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

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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), I-ELISA, Complement Fixation Test (CFT), P-ELISA, Rivanol test (RVT) and Tube Agglutination Test (TAT). In seropositive 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 Burdle, 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.

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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 TCGGTTCCGTATCAA793-809).
Primer 2 (P2 6634 CTTGCTTTTCAGGTCTCG1008-992).

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

Pathological studies: Tissue specimens taken 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: Multi focal areas of erosion and ulceration of endometrium was seen. Mild to moderate stromal diffused and multifocal accumulations of lymphocytes,

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<th>Table 1: Incidence of brucellosis in Baladi Does examined at Kafr El Sheikh governamte using different serological tests</th>
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<td>Serological tests</td>
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<tr>
<td>N (%)</td>
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<td>%</td>
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Total number of examined animals = 377

![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](image-url)
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 structureless 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 Cu, 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>
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<th>Table 2: Evaluation of different diagnostic tests in obligate slaughtered <em>Brucella</em> seropositive Baladi Does</th>
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<tr>
<td>Test</td>
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</tr>
<tr>
<td>BAPAT</td>
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<td>RBPT</td>
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<td>TAT</td>
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<td>RVT</td>
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<td>CFT</td>
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<td>L-ELISA</td>
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<td>P-ELISA</td>
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<tr>
<td>Total number of examined slaughtered animals = 33</td>
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**Table 3: Effect of brucellosis on serum copper, zinc and iron concentrations in Baladi Does (Mean±SEM)**

<table>
<thead>
<tr>
<th><em>Brucella</em> infection</th>
<th>Copper (µg dl⁻¹)</th>
<th>Zinc (µg dl⁻¹)</th>
<th>Iron (µg dl⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Seronegative (No = 5)</td>
<td>78.8±0.51</td>
<td>74.61±0.01</td>
<td>262.07±9.15</td>
</tr>
<tr>
<td>Seropositive (No = 33)</td>
<td>52.57±4.41***</td>
<td>55.23±2.86***</td>
<td>157.42±9.71***</td>
</tr>
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***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), Renakaradya 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-Martínez and Mejía-Terán, 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 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, Naroen 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; granulons 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 immunochemical 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.

REFERENCES


