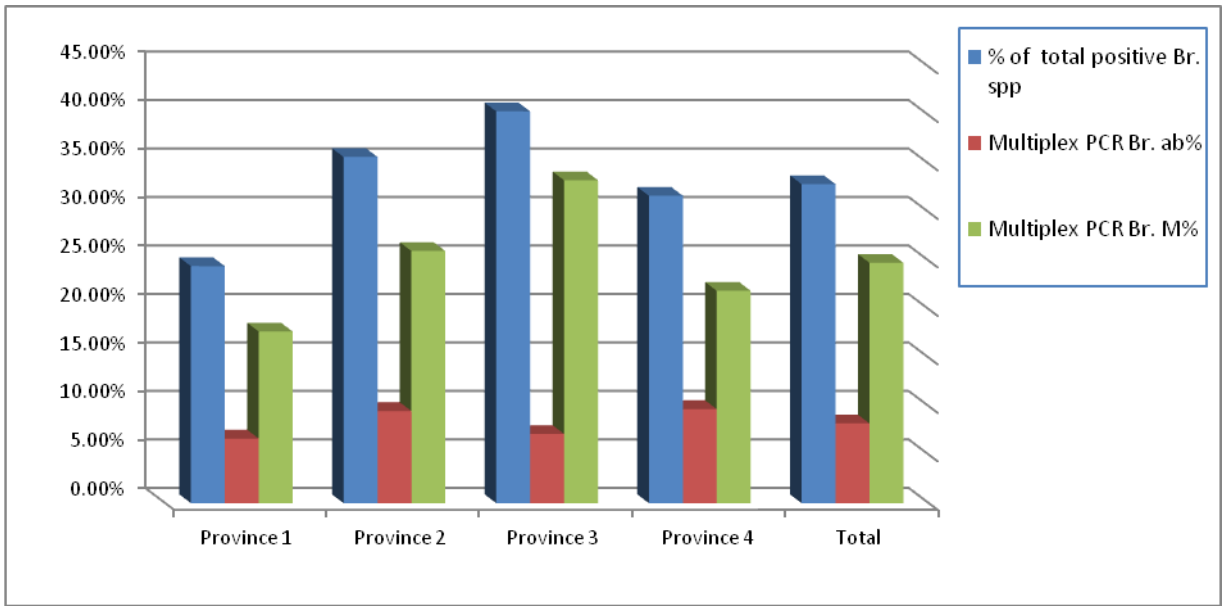


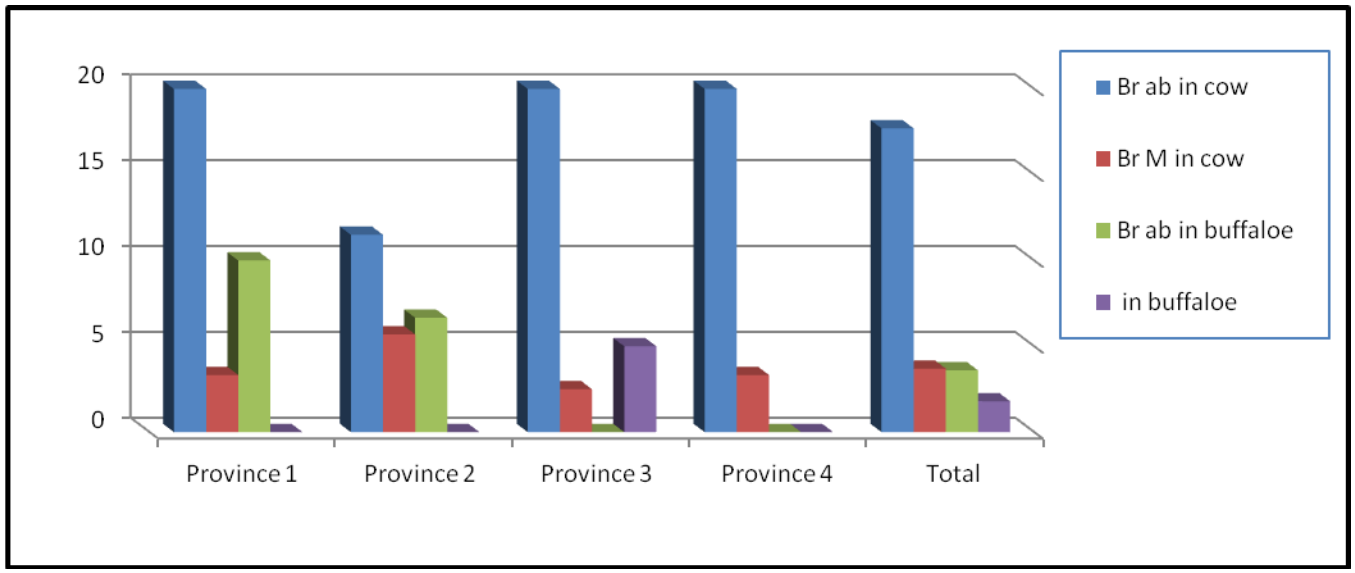
Table (4) Detection of animals brucellosis by multiplex PCR in bovine at El-Fayoum Governorate.

Localities	Multiplex PCR									
	W.B. of Cows					W.B. of Buffaloes				
	TBS	No +	+%	*B. abr*	*Br. m*	T	No +	+%	*B. abr*	*B. m*
Province 1	30	7	23.3%	6	1	10	1	10%	1	0
Province 2	35	6	17.3%	4	2	15	1	6.6%	1	0
Province 3	40	9	22.3%	8	1	20	1	5%	0	1
Province 4	30	7	26.6%	6	1	10	0	0%	0	0
Total	135	29	21.48%	24	5	55	3	5.5%	2	1

TBS: Total blood samples- No+: Number of samples positive +%: Positive percent

Table Multiplex PCR which employed three-pair primers to differentiate all *B.SPP* and brucella genus (*B. species* , *B. abortus and B.melitensis*) at 223 , 498 and 731 bp. The positive percent of PCR were (5.5 %, 21.48 %,) in buffaloes & cow respectively.

Figure (6) Differentiation of *Brucella species* by Multiplex PCR from animals blood samples



Discussion

Brucellosis is an endemic disease of humans and animals throughout the Middle East countries especially countries of the Mediterranean basin (Al-Ayed *et al.*, 2011 and Amin *et al* ., 2012). Brucellosis is still one of the most important bacterial diseases among farm animals that threat animal reproduction and production beside to its zoonotic importance(Refai, 2002).Brucellosis as a food borne and occupationally-acquired zoonosis, brucellosis has a substantial impact on public health in endemic regions (Salama Abdel Hafez *et al*., 2011). Egypt is one of the countries with high incidence of human brucellosis together with Saudi Arabia ,Palestine, Jordan, Libya and Sudan (Kwaasi *et al*., 2005).

In Eastern Mediterranean region countries such as Yemen, Oman, and Saudi Arabia higher incidence of brucellosis due to ingestion of dairy products and raw milk (Mehanna,1989).Clinical picture of human brucellosis is heterogeneous non specific which either subclinical or atypical infection in both acute or chronic stage , in hospitalization patients. ELISA as the most common clinical serological test for detection of brucellosis, available as commercial IgM and/or IgG (Maleki *et al* ., 2011).

Data presented in Table (1) and Figure (1) showed clearly that the positive percent of *antibrucella spp* ELISA IgM in male more than female live in the same area and the total positive percentage in male was (23.3%) and in female was (17.5%), higher positive percent in male may be due to male more exposure to risk factor as aborted animals, consumed row milk and dairy product from infected animals with *brucella* than female, these results were in agreement with (Samaha *et al*., 2009 and Shafee *et al* ., 2011).Also higher seropositivity of brucellosis were reported in human (Veterinarians, Shepherds, Butchers, Farm Animal Owners) in Egypt .

The total positive percentages of antibrucella IgM in sera of human male and female were (20.58%) at El Fayoum Governorate means that male and females have acute brucellosis and this finding was in agreement with (Georgios et al 2005). On other hand disagreement with (Memish *et al* 2000). They found that antibrcella IgM cannot be detected in some patients with brucellosis up to (11%).The patients did not have detectable levels of specific IgM by serological tests.

The total positive percent antibrucella IgG in male was (37.7%) and in female was (26.25%) at El fayoum Governorate respectively. However , the highest positive percent in male at Province 2 were (40.8 %) followed by (38.8%) in female at Province 4 , this result means that present number of factor favor spread of brucella in this area , this finding is in agreement with (Afifi *et al.* 2005) who studied brucellosis in Egypt , they recorded many different number of factors favor the spread of *B. SPP* infectious and facilitate the occurrence of simultaneous or multiple zoonoses. Also positive percent of SAT of *B. abortus* was (19.4%) more than *B. melitensis* was (5.88%) this result may be due to human higher exposure to the main source of *B. abortus* more than *B. melitensis* in this area , increase consumption of raw milk and milk byproducts of cow and buffaloes origin source of *B. abortus* more than sheep and goat origin source of *B. melitensis* in investigated different animals species at El-Fayoum Governorate or may be due to less accurately of SAT for detection *B. melitensis* which is considered the main prevalent biovar in Egypt Governorates as recorded by (Abd Al-Azeem *et al.*, 2012).

Comparing serological tests (ELISA and SAT) were (14.5 & 18.51%) in cows and buffaloes were (12.7 % & 14.5%) at El fayoum Governorate. It is clear that no single test is capable to identify all positive cases of brucella infected animals due to variation in sensitivity and specificity of serological tests .Also, different antibody classes in the examined sera as in Table (2) and Figure (2), pervious results explain that the SAT serological test must be confirmed by ELISA to prove that animal free from brucella infection, ELISA has been recommended as a confirmatory test and the test of choice for brucellosis by several authors, (Salama Abdel Hafez *et al*., 2011). Explained that in old standing chronic infection ELISA is often positive while the agglutination test is negative. .Also it able to detect very minutes amount of antibrucella antibodies in recent infected with brucellosis .

Multiplex PCR assay for definitively identifying *B.SPP*, *B.melitensis* and *B.abortus*, while simultaneously providing support for classical serological test (ELISA & SAT), through rapid molecular typing. Multiplex PCR showed total positive percent of brucella DNA of human (male and female) were (32.29%) .Wherever the positive percent in male was (33.5%) and female was (32.5 %) at El fayoum Governorate respectively Table (3) and Figure (3).

The PCR gave a higher figure of positive reactors compared with ELISA & SAT this results are in agreement with (Garcia-Yoldi *et al.*, 2006). When comparing serological tests (ELISA IgM , ELISA IgG & SAT) with M PCR in male , it was found that positive percentage (23.33% ,37.77% and 25.5 %) with (33.3%) and in female positive percent were (17.5% ,26.25% and, 22.5%) with (32.3%) at El fayoum Governorate. Interestingly results ELISA IgG positive reactor nearly the same positive reactor of M PCR detection of human brucellosis but other serological tests less sensitivity than multiplex PCR .The lower sensitivity of serological test may be due to lower antibody titer at the time of sample collection & early phase Sathyanarayan *et al* ., (2011) & Ibrahim *et al.*, (2012). Identification of *B.SPP* genotypes by using Multiplex PCR assays found that *B. abortus* genotype was (8.23%) lower than *B.melitensis* genotype was(24.79 %) may be due to increase infections with *B. melitensis* in human and due to high accurately of

PCR for detection *B. melitensis* by using specific primer for all *B. melitensis* Biovar which is considered the main prevalent biovar all over Egypt as in Table (3) and Figure (4 and 5) .

Table (4) and Figure (6) compared serological tests (ELISA, SAT) with M PCR in cow , we found positive percent were (18.5% , 14.8%) with (21.8%) , in buffaloes positive percent were (14.5% , 12.7%) with (5.5%) from these results obtained by the different serological tests ,it is clear that no single test is capable to identify all positive cases of brucella infected animals due to variation in sensitivity and specificity of different serological tests as the result of different antibody classes and variation in antibody classes in the examined sera according to (WHO , 2009). reported that ELISA able to detected very small amount of antibrucella antibodies in recent infected herd .

Finally, it was found that multiplex PCR base assays were able to identify and typing *Brucella spp* infection followed by ELISA and SAT.

Conclusion :

According to data obtained, it was found that the M PCR base assay is highly sensitive and specific method for detection and identification of *Brucella* organisms from human and animals blood samples than serological methods. It has several advantages over the current serological methods used to identify of brucella genotypes , fast and accurate procedures .Also, provided a deeper insight into disease epidemiology and will allow fighting back the reemergence of new strain of brucellosis in Egypt. Beside ELISA and SAT considered as the test of choice in the future for serological diagnosis of brucella infection in human and animals.

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