Journal of Reproduction and Infertility 1 (2): 33-40, 2010
ISSN 2079-2166

Pathological and Molecular Studies on Mammary Glands and Supramammary Lymph Nodes of Naturally Brucella Infected Buffalo-Cows

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Abstract: The aim of the present work was to study the histopathological changes in mammary glands and supramammary lymph nodes of buffalo- cows naturally infected with brucella and using the advanced techniques for its diagnosis. A total number of 32 brucella seropositive buffalo- cows was used in the present study. Specimens from supramammary lymph nodes and mammary glands were taken just after obligatory slaughter. Bacteriological isolation, pathological and immunohistochemistry examination as well as PCR techniques were carried out for detection of the organism in tissues. *Brucella melitensis* was isolated from 9.37% of examined cases. The most pronounced histopathological findings were lymphocytic and granulomatous mastitis in addition to marked depletion of lymphocytic cells in supra mammary L.N.. The immunohistochemical examination showed moderate to severe intracellular immuno-reactive staining of brucella antigen in the tissues of supramammary lymph node. Animals showing high titer of brucella antibodies produce positive PCR products (223bp) and (731bp) in the tissue of supramammary lymph node. It was concluded that mammary glands of brucella seropositive buffalo-cows showed pathological changes, immunohistochemistry and PCR enhance the diagnosis of the disease. Moreover, infected udder is considered as one of the main source of infection for both animal and the consumers using unpasteurised milk and milk products.

Key words: Buffaloe-cows · Brucella · Suprammary lymph node · Mammary gland · PCR · Pathology and immunohistochemistry.

INTRODUCTION

Brucellosis is one of the most important diseases worldwide, particularly in developing countries, Mediterranean countries and central Asia [1]. In Egypt, serological surveys indicated that brucella infections among buffaloes ranged from as low incidence as 0.16 - 1.49% [2 - 6] to as high incidence as 5.29 - 25.49 %. [7 - 9]. Brucellosis in buffaloes is the best known as one of the main reproductive disease, capable of causing abortion storms in the breeding season during the last third of pregnancy, retention of the fetal membranes, stillbirths and reduction in milk yield which resulting in great economic losses [10]. In ruminants, brucella has a marked affinity for lymphoid and reproductive organs, since the organism replicates to a high numbers in the gravid uterus. In non pregnant and persistently infected cows, the udder and supramammary lymph nodes are the most common site for localization [11]. Brucella infection causes lymphoplasmacytic and histiocytic interstitial mastitis, lymphofollicular hyperplasia, medullary plasmacytosis and sinus histiocytosis in lymph nodes [11-13]. It was reported that after abortion up to 80% of infected cows develop chronic infection localized in mammary glands and suprammary lymph node, and mammary gland infection may persist through out the life time of the cow [12, 14]. Currently, diagnosis of brucellosis is depending upon serological and microbiological methods. Serological tests have disadvantage of cross-reactivity with other common antigenically related bacteria and do not detect early or latent infections and bacteriological isolations are time consuming, cumbersome and of high risk to people carrying them out [15]. Moreover, immunohistochemical

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staining technique is used for direct and rapid detection of brucella antigen in the mammary gland of infected cows [12] and supramammary lymph nodes of buffaloe- cows that revealed low titer of antibodies and may contain few numbers of brucella organisms [16]. Recently, methods of molecular biology have been increasingly used in diagnosis. PCR technique has been applied for detection of b*rucell*a species in tissues and body fluids contaminated with non-viable or low numbers of brucella [17]. The aim of the present work was to study the histopathological changes in mammary glands and supramammary lymph nodes of buffalo- cows naturally infected with brucella, as well as using the advanced techniques as immunohistochemistry and PCR assay for confirmative diagnosis

MATERIALS AND METHODS

Animals and History: The present study was carried out on a total number of 32 buffalo- cows including 17 animals from governmental farm located at Ismaillia governorate. These animals suffered from reproductive disorders including repeat breading, decrease milk yield and retained fetal membranes, blood samples taken from these animals were brucella seropositive. The remaining 15 animals were seropositive brucellosis obligatory slaughtered cases from Ossim slaughtered house in Giza governorate during the period from 2004-2005.

Sample Collection: Mammary gland and supramammary lymph node tissue samples were taken from brucella seropositive cases for bacteriological, pathological and immunohistochemical examination as well as PCR assay.

Bacteriological Examination: Tissue specimens from mammary gland, supramammary lymph node were taken as freshly as possible for isolation and identification of brucella organism [18].

Polymerase Chain Reaction (PCR): Tissue specimens frozen at -20C° were thawed at room temperature and the subjected to extraction of genomic DNA [19, 20]. Four 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; [21]). Primer sequences were:

Primer 1 (B4 TGG CTC GGT TGC CAA TAT CAA). Primer 2 (B5 CGC GCT TGC CTT TCA GGT CTG).

For Brucella species whereas the expected PCR products are 223bp. Primer 3 (Bm- SP AAA TCG CGT CCT TGC TGG TCT GA). Primer 4 (IS711-SP TCG CGA TCA CTT AAG GGC CTT CAT). For *Brucella melitensis* whereas the expected PCR products are 731bp.The amplified products were visualized by electrophoresis using 2% agarose gel stained with Ethidium bromide [19].

Pathological Examination: Complete post mortem examination was done on mammary gland and supramammary lymph node of the present non pregnant culled buffalo- cows to detect any gross pathological lesions.

Parts from tissue specimens were fixed in formal saline 10% then washed, dehydrated, embedded in paraffin, sectioned at 4-5 micron thickness and stained with hematoxylin and eosin (H and E) as a routine work for histopathological studies [22]. Special stains are used for detection of specific lesions as Crossman's stain for fibrosis, Oil Red O stain for fatty changes [23] and Von Kossa stain for calcification [24].

Immunohistochemistry: Avidin-Biotin Complex Peroxidase technique was applied for detection of Brucella antigen in formalin-fixed, paraffin -embedded tissue sections of suprammary lymph nodes [25] using peroxidase detection kit purchased from Novocastra Co. UK.

RESULTS

Bacteriological Findings: Brucella was isolated from three (9.37%) out of 32 examined cases. Three isolates of brucella were obtained on culture growth mainly from supramammary lymph nodes. The cultures were smooth, glistening, transparent in appearance and pin point in size distributed all over the selective brucella agar media. The suspected colonies were picked up for direct slide agglutination test and gave positive results. Also these colonies were picked up for differentiation of brucella species by using PCR assay.

Polymerase Chain Reaction: A six samples out of a total number of a DNA extracted nine tissue samples {gave high titer of antibodies(1/80) by using tube agglutination test }of supramammary lymph node yielded positive PCR product (223bp), as shown in (Fig.1), using of a brucella species primers specific for 31 kD outer membrane protein gene and a positive PCR product (731bp), using primers specific for *Brucella melitensis*.

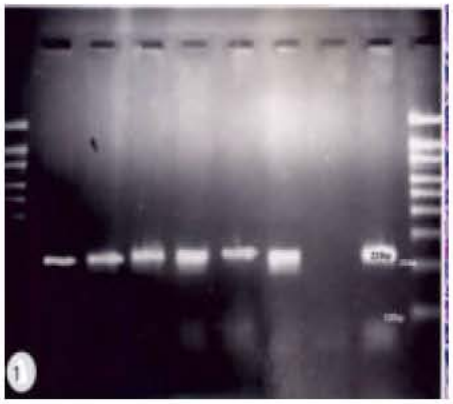

Fig. 1: PCR amplification of *Br. Melitensis* DNA from tissue samples. Lane 1 and 10: Molecular weight marker (100 bp). Lane 2-7 tissue samples DNA PCR (223). Lane 8 control negative; Lane 9 control positive with *Br. Melitensis DNA*.

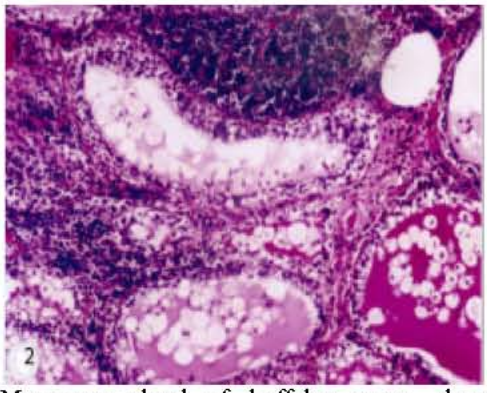

Fig. 2: Mammary gland of buffalo- cows, showing massive focal aggregation of lymphocytes among the secretory acini and intra luminal aggregation of lymphocytes. (H and E, X 100).

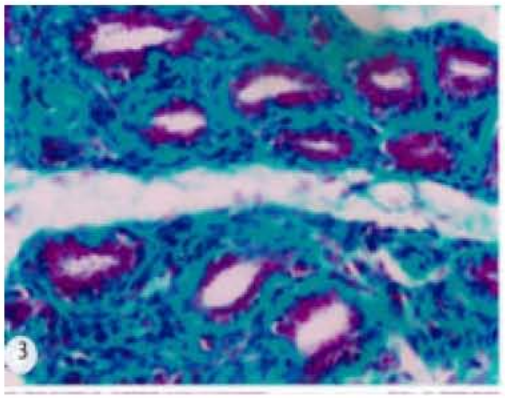

Fig. 3: Mammary gland of buffalo- cows, showing severe peri-acinal thickening due to fibrous proliferation leading to atrophy of the mammary acini. (Crossman's stain, X 200).

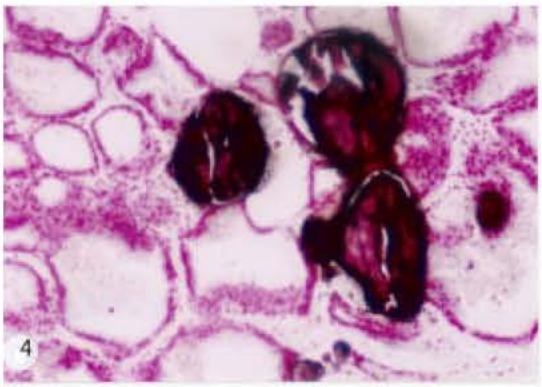

Fig. 4: Mammary gland of buffalo- cows, showing black brown coloration of calcium deposits completely occupied some acini.(Von Kossa stain, X 100).

Pathological Findings

Macroscopical Findings: No obvious gross lesions were found in mammary glands of brucella infected buffalo-cows. However, the glands were hard in texture. On cut section, the milk secretion was scanty and slightly turbid with yellowish coloration. The gross examinations of the supra mammary lymph nodes of most of examined cases revealed that these nodes were relatively small in size and firm in consistency. However, few cases appeared enlarged and edematous.

Histopathological Findings

Mammary Glands: The microscopical examination of most of examined cases revealed lymphocytic mastitis which is characterized by massive peri-acinal and intra acinal infiltration of mononuclear inflammatory cells mainly lymphocytes (Fig. 2). The epithelium lining of some of secretory acini showed vacuolar degeneration. Cystic dilatation of some acini was noticed.

In one case, chronic mastitis was observed. The mammary glands exhibited interlober and interlobular fibrous connective tissue proliferation. The secretory acini appeared small and atrophied with narrowing lumen and associated with infiltration of inflammatory cells mainly lymphocytes. Sections stained with Crossman's stain revealed increased collagen stroma and small atrophic alveoli (Fig.3).

In other examined cases, some acini showed papillary hyper plastic projections toward the lumen meanwhile; other acini revealed degeneration and desquamation of the epithelium lining into the lumen. Extensive calcification of some acini was observed. Sections stained with Von Kossa stain revealed brownish black coloration occupied most of the lumen of acini (Fig.4).

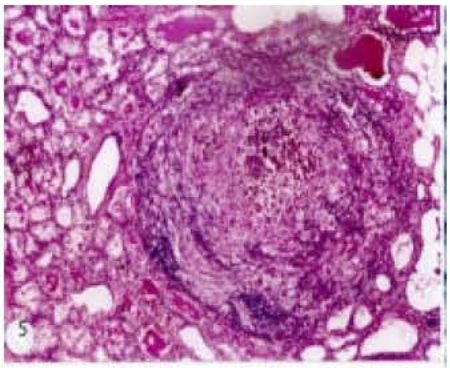

Fig. 5: Mammary gland of buffalo- cows, showing granulomatous mastitis characterized by presence of granuloma. (H and E, X 40).

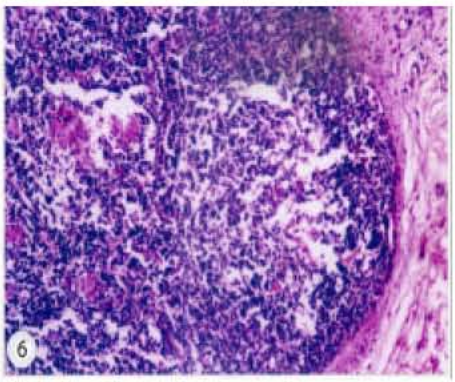

Fig. 6: Supra mammary lymph node of buffalo- cows, showing marked lymphoid depletion in the sub cortical lymphoid follicles. (H and E, X 100).

In one case, granulomatous mastitis was seen. There were multiple granuloms distributed throughout the glandular parenchyma. They were composed of central area of caseous necrosis associated with massive calcification surrounded with a zone of mononuclear inflammatory cells mainly macrophages and plasma cells and encircled by fibrous connective tissue capsule (Fig.5)

Supra Mammary Lymph Node: The microscopical examination showed prominent fibrous proliferation of connective tissue capsule and trabecullae leading to disappearance of the paracortical area in some cases. There was a marked depletion of lymphocytic cells in the cortical and paracortical follicles (Fig.6). Peritrabecullar vacuolations around the C.T. trabecullae was seen. The trabecullar sinuses were dilated and impacted with erythrocytes in addition to few macrophages and Langhans giant cells which are multinucleated, arranged in a horse shoe shape. Section of these cases stained with Oil Red O stain revealed positive result (red globules of

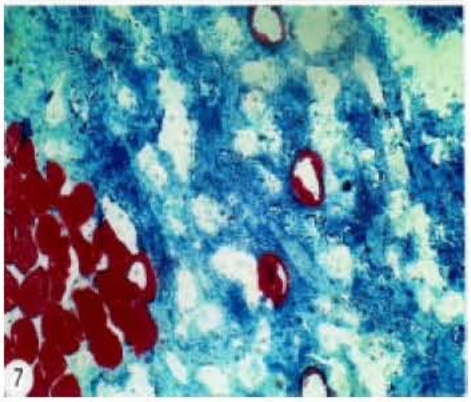

Fig. 7: Supra mammary lymph node of buffalo- cows, showing deposition of the reddish fat globules in between the trabecullae either single or in clusters.(Oil Red O, X 100).

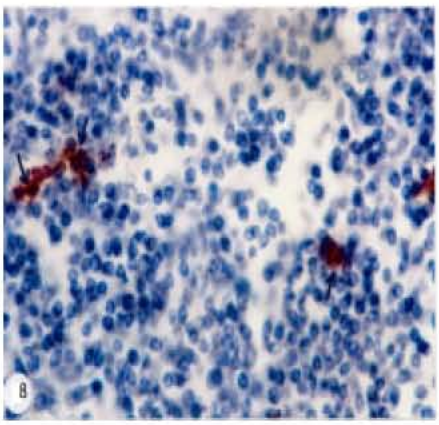

Fig. 8: Supra mammary lymph node of Buffalo cows, showing golden brown deposition of *Br. melitensis* antigen among lymphocytes and macrophages and also found intracellular in some of macrophages (arrows) in the para cortical area. Indirect peroxidase technique. (DAB,X200).

adipocytes in the medulla of lymph node either in clusters and or single) (Fig.7). In other cases the germinal centers were replaced by deposition of homogenous eosinophilic structureless mass of hyalinization in addition to lymphocytic cell necrosis was observed.

Immunohistochemistry Findings: Strong positive immuno-reactive staining of *Brucella melitensis* antigen was detected in between macrophages in the germinal centers of lymphoid follicle of the supramammary lymph node. Moreover, extracellular multiple clusters of moderate to severe brucella antigen immunostaining were observed in the cortex (Fig. 8). Intracytoplasmic and extracellular immunostaining in some areas was intense

and interfere with the visualization of cellular structures details. In all positive cases deposition of golden brown chromogen pigment at the site antigen antibody reaction was seen within the cytoplasm of mononuclear cells and detected extracellually among the lymphocytes in the germinal centers of the lymphoid follicles.

DISCUSSION

Brucellosis in buffaloes is the best known as one of the main reproductive disease, capable of causing abortion storms in the breading season during the last third of pregnancy, retention of the fetal membranes, stillbirths and reduction in milk yield which resulting in great economic losses [10]. In the current investigation, brucella organisms were isolated from supramammary lymph node of three cases (9.37%) out of a total number of 32 cases. These findings come in accordance with Essmail *et al.*[16] who isolated *B. melitensis* from supramammary lymph nodes of 3 out of 16 cases (19 %) of naturally infected buffalo- cows. In this respect, isolation of brucella organism is often difficult from the tissue samples [26]. On the other hand, a high rate of isolation of brucella organism from the udder and supra mammary lymph node was reported [27]. Tissue samples which give negative results may be due to the time required for culturing field specimens can be long and/or tissues are contaminated with a low number or non viable brucella organism. Despite of brucella isolation and identification are a direct and reliable methods for diagnosis, they are time consuming, cumbersome and represent human health hazard [17].

In the present work, PCR assay gave high positive results (66.6 %.) in six cases out of a total number of nine samples (gave high titer of antibodies1/80 by using tube agglutination test) of supramammary lymph node tissues. Similar results were reported in goats by Gupta *et al.* [28] who detected *Brucella melitensis* DNA in supramammary lymph nodes and udder and in cattle [29 -31] whereas *Brucella abortus* DNA was detected in milk and supramammary lymph nodes. PCR assay is a useful technique for detection of brucella DNA in tissues and body fluids contaminated with non-viable or low numbers of bacteria [32, 33]. Also, PCR has facilitate detection of bacteria particularly slow growing, difficult or time consuming to grow and difficult in differentiation of species or strains [17]. It is a sensitive, specific, rapid, relatively inexpensive method and diagnostic tool for detecting *Brucella* antigen [31].

The diversity of expression of the pathological lesions of domestic animals is known to be influenced by species and strain of brucella, immune status of animal host, route of exposure, sexual maturity, infection rate and virulence of the organism [34]. Also, it was reported that the disease expression in pathological lesions depends upon the ability of brucella to survive and persist intracellularly within professional and none professional phagocytic cells [35]. Moreover, the intracellular survival mechanism is due to the inhibition of phagolysosomal fusion [36, 37].

In the present study, the histopathological findings of mammary gland were characterized by interstitial lymphocytic mastitis in addition to extensive calcification in some acini. These findings comes in accordance with other previous observations in goats [11, 38, 39] and cows [13]. On the other hand, occurrence of granulomatous mastitis of the present work indicated the chronicity of the condition and reflects the nature of the persisting infection [34]. Wherever the organisms are localized, a granuloma develops where the phagocytes that are attracted by the bacilli engulf them. The bacteria multiply within the cytoplasm of the phagocytes, which are transformed into epithelioid cells. Around these cells lymphocytes, Langhans giant cells and plasma cells accumulate [40]. It has been found that the transportation of brucellae by phagocytic cells was the primary mechanism by which the brucella enter to mammary gland [11].

In the present study, the most pronounced histopathological changes of supramammary lymph node are thickening of C.T.capsule and trabecullae, marked lymphoid depletion and presence of fat vacuoles. The occurrence of a marked depletion of lymphocytic cells in the cortical and paracortical follicles of supramammary lymph node was coincided with observations reported by previous studies [41] explained that the lymph nodes draining mammary glands and reproductive tract in the late stages of infection develop chronic granulomatous lymphadenitis which is usually associated with cortical and paracortical lymphoid depletion and germinal center expansion. Also, the occurrence of lymphoid depletion indicates immunodeficiency due to localization and replication of the microorganism in the macrophages and lymphocytes [42]. Concerning to presence of fatty infiltration in lymph node, this finding agrees with results reported by El-Mahdy *et al.* [43] who stated that this condition was encountered in lymph nodes draining area of fat necrosis.

The present work revealed presence of positive immunostaining brucella antigen in supramammary lymph node. Similar results were reported in cows, goats and mice inoculated with *Br.abortus* [44], in mammary gland tissues of naturally infected cows with *Br. abortus* [12] and in adult female buffaloes naturally infected with *Brucella melitensis* [16]. They concluded that this technique is sufficiently sensitive for detecting brucella antigens in formalin-fixed, paraffin embedded tissues and could be a complementary tool to serological and bacteriological examinations for diagnosis of brucellosis. In this respect, it was reported that immunoperoxidase technique may enhance diagnosis capabilities of brucellosis particularly in chronic infection and is an efficient mean for detecting brucella organisms when are inherently slow or difficult to diagnose by isolation or culture from tissues obtained from field cases due to contamination [45]. In addition to, this technique is relatively rapid and enables detection of dead and/or low numbers of bacteria [46]. Although, cross-reaction of the polyclonal antibodies with other microorganisms such as *Yersinia enterocolotica* and *E. coli* can not rule out. Also, immunohistochemical staining has been used to study and assist in the understanding of the pathogenesis of brucella organism as the quantity, tissue and cellular locations and distribution of the agent can be visualized [13, 44].

It could be concluded that mammary gland of brucella seropositive buffalo- cows showed pathological changes, immunohistochemistry and PCR enhance the diagnosis of the disease. Moreover, infected udder is considered one of the main source of infection for both animal and the consumers of using unpasteurised milk and milk products.

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