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MOLECULAR CHARACTERIZATION IN COMPARISON TO SEROLOGICAL AND CULTURAL TECHNIQUES FOR DIAGNOSIS OF BRUCELLA INFECTION IN ASYPTOMATIC CATTLE AND BUFFALOES

BY

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ABSTRACT

As a prompt and accurate diagnosis is important for undertaken an effective control measure, the present study was undertaken to evaluate some diagnostic methods for Brucella infection in dairy animals. Three hundred serum samples from dairy cattle (n = 200) and buffaloes (n = 100) from different localities in Dakahlia and Dammitta governorates were collected with unknown history of vaccination or infection and the sera were examined using Rose Bengal Plate Test (RBPT), Enzyme Linked Immunosorbant Assay (ELISA), Standard Tube Agglutination Test (STAT) and Complement Fixation Test (CFT), Among the total 300 serum samples, Brucella antibodies were detected in 129 (43%), 138 (46%), 115 (37.33%) and 96 (32%), respectively. Milk samples were also collected from the same animals and analyzed by Milk Ring Test (MRT) and ELISA for brucella antibodies and culturing has also done for isolating Brucella organisms, meanwhile polymerase chain reaction (PCR) was made for detecting the Brucella DNA. Positive rate was 114 (38%), 97 (32.33%) 27 (9%) and 82 (27.33%), respectively. *Brucella melitensis* was recovered only from milk samples. In conclusion, ELISA technique is suitable for large scale screening as it detected a higher seropositivity. PCR is more sensitive and more specific in comparison to the conventional technique and it can be used in clinical samples directly. *B. melitensis* biovare3 is the most existence type in Egypt now.

Key wards: Brucella, Rose Bengal Plate Test, Enzyme Linked Immunosorbant Assay, Polymerase Chain Reaction, Milk Ring Test

INTRODUCTION

Brucellosis is one of the major zoonotic infections worldwide ***(Pappas et al., 2006).*** It is caused by gram-negative coccobacilli of the genus *Brucella* and affects cattle, sheep, goats, and other livestock ***(Corbel, 1997; Solera et al., 1997).*** Since the discovery of *Brucella melitensis* by David Bruce in 1887, several species have been identified, such as *B. abortus, B. melitensism B. suis*, *B.neotomae*, *B. ovis*, and *B. canis* ***(Corbel, 1997 and Garin-Bastuji et al., 1998***).Although brucellosis has been controlled in most industrialized countries, it remains a major problem in the Mediterranean region, western Asia, Africa, and Latin America ***(Pappas et al., 2006***). It can cause appreciable economic losses in the livestock industry because of abortions, decreased milk production, sterility, veterinary care and treatment costs ***(Corbel, 1997***). Brucellosis was first reported in Egypt in 1939 ***(Refai, 2002).*** Control programs for brucellosis in Egypt have used 2 methods: vaccination of all animals and slaughter of infected animals with positive serologic results.

The difficulty of accurately detecting all infected animals, especially carriers, is a major limitation of these programs. To enhance efficiency of brucellosis-specific prophylaxis, early detection of brucellosis by highly sensitive and specific methods is needed. Egypt has mixed populations of sheep, goats, cattle, and buffaloes. The number of buffaloes in Egypt is higher than in any other country in the Near East region ***(Refai, 2002)***. In addition to high prevalence rates of *B. melitensis* infections in sheep and goats, *B. melitensis* infections of cattle and buffaloes have increased in Egypt ***(Refai, 2002).***

The present investigation aimed to determine the prevalence of brucellosis in asymptomatic cattle and buffaloes at Dakahalia and Damitta governorates, in Egypt and to find a good test for screening of Brucella infection.

MATERIAL AND METHODS

Samples.

300 Serum and 300 milk samples were collected from asymptomatic 200 cattle and100 buffaloes of unknown history of infection or vaccination from different farms at Dakahlia and Damitta governorates, Egypt.

Serological Examination:

B. abortus cell suspensions for RBPT, TAT, CFT and MRT were provided by Veterinary Serum and Vaccine Institute, Abbassia, Cairo, Egypt. RBPT, TAT, CFT and MRT were carried out according to ***Alton et al.(1988)*** The iELISA was done and results were interpreted according to the instructions of the manufactures using Brucella- Ab I-ELISA kit obtained from Svanova Biotech AB, Science Park, SE-751 83 Uppsala, Sweden.

Bacteriological Examinations

Isolation and identification of Brucella species from all collected milk samples was carried out according to ***Alton et al.(1988***)

Molecular detection of brucella from milk

Extraction and purification of Brucella DNA from milk samples was done as described by ***Leal-Klevezas et al.(1995).*** Briefly, 400 mL of lysis solution (2% Triton X-100, 1% sodium dodecyl sulphate, 100mM NaCl, 10mM Tris-HCl [pH 8.0]) and 10 mL of proteinase K (10mg/mL) were added to 400 mL taken from the fatty top layer of each milk sample. The contents were mixed thoroughly and incubated for 30 min at 50°C. Then, 400 mL of saturated phenol (liquid phenol containing 0.1% 8-hydroxyquinoline, saturated and stabilized with 100mM Tris-HCl [pH 8.0] and 0.2% of 2-mercaptoethanol) were added. The contents were mixed thoroughly and centrifuged at 8000 g for 5 min and the aqueous layer was transferred to a fresh tube. An equal volume of chloroform-isoamyl alcohol (24: 1) was added, mixed thoroughly and centrifuged at 8000 g for 5 min. The upper layer was again transferred to fresh tube, and 200 ml of 7.5M ammonium acetate was added and mixed thoroughly. The contents were kept on ice for 10 min and centrifuged at 8000 g for 5 min. Before the aqueous content was transferred to a fresh tube two volumes of 95% ethanol were added. The contents were mixed and the tubes were stored at 20°C. DNA was recovered by centrifuging the samples at 8000 g for 5 min, the pellets were rinsed with 1ml of 70% ethanol, dried and resuspended in 20 ml of TE buffer (10mM Tris-HCl [pH 8.0], 1mM disodium EDTA). DNA concentrations were determined by measuring their wavelengths at A260. Finally the DNA extraction was stored at 20°C until they used.

PCR Amplification

The forward (F) and reverse (R) primers of *omp2a* gene were F5′-GGCTATTC AAA AT- TCTGGCG-3′ and R 5′-ATCG ATT CTC AC- GCTTTCGT-3′,respectively.PCR

amplification was performed by the method of ***Mullis and Faloona (1987)***. A typical reaction mixture contained 50 mM KCl, 1.5 mM MgCl2, 0.1% (wt/vol) Triton X-100, 0.2 mg of bovine serum albumin (fraction IV; per ml, and 10 mM Tris-HCl (pH 8.5). Each reaction mixture was supplemented with 100 mM each of the four deoxyribonucleotides, 100 ng of sample DNA, and each oligonucleotide primer. Reactions were initiated by adding 0.5 U of Taq polymerase. Following hot start treatment at 95°C for 3 min, PCR was performed with an Eppendorf thermocycler as follows: 40 cycles of PCR, with 1 cycle consisting of 1 min at 95°C for DNA denaturation, 2 min at 58°C for DNA annealing, and 3 min at 70°C for polymerase mediated primer extension. 38 cycles consisting of 1 min at 95°C and 2 min at 58°C and 3 min at 70°C. The last cycle samples at 95°C for 1 min and then for another 2 min at 58°C and 10 min at 70°C. Ten µl of the amplified product was analyzed by electrophoresis in 1.5% agarose gels in TEA buffer (20 mM Tris-acetate, 1 mM EDTA [pH 8.0]).

Positive and Negative Controls

DNA was also extracted from vaccines strains (*B.abortus* S19 and *B.melitensis* Rev.1). These extracted DNA were used in PCR reaction as positive control. In negative control tubes of water was added instead of DNA sample.

Statistical analysis

Sensitivity and specificity of the used diagnostic tests was studied according to ***(Nielsen, 1990)*** using the bacterial isolation diagnostic method as a gold standard.

RESULTS

Table (1) Efficacy of each serological test used for detection of brucella antibodies in serum of asymptomatic cattle and buffaloes.

Test	Cattle (200)		Buffaloes(100)		Total	
	Positive	%	Positive	%	Positive	%
ELISA	108	%54	30	%30	138	46%
RBPT	100	%50	29	%29	129	%43
STAT	90	%45	25	%25	115	%38.33
CFT	70	%35	26	%26	96	%32

ELISA (Enzyme Linked Immunosorbant Assay), RBPT (Rose Bengal Plate Test), STAT (standered Tube Agglutination Test), CFT (Complement Fixation Test). Percentages were calculated according to the number of examined samples in each group.

Table (2) efficacy of each serological test used for detection of Brucella antibodies in milk of asymptomatic cattle and buffaloes.

Animals	Tested	MRT Positive	Percentage	ELISA Positive	Percentage
Cattle	200	84	%42	72	%34
Buffaloes	100	30	%30	25	%25
Total	300	114	%38	97	%32.33

MRT (Milk Ring Test) ELISA (Enzyme Immunosorbant Assay)

Percentages were calculated according to the number of examined samples.

Table (3) Recovery of *B. melitensis* in milk of cattle and buffaloes

Animal species	Examined number	Positive	Percentage
Cattle	200	23	11.5%
Buffaloes	100	4	4%
Total	300	27	9%

Detection of Brucella DNA in Milk Samples by PCR

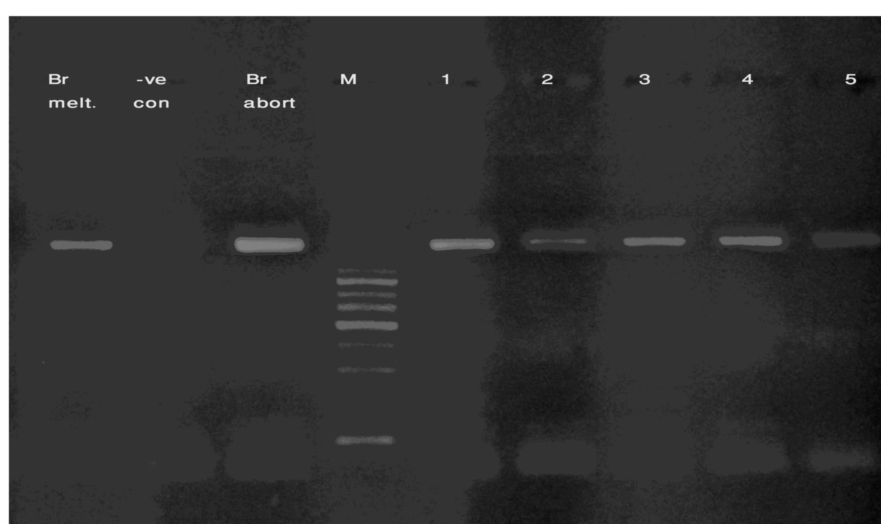

Figure (1): PCR product of *omp2a* gene amplification, *Brucella* spp. lane 1,2,3,4,5 samples and reference strains *B. abortus S19 and* *B. melitensis* H38 strain and –ve control.

Table (4) Sensitivity and specificity of different serological tests in comparison to molecular techniques for diagnosis of Brucella in milk and serum samples.

Test	Sensitivity	Specificity	Posit. predictive value	Negat. predictive value
RBPT	0.88	0.62	0.19	0.99
ELISA	1	0.58	0.19	1
TAT	0.9	0.66	0.21	0.98
CFT	0.9	0.7	0.24	0.98
MRT	0.92	0.67	0.21	0.98
MELISA	0.88	0.73	0.24	0.98
PCR	0.92	0.78	0.30	0.99

DISCUSSION

Control of brucellosis depends mainly upon elimination of infected animals. The most effective plan for elimination of the disease is the detection of infected animals by periodic testing of milk or blood for specific antibody and elimination of positive reactors ***(Nielsen, 1990***). Diagnosis of brucellosis in animals is based mainly on clinical signs, serological and bacteriological investigations. The obtained results revealed that for RBPT, ELISA, SAT and CFT, 138 (46%), 129 (43 %), 115 (38.33%) and 96 (32%) were positive respectively (Table 1). Similarly, higher seropositivity by ELISA as compared to RBPT and STAT were also recorded by ***(Rao et al., 1999)*** in cattle and buffaloes. ELISA has been shown to be highly sensitive and of equal or greater specificity than CFT and RBT and suitable test for large scale screening to Bovine Brucellosis ***(Saravi et al., 1995***). Moreover, latent infection could be detected earlier by ELISA than other serological tests as it detected all classes of antibodies. However RBPT assay can detect antibodies of classes IgG1 and IgM against surface antigen lipopolysaccharides (LPS) of smooth Brucella ***(Davies, 1971***), it was also able to detect high number of positive samples. Indeed, this test is internationally acknowledged as the choice for the screening of brucellosis (***Garin-Bastuji et al., 1998)***. Due to cross reactivity between these antigen with other bacterial species including *Yersinia enterocolitica* 0:9 and *E. coli* serotype O:57 ***(Chukwu, 1985* and*Kittelberger et al., 1998),*** RBPT may suffer higher rates of false positive results than other serological tests.

The complement fixation test is technically challenging because a large number of reagents must be titrated daily and a large number of controls of all reagents is required. It is also an expensive test again because of large number of reagents needed and because it is labour intensive. However, since only IgG1 isotype of antibody fixes complement well, the test specificity is high.

The prevalence rates of brucellosis using MRT was 42 % and 30% for cattle and buffaloes respectively with an overall incidence 38%(Table 2). ***Ibrahim et al., (2012)*** detected a higher incidence rate of brucellosis using MRT, 51% and 49.8%, for cattle and buffaloes respectively. This may be contributed to that they collected milk samples from endemic populations showing a problem of abortion. The prevalence rates of brucellosis in our study using milk ELISA in cattle and buffaloes was 34% and 25% with overall prevalence rate 32.33% ***Ibrahim et al., (2012)*** detected much higher percentage 50%, 39.6%, with an overall incidence 42.8%

Bacteriological examination of milk samples(Table, 3) revealed the recovery of *Brucella* isolates from only 27 (9%) cases. This indicated that the sensitivity of serological test was higher than that of the culture method. The same conclusion was reached by ***Hamdy and Amin(2002)*** who suggested that the most specific diagnostic test involves isolation of the causative organism, but this suffers from the drawback of requiring a long incubation period and low sensitivity especially in the chronic stage of the disease. Moreover, the culture material must be handled carefully as the *Brucella* organism is a class III pathogen. All the 27 isolates identified as *B. melitensis* biovar 3 no *B. melitensis* Rev 1 strain isolates and these results differ from the results obtained by ***Helmy and Zaki (2007)*** who isolated *B. melitensis* Rev 1 strain which may be due to previously vaccination with Rev. 1 vaccine or due to Rev. 1 horizontal infection.

In the present study Brucella DNA was detected by using a primer pairs Brucella *2aA and 2aB* encoding omp2a gene. Out of 300 bovine milk samples a total of 82 (27.33%) from 61 cattle and 21 buffaloes were giving a PCR products of 1100bp size and the remaining 218 (72.67%) milk samples failed to produce the targeted amplification.

Among the different methods used for Brucella detection, tests used for detection of Brucella antibodies in serum were resulted in highest number of positivity followed by tests detected Brucella antibodies in milk, PCR assays then cultural isolation. Similarly, ***Leal-Klevezas et al. (1995)*** found higher sensitivity of serological tests than that of semen culture

and semen based PCR assay. They also found no advantage of using PCR methods over standard serological and bacteriological methods for diagnosis of brucellosis.

Likewise, some of the bovines showing antibodies both in serum as well as in milk could not reveal the presence of Brucella in milk either by PCR or cultural isolation. This might be due to the previous exposure and possibility of periodic shedding or no shedding of the Brucella in milk ***Hamdy and Amin (2002)***. Brucella gene target which located in various tissues at a given time might have affected the detection of Brucella in semen by PCR ***Helmy and Zaki (2007***) However, seropositivity due to possibility of cross-reacting antibodies could not be overlooked ***Nielsen(2002***). Higher prevalence of brucellosis in cattle than that in buffalo agrees with ***Samaha et al.(2008)***, the higher prevalence in cattle may be attributed to the species specificity or possibly the higher fat percentage in buffalo milk may be the obstacle in the detection of positive cases.

Brucella could not be recovered in cultural isolation from a lot of PCR positive samples, which might be due to the slow growth and fastidious nature of the organisms. Even, types of cultural medium and selective supplements may affect the recovery rate of the Brucella from the specimens ***Farrell and Robertson (1972)***. It was evident that all samples which were positive to culture and PCR assay were positive also to the MRT. Although the MRT is sensitive, it nearly detected low numbers of positive animals 113 (37.67 %) than most those detected by blood serological tests. This could be due either to the stage of infection where the levels of the agglutinins were not high enough to be excreted in milk, or to the irregularity in the filtration of the agglutinins from blood ***Pat and Panigrahi (1965).***

By using culture examination as a gold standard test the sensitivity of different methods were shown in Table (4) in which serum ELISA showed highest sensitivity in comparing to the other methods used for Brucella diagnosis. And the highest specificity detected by PCR technique which used for detection of Brucella DNA in milk.

CONCLUSION

ELISA can be used as a suitable screening test for diagnosis of brucellosis in cattle and buffaloes. PCR is more sensitive and more specific in comparison to the conventional techniques and it can be used for clinical samples directly.

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