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Investigations on Brucellosis in Egyptian Baladi Does with Emphasis on Evaluation of Diagnostic Techniques

K.A. Abd El-Razik, H.M. Desouky and W.M. Ahmed
Department of Animal Reproduction and A.I. National Research Center, Dokki, Giza, Egypt

Abstract: Investigations were carried out on caprine brucellosis in a costal area in Egypt. A total number of 577 *Baladi* Does was examined for *Brucella* infection using different serological tests. Specimens were taken from seropositive obligatory slaughtered Does (No = 33) for *Brucella* isolation, histopathological examination, Polymerase Chain Reaction (PCR) assay and determination of serum copper (Cu), zinc(Zn) and iron (Fe) concentrations. Results indicated that the incidence of brucellosis was 3.0-5.0%, by using the different serological tests. Buffered Acidified Plate Antigen Test (BAPAT) is of the highest sensitivity followed by Rose Bengal Plate Test (RBPT), L-ELISA, Complement Fixation Test (CFT), P-ELISA, Rivanol test (RVT) and Tube Agglutination Test (TAT). In seropostive Does, *Brucella melitensis* biovar 3 was isolated from 78.78% and PCR yielded expected products in 81.81%. Moreover, granulomatous endometritis, lymphocytic mastitis and lymphoid depletion in both lymph nodes and spleen were evident together with significant ($p<0.001$) decreases in serum Cu, Zn and Fe concentrations. In conclusion, more attention should be paid to goat in brucellosis epidemiology in the application of national program of brucella control and eradication.

Key words: Goat-brucella-serology-PCR-pathology-trace elements

INTRODUCTION

Brucellosis is a major zoonotic problem in many countries and the eradication of this disease in animals is a necessary step to control the disease in man (Corbel, 1997; Reviriego *et al*., 2000). Also, it is considered as one of the most economically important reproductive diseases of livestock, leading to abortion, sterility and decreased productivity (Leal-Klevezas *et al*., 1995).

Currently, diagnosis of brucellosis is based on serological and microbiological tests. Serological methods are not always sensitive or specific (Diaz-Aparicio *et al*., 1994), mainly due to cross-reactivity with other antigens (Perry and Bundle, 1990). Isolation and identification are the most reliable methods of diagnosis in brucellosis. Despite they are not always successful, are cumbersome and represent a great risk of infection for technicians (Lopez-Merino *et al*., 1991). Recently, methods of molecular biology have been increasingly used in diagnosis. PCR is particularly useful in detection of brucella DNA in tissues and body fluids contaminated with non-viable or low numbers of brucella. (Leal-Klevezas *et al*., 2000).

The objective of this study was to throw lights on caprine brucellosis in one of coastal Egyptian governorate, whereas goat keeping and breeding are extensively practiced. Evaluation of the different used diagnostic tests in relation to the recently used techniques such as PCR was another target.

MATERIALS AND METHODS

This Study was done in corporation with the Egyptian Veterinary Service Organization (Brucella Test and Slaughter Program) during the period from July 2004 to June 2005.

Animals: A total number of 577 Baladi Does were used in this study. Does were reared in forms of small flocks at different localities in Kafr El-Sheikh Governorate (lies on the coast of the Mediterranean Sea). Case history and/or owner complain were recorded. Among these animals, 33 head were obligatory slaughtered due to their positive brucella titers.

Samples collection: Blood samples were collected from jugular veins of all animals. Serum samples were kept frozen (-20°C) till analysis.

Tissue specimens were taken from supra-mammary lymph nodes, spleen, uterus and mammary glands of obligatory slaughtered animals.

Corresponding Author: K.A Abd El-Razik, Department of Animal Reproduction and A.I. National Research Center, Dokki, Giza, Egypt Tel/Fax: 0123670455

Serological examination: Seroprevalence of brucellosis was investigated using different serological tests. BAPAT; (Anon, 1984), RBPT (Morgan *et al*., 1978), as well as TAT, RVT and CFT (Alton *et al*., 1988). At the same time, ELISA was carried out using crude lipopoly saccharide (LPS) and periplasmic protein (SBP50) antigens of *Brucella abortus* strain 19. The optimum antigen concentration and serum dilution rate were determined according to checker Board titration (Narayanan *et al*., 1983). ELISA reading equal to or higher than double folds reading of negative control was considered positive (Bassiri *et al*., 1993).

Bacteriological examination: Tissue specimens from supramammary lymph nodes and spleen were subjected to brucella isolation and identification according to the method recommended by Alton *et al.* (1988).

PCR: Tissue specimens from supramammary lymph node and spleen were frozen at-20°C. Samples were thawed at room temperature and the extraction of genomic DNA was done using CTAB/NaCl solution (hexadecyltrimethyl ammonium bromide/NaCl solution) according to Ausubel *et al.* (1988). Two oligonucleotide primers were used for amplification of brucella DNA prepared according to the sequences of highly preserved region that coding for outer membrane protein (OMP2; Baily *et al*., 1992).

PCR products are 216 base pairs. The used programme was the same of Baily *et al.* (1992) and primer sequences were:

Primer 1 (p1 6633 TCGGTTGCCAATATCAA793-809).
Primer 2 (P2 6634 CTTGCCTTTCAGGTCTG1008-992).

The amplified product was resolved using 1.5% agarose gel electrophoresis that is stained with ethidium bromide.

Pathological studies: Tissue specimens taking from uterus, mammary glands, supramammary lymph nodes and spleen were fixed in 10% neutral buffered formalin, dehydrated, embedded in paraffin wax, sectioned and stained with haematoxylin and eosin (H and E) as outlined by Bancroft *et al*. (1996).

Analysis of serum Cu, Zn and Fe concentrations: Serum Cu, Zn and Fe concentrations were determined in the diluted sera of obligatory slaughtered animals by atomic absorption spectrophotometer, model Perkinelmet 2380 according to Varly *et al*. (1980). Also, blood samples (No = 15) were collected from the jugular veins of comparable brucella seronegative Does in same flocks.

Statistical analysis: Data were statistically analyzed according to Snedecor and Cochran (1980).

RESULTS

According to case history and/or owner complains, Does in this experiment suffered from reproductive disorders such as repeat breeding, infertility and abortion and were in close contact with cattle and buffaloes.

Serological investigation: Table 1 shows the highest incidence of positive reactors was given by BAPAT (5.71%) and the lowest incidence was given by TAT (3.11%).

Bacterial isolation: *Brucella melitensis* biovar 3 was isolated in 26 out of the 33 positive reactors.

PCR: PCR was indicative of brucellosis in 81.81% of the positive slaughtered goats as shown by the typical PCR product specific for *Brucella* (216 base pair) (Fig. 1)

Histopathological findings

Uterus: Multifocal areas of erosion and ulceration of endometrium was seen. Mild to moderate stromal diffused and multifocal accumulations of lymphocytes,

Table 1: Incidence of brucellosis in Baladi Does examined at Kafr El-Sheikh Governorate using different serological tests

	Serological tests						
	BAPAT	RBPT	TAT	RVT	CFT	L-ELISA	P-ELISA
N°	33.00	32.00	18.00	23.00	26.00	32.00	21.00
%	5.71	5.54	3.11	3.98	4.50	5.54	3.63

Total number of examined animals = 577

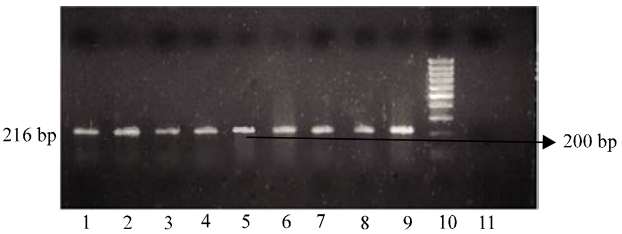

Fig. 1: Electrophoretic pattern of PCR product 216 bp in 1.5% agarose gel stained with Ethidium bromide. Lanes 1-7 positive tissue samples DNA PCR. Lane 8 and lane 9 positive controls (*Br. abortus* and *Br. melitensis* respectively). Lane 10, DNA marker and Lane 11, negative control

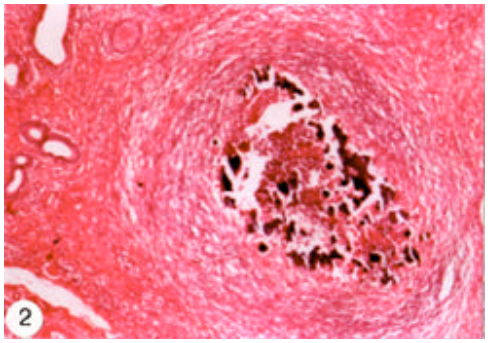

Fig. 2: Uterus, showing presence of calcified granuloma embedded in the endometrial stroma(H&E,X40)

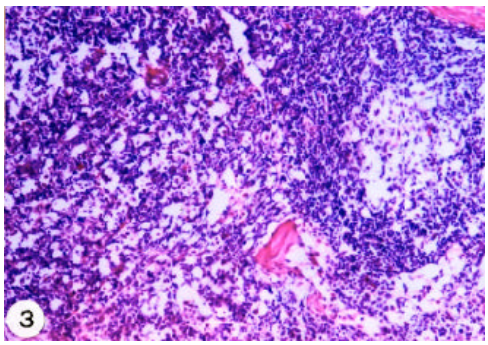

Fig. 3: Supramammary lymph node, showing lymphocytic cell depletion in follicles and cortex(H&E,X100)

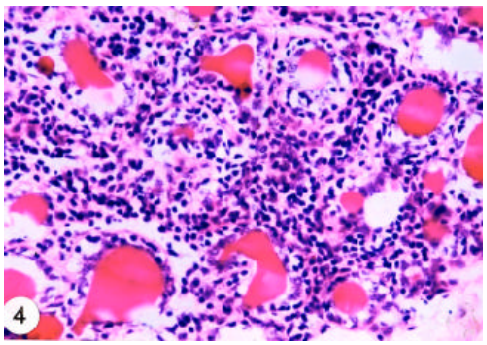

Fig. 4: Supramammary gland, showing, diffuse aggregations of mononuclear cells among the secretory acini(H&E,X200)

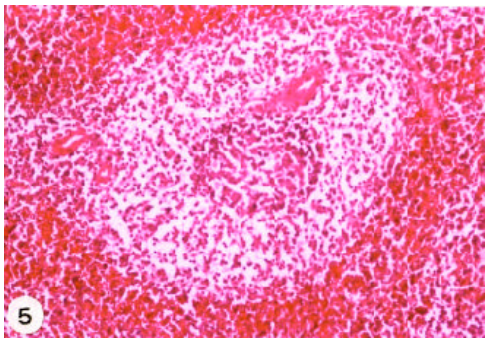

Fig. 5: Spleen, showing depletion of lymphoid tissue of white pulp(H&E,X100)

macrophages and plasma cells were noticed. 6 out of 33 cases showed the presence of granulomatous lesions consisting of central area of caseous necrosis together with calcification and surrounded by inflammatory cells and finally encircled with thick fibrous connective tissue capsule (Fig. 2). The uterine glands revealed marked cystic dilatation associated with periglandular fibrosis and inflammatory cell infiltration.

Supramammary lymph nodes: The lymphoid cells in follicles, cortex and paracortical zone appeared necrotic (Fig. 3). Germinal centers of most follicles revealed deposition of homogenous eosinophilic structurless material accompanied with individual lymphocytic cell necrosis (Fig. 4).

Mammary glands: prominent multifocal and diffuse aggregations of mononuclear cells in the intralobular connective tissue were observed (Fig. 4). Alveoli in the affected lobules were progressively decreased in size. In some cases, infiltrated inflammatory cells replaced alveoli and formed lymphoid follicles in few foci.

Spleen: Depletion of lymphoid tissue in the external zone of Malpighian corpuscles was seen. Moreover, the lymphocytes appeared pyknotic and smaller in size (Fig. 5).

Evaluation of different *Brucella* diagnostic test: Evaluation of the different tests used for diagnosis of brucellosis was recoded in Table 2. It is evident that the most comparable serological tests with bacterial isolation and PCR were CFT and P-ELISA. Histopathological examination was of lesser value for diagnosis of brucellosis.

Serum Cur, Zn and Fe concentrations: As shown in Table 3, *Brucella* infection induced significant ($p<0.001$) decreases in serum Cu, Zn and Fe concentrations as compared to seronegative comparable animals.

Table 2: Evaluation of different diagnostic tests in obligatory slaughtered *Brucella* seropositive *Baladi* Does

Test	Positive	Histopath	Bact. Isol. (granulomas)	PCR
BAPAT	33	6 (18.18)	26(78.78)	27(81.81)
RBPT	32	6(18. 75)	26(81.25)	26(81.25)
TAT	18	5(27.77)	14(77.77)	14(77.77)
RVT	23	5(21.73)	20(86.96)	18(78.26)
CFT	26	5(19.23)	26(100.00)	26(100.00)
L-ELISA	32	6(18.75)	25(71.43)	25(78.13)
P-ELISA	21	4(19.04)	21(100.00)	21(100.00)

Total number of examined slaughtered animals = 33

Table 3: Effect of brucellosis on serum copper, zinc and iron concentrations in Baladi Does (Mean±SEM)

Brucella infection	Copper ($\mu g\ dL^{-1}$)	Zinc ($\mu g\ dL^{-1}$)	Iron ($\mu g\ dL^{-1}$)
Seronegative (No = 5)	78.82±0.51	74.61±1.02	262.07±9.15
Seropositive (No = 33)	52.57±0.41***	53.23±0.26***	157.42±0.71***

*** Significant at $p<0.001$

DISCUSSION

Caprine brucellosis received limited research interest in Egypt despite its zoonotic importance and economic losses. So sensitive and rapid diagnostic tools for its control must be applied. In the same time, brucellosis epidemiology in small ruminants, especially goats is complex (Blasco and Barberan, 1990; Reviriego *et al*., 2000) because several extrinsic factors (flock management, ecological conditions and socio-economic factors) play an important but poorly defined role.

The current investigation indicated that 3-5% of Does reared at Kafr El-Sheikh area were brucella positive by different serological tests and were suffering from reproductive problems. Varying low incidence of infection (0.58-4.90%) was recorded by Montasser *et al.* (1999), Reviriego *et al.* (2000), Renukaradhya *et al.* (2002) and Refai (2003). On the other hand, high incidence 6.6-32.8%) was reported by Ghosh and Nanda (1988), Mikolon *et al.* (1998) and Akhtar (1992). Variations in the incidence of infection is related to, the course of the diseases, locality, rate of exposure, reproductive status, sex, the improvements in diagnostic techniques, vaccination strategies and the enforcement of a national eradication policy (Nada *et al*., 1992; Luna-Martinez and Mejia-Teran, 2002). However, attention should be paid to combat this infection, as it is a risk factor for man and other contact farm animals (Reviriego *et al*., 2000). Evaluation of the used serological tests in this study indicated that BAPAT is of the highest sensitivity, followed by RBPT and L-ELISA, CFT, P-ELISA, RVT and TAT. It was reported that BAPAT is a screening test based on its efficiency, simplicity and cost (Ghazi, 1996; and Mikolon *et al*., 1998) as well as the low final antigen concentration after the addition of serum (Alton *et al*., 1988). RVT depends mostly upon the IgG antibodies, yet detected lower number of reactors due to it fails to detect animals in the incubation period or animals at the beginning of infection (Angus and Barton, 1984).

From the obtained results it is evident that CFT detected more positive animals than the TAT. This condition could be explained on the base of the chronic stage of infection in such Does whereas, it was reported that CFT antibody titers persist for diagnostically significant levels for longer time than those of TAT (Sting and Ortmann, 2001). Moreover, lower sensitivity of TAT

was attributed to the high incidence of suspicious cases as well as to the prozone phenomena (Alton *et al*., 1988). In the current study, seropositivity of L-ELISA was the same as RBPT and was superior to that of CFT and TAT as reported by Sting and Ortmann (2000). This indicating that if ELISA is well standardized, it yields good screening results that can be used either alone or in addition to RBPT (Jacques *et al*., 1998). However, the increase in sensitivity of ELISA technique is associated almost with decrease in its specificity. Similar results were reported by Nielsen *et al*. (1996). In the same time, the low sensitivity of P-ELISA in comparison to that of L-ELISA is related to the difference in the binding of protein bands to ELISA polystyrene plates (Shringi *et al*., 2002). In this study, *Br. melitensis* biovar 3 was isolated and identified in only 26 out of 33 obligatory slaughtered Does. Negative culture results may be due to the presence of microbial contaminants in the samples and loss of viability of the organism before culturing (Blasco, 1992). PCR assay gives higher positive results (81.81%) than the bacterial isolation. This proves the efficiency of PCR as a reliable, highly sensitive and specific test for accurate detection of *Brucella* sp. DNA (Gallien *et al*., 1998 and Ibrahim *et al*., 2002). The difference between the serological results from one side and both the PCR and bacteriological isolation from the other side may be explained on the basis of cross reactivity of *Brucella* sp. lipopolysaccharide and various organisms of other genera as *Yersinia enterocolitica* O: 9 (Pouillot *et al*., 1998). On the other hand, the presence of polymerase inhibitors could account, at least in part, for PCR-negative results in samples that were culture positive (Rolfs *et al*., 1992).

The histopathological findings in brucella reactors in this study were characterized by granulomatous endometritis and lymphocytic mastitis and lymphoid depletion in spleen and lymph nodes. Also, such Does yield PCR products. Occurrence of granulomatous lesions indicated the chronicity of the condition and reflect the nature of the persisting infection (Adams, 2002). In this respect, it was reported that the disease expression of pathological lesions depends upon the ability of *Brucella* to survive and persist intracellularly within professional phagocytic cells (Liautard *et al*., 1996). Moreover, the intracellular survival mechanism is due to the inhibition of phagolysosomal fusion (Arenas *et al*., 2000; Naroeni *et al*., 2001). The present lymphoid depletion indicates immunodeficiency due to localization and replication of the microorganism in the macrophages and lymphocytes (Cheville *et al*., 1996). Histopathological examination is of low value for diagnosis of *Brucella*, whereas the pathognomonic lesion; granuloms formation was not observed in all examined Does. In this respect it was recorded that the diversity of pathological lesion are influenced by route of exposure, immune status of the host, sexual maturity, infection rate and virulence of the organism (Adams, 2002).

Seropositive *Brucella* Does herein revealed significant decrease in serum Cu, Zn and Fe concentrations ($p<0.001$). The significant decrease in both serum Cu and Zn concentrations could be attributed to the chronicity of the inflammatory process and the condition was in line with the finding of Ghazi *et al*. (2001) in bovines suffering from endometritis. Serum Cu and Zn concentrations were reported to alter by the effects of some cytokines as host defense elements of organism during brucellosis (Kaufmann, 1997). In the same time, the immunobiochemical mechanism activated by the host against brucellosis affect the metabolism of its own trace elements (Araya *et al*., 1989). Silvia-Cerone *et al*. (1995) reported that antibody titers against *Brucella abortus* and peripheral blood mononuclear cells were significantly lower in Cu deficient heifers. On the other hand, the present decrease in iron concentration was in agreement with the findings of Nada *et al*. (1992), El-Sawalhy *et al*. (1996) and Ghazi *et al*. (2001). This decrease could be attributed to the disturbance in the function of the spleen following to the pathological lesions (Nada *et al*., 1992).

In conclusion, raising goats with large dairy animals is a faulty traditional practice, whereas it may be a source of *Br. melitensis* infection for man and animals in Egyptian villages. Therefore, more focusing should be paid to goat in the national eradication program. Finally, caprine brucellosis still needed further investigations, especially for evaluation of diagnostic tests and biochemical changes during the course of the infection.

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