

Pathological, Immunohistochemical, and Bacteriological Findings in the Mammary Glands and Supramammary Lymph Nodes of Cows with a History of Abortion due to *Brucella abortus*

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Abstract: The present study examined the mammary glands and supramammary lymph nodes of cows with a history of abortion due to *Brucella abortus*, and presents the histopathological, immunohistochemical, and bacteriological results. The study included 11 *Brucella*-seropositive cows that aborted during their first gestation. Histopathologically, the mammary glands showed lymphoplasmacytic and histiocytic interstitial mastitis. The lymph nodes had lymphofollicular hyperplasia and medullary plasmacytosis. Immunohistochemistry results showed brucellar antigens, predominantly in the cytoplasm of macrophages, and neutrophils in the intralobular interstitium and periductal stroma in the udders of 3 cows. Desquamated alveolar epithelium also indicated intense immunopositivity. In the lymph nodes, macrophages containing the antigen were frequently observed in the medullary region. *B. abortus* biotype 3 was isolated from the udders and supramammary lymph nodes of 4 cows. Although the most specific procedure for diagnosing the disease is isolation of the causative organism, for suspected cases in which the bacteriologic culture is negative or the material is fixed in formalin, immunohistochemistry may be used as a diagnostic tool for the detection of *Brucella* organisms.

Key Words: *Brucella abortus*, cow, mammary gland, supramammary lymph node, immunohistochemistry

***Brucella abortus*'a Bağlı Atık Yapmış İneklerin Meme Bezi ve Supramammar Lenf Dğümlerinde Patolojik, İmmunohistokimyasal ve Bakteriyolojik Bulgular**

Özet: Bu çalışmada, *Brucella abortus*'a bağlı atık yapmış ineklerin meme bezi ve supramammar lenf dğümleri patolojik, immunohistokimyasal ve bakteriyolojik olarak değerlendirildi. Çalışma materyalini, ilk gebeliklerinde atık yapmış *Brucella*-seropozitif 11 inek oluşturdu. Histopatolojik olarak, meme bezlerinde lenfo-plazmositik ve histiyositik intersitisyel mastitis gözlemlendi. Lenf dğümlerinde folliküler hiperplazi ve medullar plazmasitozis görüldü. İmmunohistokimyasal olarak, 3 ineğin meme bezinde intralobular intersitisyum ve periduktal stromada özellikle makrofaj ve nötrofil lökositlerin sitoplazmasında *brucella* antijeni tespit edildi. Ayrıca, alveolar lumende deskuamatif alveolar epitelyumun şiddetli immunopozitif sitoplazmik reaksiyonu gözlemlendi. Lenf dğümlerinde, antijen bulunan makrofajlara sıklıkla medullar bölgede rastlandı. Dört ineğin meme bezi ve supramammar lenf dğümlerinden *B. abortus* biyotip 3 izole edildi. Brusellozisin kesin tanısında en güvenilir yöntem etken izolasyonu olmakla beraber, bakteriyel kültürün negatif olduğu veya dokunun formalin ile tespit edildiği durumlarda, *brucella* antijenlerinin ortaya konulması için immunohistokimyasal yöntemlerin teşhis aracı olarak kullanılabilceği sonucuna varılmıştır.

Anahtar Sözcükler: *Brucella abortus*, inek, meme bezi, supramammar lenf dğümü, immunohistokimya

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Introduction

Brucellosis is a zoonotic disease of major economic and public health significance. It is primarily identified as a contagious occupational disease of farm and abattoir workers, veterinarians, and others that work with animals, but is also associated with foodborne transmission. Increasingly, sporadic cases and outbreaks are appearing among consumers of unpasteurized milk or milk products, especially cheese, from cows, sheep, and goats (1-3).

In many mammalian species, *Brucella* spp. are a facultative intracellular pathogen of the monocyte-macrophage cell line. In pregnant cattle, brucellosis is characterized by initial replication of *B. abortus* in the lymph nodes, followed by bacteremia and rapid multiplication of the organism in fetal tissues, with subsequent abortion during the second or third trimester of gestation (4,5). It is reported that after abortion up to 80% of infected cows develop chronic infection localized in the mammary glands and supramammary lymph nodes, and mammary gland infections may persist throughout the lifetime of the cow (3,6-11). Infected mammae can serve as a focus of infection from which *Brucella* disseminates to other tissues in the host (12).

Although there are a number of countries that have been declared brucellosis-free, the disease remains widespread in many parts of the world. The prevalence is highest in Mediterranean countries, and in Central and South America (1). In the region of Kars, Turkey, a major ruminant breeding area, brucellosis causes a high proportion of abortions in cows, sheep, and goats. Additionally, the disease is frequently diagnosed in humans living in the region who work with animals, and as a result of the consumption of unpasteurized milk and cheese. Likewise, infected mammae intermittently or continuously excrete *Brucella* into the milk throughout lactation (9,11,13). Thus, it might be important to identify and diagnose disease-affected animals excreting such zoonotic bacteria, for both human and calf health. The aim of the present study, therefore, was to examine the histopathological, immunohistochemical, and bacteriological findings in the mammary glands and supramammary lymph nodes of cows with a history of abortion due to *B. abortus*, and to establish whether or not *B. abortus* was localized in the sites shortly after abortion.

Materials and Methods

Animals and History

The study included 11 cows with a recent history of abortion from the farm of the Faculty of Veterinary Medicine, University of Kafkas, Kars, Turkey. During the 2002 winter season 11 cows in their first gestation, which were purchased from an animal market about 1 year earlier and, according to statements made by the sellers, had not been vaccinated against brucellosis, aborted in the last trimester of gestation. All the aborted animals had a retained placenta. Blood samples taken from the cows for serological testing were *Brucella*-seropositive and the animals were brought to the abattoir for slaughter.

Histopathology

Tissues samples for light microscopical examination and immunohistochemical staining were taken from each mammary lobe and its supramammary lymph nodes, particularly from the dorsal, middle, and ventral portions of the udders. All samples were fixed in 10% buffered formalin, processed routinely, and stained with hematoxylin and eosin (H&E).

Immunohistochemistry

Immunostaining of brucellar antigen in the tissues was performed using an avidin-biotin-peroxidase-complex (ABC) technique (14). For immunohistochemical analysis, sections 4 µm thick were deparaffinized in xylene and hydrated through graded alcohols. Endogenous peroxidase activity was blocked by incubation with 3% H₂O₂ in methanol for 15 min. After washing the slides with phosphate-buffered saline (PBS), all sections were incubated with 5% normal goat serum (Dako, Carpinteria, USA) for 30 min at room temperature in order to block non-specific binding. The slides were then incubated overnight at 4 °C with rabbit anti-*B. abortus* polyclonal antibody (Difco Lab., Detroit, MI, USA) diluted 1:50 in PBS. After washing 3 times with PBS, the sections were incubated for 30 min at room temperature with biotinylated goat anti-rabbit immunoglobulin G (Dako, Carpinteria, USA) diluted 1:200 in PBS. The sections were then incubated with streptavidin peroxidase complex (ABC; Dako, Carpinteria, USA). Immunostaining was obtained using 3,3 diaminobenzidine as the chromogen. Hematoxylin was used as the counterstain. Control

sections were incubated with normal rabbit serum instead of primary antibody.

Bacteriology

The mammary glands and supramammary lymph nodes taken from all the seropositive cows were cultured on blood agar plates containing 7% (V/V) defibrinated sheep blood (Oxoid, CM 271) and *Brucella* medium (Oxoid, CM 169) supplemented with *Brucella* selective supplement (Oxoid, SR209E). The cultures were incubated in duplicate at 37 °C for 5-7 days, aerobically and micro-aerobically (Micro-aerobic kit, Merck, Anaerocult C). Suspected *Brucella* colonies were identified by colony morphology and growth characteristics, Gram stain, catalase, urease, oxidase, and H₂S production, CO₂ requirement, growth in the presence of thionine, and basic fuchsin and slide agglutination testing with monospecific A and M antisera. *Brucella* strains were biotyped according to the method of Ribeiro and Herr (15). Field strains were distinguished from the vaccine strain (*B. abortus* S 19) by their ability to grow on media containing penicillin (5 IU/ml). L-arabinose and D-ribose were used to differentiate isolated strains from *B. melitensis* (15).

Results

Gross Findings

All of the mammary glands and the supramammary lymph nodes were examined following slaughter, and subcutaneous edematous swelling was detected in 5 cows. In general, the parenchyma, cistern, and large lactiferous ducts of the mammary glands had many pin-point-sized granulomas and fibrosis. The supramammary lymph nodes, in general, were enlarged and edematous. When the lymph nodes were sectioned, cut surfaces were wet from a serous effusion and the cortico-medullary regions were not distinguishable. Additionally, in 2 animals petechial hemorrhages were observed on the subcapsular and cut surfaces of the nodes.

Histopathology

The results of the histopathological, immunohistochemical, bacteriological, and serological analyses are shown in the Table. Infected mammary glands, in general, had lymphocytic and histiocytic, lobular, and periductal interstitial mastitis. In the affected lobules, the intralobular interstitium and the alveolar epithelial layer were often infiltrated with macrophages

Table. Histopathological, immunohistochemical, bacteriological, and serological results.

Case No.	Histopathology*					Immunohistochemistry**		Bacteriological Culture***		Serology
	Mammary Glands		Supramammary Lymph Nodes			Mammary Glands	Supramammary Lymph Nodes	Mammary Glands	Supramammary Lymph Nodes	
	LPHI	LFF	EN	LFH	MP					
1	+	-	-	+	-	-	-	-	-	+
2	+	-	-	+	-	-	-	-	-	+
3	+	-	-	+	-	-	-	-	-	+
4	+++	+++	++	+++	++	+++	+++	+	+	+
5	+	+	-	-	-	-	-	-	-	+
6	++	++	-	+	-	-	-	-	-	+
7	-	-	-	-	-	-	-	-	-	+
8	+++	+++	+	++	+	-	-	+	+	+
9	+++	+++	++	++	+	-	-	-	-	+
10	++	++	+	++	+	++	+	+	+	+
11	+++	+++	++	+++	++	+++	+++	+	+	+

*-: No lesion found; +: mild; ++: marked; +++: severe. **Brucellar antigen. -: Negative; +: low; ++: moderate; +++: abundant. ***Culture results; -: culture negative; +: culture positive. LPHI: lymphoplasmacytic and histiocytic infiltration; LFF: lymphoid follicle formation; EN: epithelial necrosis; LFH: lymphofollicular hyperplasia; MP: medullary plasmacytosis. SAT: serum agglutination test.

and lymphocytes (Figure 1). Lobules severely affected by the disease had neutrophil leukocyte aggregations, along with a small number of macrophages and lymphocytes in the alveolar and ductal lumina. Lymphoid follicles were often observed in the intralobular interstitium and periductal stroma (Figure 2). Occasionally, mast cells were distributed throughout the interstitium, primarily in the perivascular spaces. There were also marked increases in the number of plasma cells in the mammary glands. In such cases, dense lymphocytic accumulation with macrophages expanded the intralobular septa and often displaced alveoli. In the intralobular interstitium infiltrated by mononuclear cells, Russell's bodies, composed of small round eosinophilic cytoplasmic droplets, were often detected in plasma cells. In these elements the appearance and location of the nucleus varied, making it difficult to distinguish. Individual and small foci of neutrophils and a few eosinophils were randomly distributed in the mammary gland interstitium. Periductal stromas were infiltrated with numerous lymphocytes, macrophages, a few plasma cells and eosinophils, and a small number of neutrophils. Occasionally, the intra- and interlobular ducts were filled with macrophages, neutrophils, lymphocytes, and cell debris. Changes in the cistern were characterized by cellular debris, epithelial hyperplasia, and squamous metaplasia, along with periductal mononuclear cell infiltration and fibrosis.

The supramammary lymph nodes commonly had marked lymphofollicular hyperplasia. The para-cortical

areas were expanded and had increased numbers of lymphoid cells. The medullary region was generally infiltrated by plasmacytes and macrophages. Russell's bodies, with cytoplasmic eosinophilic globes, were detected in the nodes. Minor hemorrhaging, necrosis, and a small number of neutrophils were observed mainly in the medullary region. In some of the cows, diffuse eosinophil infiltration was observed around the perivascular, capsular, and trabecular areas.

Immunohistochemistry

In the mammary glands, *Brucella* antigens were detected in 3 of the cows with a history of abortion. Brucellar antigen stained brown and finely granular, and was detected predominantly in the cytoplasm of macrophages and neutrophils located in the intralobular interstitium, alveolar and ductal epithelium, and periductal stroma (Figure 3). Brucellar antigen in macrophages ranged from single to aggregates that filled the cytoplasm. In some cases, alveolar epithelium with intense immunopositivity was desquamated into the alveolar lumina (Figure 4). Occasionally, extracellular brucellar antigen was detected in the interstitium, and in the alveolar and ductal lumens of the mammary glands. The quantity of antigen was less than that of intracellular brucellar antigens, and was mixed with leukocytes, cellular debris, and lipid droplets in the duct lumens.

Brucellar antigens were stained in the sinusoidal macrophages of the supramammary lymph nodes of the cows with mammary glands that were positive for the antigen. Macrophages with phagocytized antigens were

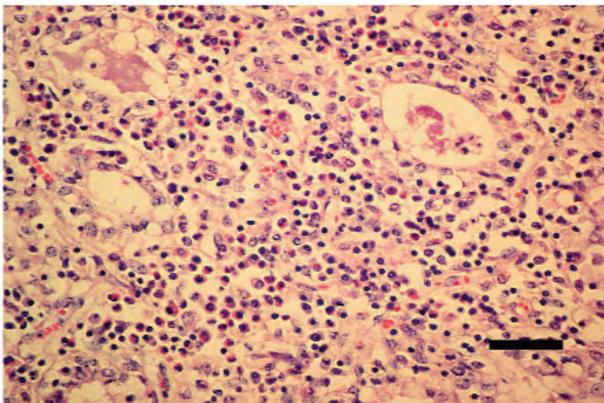


Figure 1. Diffuse cellular infiltration composed mainly of macrophages, lymphocytes, plasmacytes, and neutrophils in the intralobular interstitium (H&E, bar = 51 μ m).

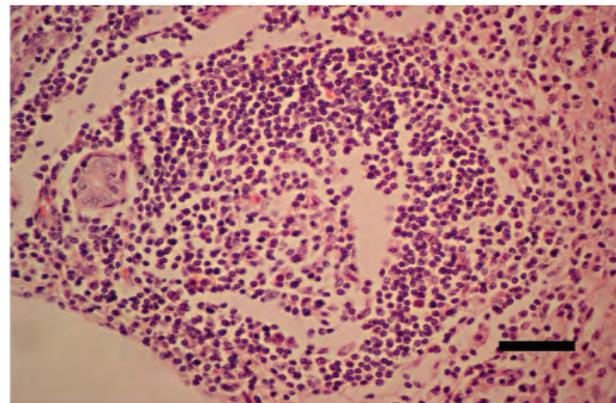


Figure 2. Lymphoid follicle formation around the intralobular duct of the mammary gland (H&E, bar = 166 μ m).

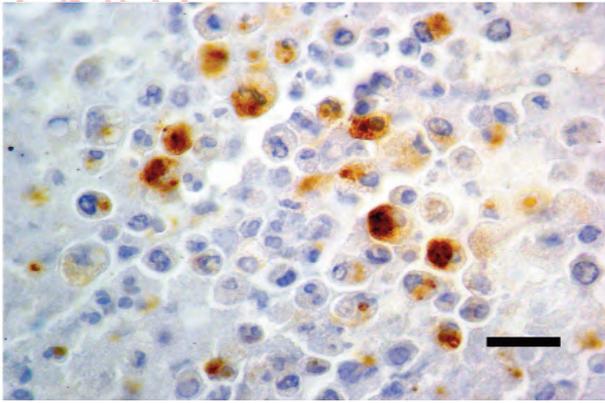


Figure 3. Positive immunoreactivity to the anti-*B. abortus* polyclonal antibody of macrophages and neutrophils in the intralobular interstitium of the mammary gland (ABC, bar = 11 µm).

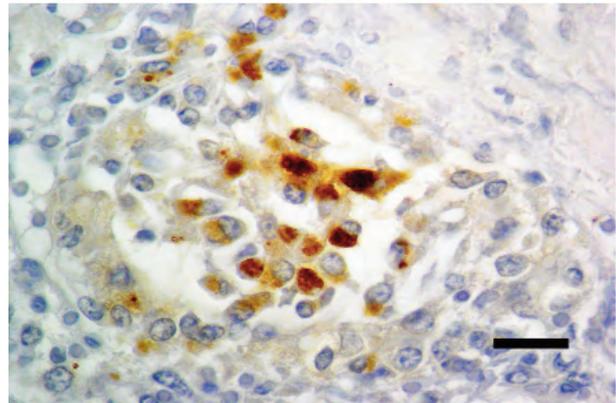


Figure 4. Epithelial cells desquamated into the alveolar lumina of the mammary gland reveal dense intracytoplasmic staining for *Brucella* antigens (ABC, bar = 11 µm)

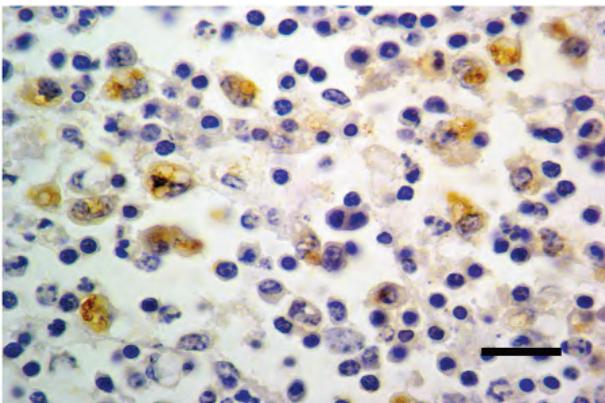


Figure 5. Positive immunoreaction to the anti-*B. abortus* polyclonal antibody of macrophages in the supramammary lymph nodes (ABC, bar = 11 µm).

detected mainly in the medullary or cortico-medullary regions (Figure 5). Cells containing antigen were present in moderate numbers in the lymph nodes, and there was less brucellar antigen in the macrophages than in the mammary glands.

Bacteriology and Serology

According to standard tube agglutination testing, all of the cows with a history of abortion were *B. abortus*-seropositive and had a high antibody titer (1/160-1/1280). *B. abortus* biotype 3 was isolated from the udders and supramammary lymph nodes of 4 of the 11 cows sampled. Cultures from both the udders and the lymph nodes yielded moderate growth of *B. abortus*.

Discussion

In the present study the mammary glands of some cows with brucellosis had minor gross lesions comprised of multiple small granulomas in the cistern and lobar ducts, and an increase in connective tissue. In particular, the supramammary lymph nodes were severely enlarged, identical to findings reported in goats infected with *B. abortus* (9,12). The severity of the mammary gland lesions might have been due to the number of *Brucella* involved (12) and that marked gross lesions in the mammary glands were not produced by *Brucella* spp., or that only minor changes occurred. Nonetheless, it should be stated that the lesions observed in the present and other studies (12) were not characteristic of brucellosis, either in cows or other animal species.

The present study observed that histological lesions in the mammary glands were primarily characterized by lymphoplasmacytic and histiocytic interstitial mastitis, which is compatible with previous results reported in cows, goats, and sheep with brucellosis (9,12,16,17). Yet, leukocytic infiltration of the ducts and duct segmental squamous metaplasia are not always associated with *Brucella*-induced mastitis in cattle and goats, as stated by Meador et al. (9). It should be noted that similar inflammatory reactions might occur due to other bacterial infections of the mammary glands. Lymphofollicular proliferation and lymphoid follicle formation in the periductal and intralobular interstitium of the mammary glands, as observed in the present study, have also been

documented in *Brucella*-infected goats and it was reported that such lesions are more severe in chronically infected goats than in those during the early period of infection (12). Nevertheless, it is possible that the occurrence of lymphoid follicles in the mammary glands of the cows might have been due to prior antigenic stimulation from other agents, as reported by Meador et al. (12).

Specific identification with light microscopy of *Brucella* organisms in histologic sections is difficult and non-specific when using conventional staining procedures. Immunoenzymatic histologic techniques offer a sensitive method for the identification of antigens in tissue and eliminate many of the shortcomings of fluorescent antibody techniques (18,19). Studies using immunohistochemical techniques have documented that brucellar antigens are intensely stained within the cytoplasm of phagocytic cells (9,12,16,18-20). Likewise, macrophages play a central role in the defense against *Brucella*, and phagocytosis and death due to macrophages are among the initial innate defenses of the body against infectious agents (7,10). Similarly, in our study, brucellar antigens in particular stained in the cytoplasm of macrophages and neutrophils located in the intralobular interstitium, and in the ductal and alveolar lumina in the mammary glands of 3 cows with rare extracellular positivity.

Brucella spp. are capable of surviving and replicating in epithelial cells, as in phagocytic leukocytes (4,9,18,20,21). Accordingly, in the present study the alveolar and ductal lumens were filled with desquamated secretory epithelial cells that showed intense positivity. Intensive staining of the ductal and alveolar secretory epithelial cells might have been due to a high quantity of the antigen in the mammary glands. As stated by Meador et al. (12), it may be that intra-epithelial localization of *Brucella* cannot be ruled out, although it is not an essential event for the development of brucellar mastitis. Immunohistochemistry clearly revealed that abortion in these animals was caused by *Brucella* spp. and that the causative agent might have been excreted via colostrum and milk from the infected mammary glands of the animals.

The present study observed that staining of brucellar antigen in the supramammary lymph nodes occurred primarily in the cytoplasm of sinusoidal macrophages; however, the quantity of antigen that reacted with the antibody was less than that in the macrophages of the mammary glands, in accordance with previous findings

that macrophages containing antigen are infrequent in the lymph nodes (9,12,22). In ruminant brucellosis, it was reported that dissemination of *Brucella* from the mammary glands to the supramammary lymph nodes occurs primarily in infected mammary macrophages migrating in the lymphatics (9,12). Thus, both the small number of immunopositive macrophages and the small quantity of antigen in their cytoplasm might have been due to the migration of *Brucella*-containing macrophages from the mammary glands into the lymph nodes (9,12,21).

Bacteriological methods have the advantage of detecting the organism directly and limit the possibility of false-positive reactions; moreover, bacterial culturing is more sensitive than immunohistochemical and serological testing (18). Likewise, it was reported that 9 out of 14 caprine fetal organs in which *B. abortus* was isolated were determined to be positive by the immunoperoxidase technique (18); however, culture material must be handled carefully, as the *Brucella* organism is a class III pathogen (3,18,23). In the present study *B. abortus* biotype 3 was the only causative agent and was cultured from the udders and supramammary lymph nodes of 4 of the 11 cows that were sampled. This is consistent with the finding reported by Hamdy and Amin (3) that *B. abortus* biotype 3 is commonly isolated as a causative agent from the organs of cattle with a history of abortion.

Serological tests recommended as a means of indirectly diagnosing the disease are associated with occasional false-positive results (3). Although all of the 11 cows with a history of abortion were *Brucella*-seropositive according to the serum agglutination test, immunohistochemistry and bacterial culture detected fewer *B. abortus* positive cases than did the serological test. The differences between serological and culture techniques, and those of immunocytochemistry might be due to the development of an immune response following an infection and the consequent clearance of the bacteria from the tissues, or might indicate a false-positive serological test result.

In conclusion, the present study shows that *Brucella* agents localized in the mammary glands and supramammary lymph nodes following abortion, and that positive staining of brucellar antigen in the mammary glands was useful in the diagnosis of the disease. Nonetheless, it should be noted that the most specific diagnostic procedure for the diagnosis of brucellosis is the

isolation of the causative organism. Nevertheless, in suspected cases in which the bacteriologic culture is negative or the material is fixed in formalin, as bacterial culture detects only living microorganisms,

immunohistochemical techniques may be recommended as a supplemental diagnostic tool for the detection of *Brucella* organisms in the tissues of disease-affected animals.

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