

Induction of mastitis in Awassi lactating ewes with a slime producing *Staphylococcus aureus*

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Abstract

The aim of this study was to investigate the clinico-pathological changes in the mammary gland of Awassi ewes inoculated with a slime producing *Staphylococcus aureus*. Five ewes were examined clinically by visual inspection and palpation of the teats and udder halves for local and generalized reaction and milk samples were collected in sterile test tubes aseptically for bacterial examination and California mastitis test (CMT) to confirm that these ewes were free from intramammary infection prior to inoculation. The ewes were inoculated with one mL PBS containing 7×10^2 CFU of slime producing *Staph. aureus* through the teat canal. Post inoculation, all the ewes were examined clinically and bacteriologically daily for appearances of clinical signs, then the ewes were slaughtered 6 days later, one centimeter cubes from different parts of the mammary gland were obtained and fixed in 10% buffered formalin, processed, embedded in paraffin, sectioned, and stained with Haematoxylin and Eosin (H&E) and examined for presence of pathological lesions. The ewes showed systemic disturbances and developed mastitis within 24 h post inoculation, the body temperature, pulse and respiratory rates were elevated accompanied with depression and loss of appetite. The main lesions in the udder characterized by fibrosis of interstitial tissues with polymorphonuclear neutrophils (PMNs) and mononuclear leukocyte infiltration as well as suppuration in the wall of necrotic milk ducts in addition to abscess surrounded by fibrous connective tissue. This study clearly showed that the slime producing *Staph. aureus* induced severe mastitis in lactating Awassi ewes, and is the first description of some clinico-pathological features of the ewes mastitis experimentally induced by this organism in Iraq.

Key words: *Staphylococcus aureus*, slime, mastitis, Awassi ewes, histopathology.

أحداث التهاب الضرع في النعاج العواسي الحلوبة بالمكورات العنقودية الذهبية المنتجة للغراء

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الخلاصة

تهدف هذه الدراسة الى تقصي التغيرات السريرية والمرضية في الغدد اللبنية للنعاج العواسي الحلوبة المحقونة بالمكورات العنقودية الذهبية المنتجة لمادة اللزج. فحصت خمسة نعاج سريريا بوساطة الفحص العياني والجس باليد للحلمات والضرع وفحصت نماذج الحليب منها باختبار كاليفورنيا والفحص البكتيري للتأكد من خلو هذه الحيوانات من التهاب الضرع قبل الحقن. حقنت النعاج بجرعة 7×10^2 خلية مكونة للمستعمرات من المكورات العنقودية الذهبية المنتجة لمادة اللزج داخل قناة حلمة الضرع. بعد الحقن فحصت النعاج سريريا وجرثوميا لظهور العلامات المرضية وبعد 6 أيام من الحقن ذبحت النعاج وجمعت نماذج من غدها اللبنية بحجم 1 سم³ وثبتت بمحلول 10% فورمالين للفحص النسيجي. اظهرت النعاج علامات مرضية جهازية متمثلة بارتفاع درجة حرارة الجسم وسرعة التنفس والنبض مع خمول وفقدان الشهية وعلامات التهاب الضرع بعد الحقن. واتسمت الآفات النسيجية بالتليف وارتشاح الخلايا البيضاء فضلا عن تقبح الانسجة اللبنية. بينت هذه الدراسة بأن المكورات العنقودية الذهبية المنتجة لمادة اللزج أحدثت التهاب ضرع شديد في النعاج العواسي وهي أول وصف لبعض الملامح السريرية لالتهاب الضرع التجريبي في النعاج المتسبب بهذه الجرثومة في العراق.

الكلمات المفتاحية: المكورات العنقودية الذهبية ، الغراء ، التهاب الضرع ، نعاج عواسي ، التغيرات المرضية النسيجية

Introduction

Ewe's mastitis is a major cause of substantial economic losses in dairy sheep flocks throughout many regions of the world including Iraq (1,2, and 3). Decreased milk production accounts for approximately 70% of the total cost of mastitis (4). In addition, mastitis may have some public health importance, since occasionally milk harboring human pathogens may cause infection to the consumers of raw or inadequately heated milk (5). Although a wide range of microorganisms can cause mastitis, most cases are reported to be due to *Staphylococci* (6). *Staph. aureus* is one of the most prevalent major mastitis pathogen in dairy herds and associated with persistent infection (7,8). Persistent intramammary infection may be related to the ability of this pathogen for slime production (9). The slime is a viscous extracellular polysaccharide layer, which is considered to one of the virulence factors that facilitate the adherence and colonization of *Staphylococci* on the mammary gland epithelium, also contributing to the avoidance of the immunological defenses and to the difficulty of pathogen eradication, leading to recurrent or persistent infections (10,11). When the infection persists and the channels are blocked, the milk within the alveoli increases the pressure there, the secretory cells lose their function to synthesize the milk and the cells begin to atrophy, substances released by white blood cells cause destruction of cellular structures, which are replaced by scar tissue (12). Histological examination has been widely used for assessing the damage of the mammary glands tissues caused by mastitis pathogens. The rabbits and mouse has been used extensively as a model of intramammary inoculation in studying this disease (13), while few studies have been published to describe histopathological changes in experimentally induced staphylococcal mastitis in dairy animals. The aim of this study was to investigate the clinico-pathological changes in the mammary gland of Awassi ewes inoculated with a slime producing *Staph. aureus*.

Materials and methods

1-Animals: The experiment was conducted during the year 2014 on five clinically healthy lactating ewes of the Awassi breed aged 2 to 3 years which were given food and water *ad libitum*. The ewes were kept under observation for 2 weeks in clean building before starting the experiment, their lambs were weaned 3-4 weeks after lambing and subsequently, the animals were hand-milked twice daily. The ewes were examined clinically by visual inspection and palpation of the mammary glands and teats. Milk samples were collected in sterile test tubes aseptically after discarding the fore milk for bacterial analysis and CMT to confirm that these ewes were free from intramammary infection prior to inoculation (14, 15).

2-Bacterium: A slime producing *Staph. aureus* bacterium, recently isolated from a case of clinical mastitis in ewes was used for induction of mastitis in the lactating ewes. This isolate was kindly provided by Dr. Khalid Mahmood Hammadi - Department of Internal and Preventive Veterinary Medicine /University of Diayla, Iraq, its slime production and cultural characteristics were described in details previously (9).

3-Intramammary inoculation: The slime producing *Staph. aureus* was grown on blood agar and checked for purity, then it was inoculated into soya-broth and incubated aerobically at 37 °C for 6 h. Serial dilutions of the broth culture into phosphate buffer-saline (PBS), pH=7.2, were carried out (16), one mL of PBS containing 7×10^2 CFU of *Staph. aureus* was infused through the teat canal. Post inoculation, all the ewes were clinically examined daily for appearances of clinical signs including temperature, pulse and respiration rates and any abnormal changes in the mammary glands and teats. Milk samples were collected in sterile test tubes aseptically after discarding the fore milk and examined by bacterial analysis (14) and California mastitis test (CMT) (15).

4-Histopathological examination: The animals were slaughtered at intervals (3-6 days) after inoculation, one centimeter cubes from different parts of the mammary gland were taken and fixed in 10% buffered formalin, dehydrated in ascending

concentrations of ethanol and cleared in xylene followed by embedding in paraffin. Sections (5µm) were prepared from each

tissue block and stained with Hematoxylin-Eosin stain (H&E) for histological examination as described previously (17).

Results

Clinical and bacteriological finding:

All ewes were found to be clinically healthy during the period from lambing to inoculation, no abnormal clinical findings were present in the mammary gland and the teats of all ewes, the CMT was negative and no bacteria were isolated from the milk samples prior inoculation. The inoculated ewes showed signs of subclinical mastitis within 24 h post inoculation, and then, they showed high increase in body temperature ($40.6 \pm 0.21^\circ\text{C}$). Pulse rate (114.4 ± 3.3 beat/min) and respiration rate (48 ± 2.9 breath/min). The inoculated halves of the udders were hot, swollen, and tender, and the secretion of milk was greatly reduced and changed to a straw yellow fluid and later becoming purulent. These ewes were slaughtered 3-6 days after inoculation. A slime producing *Staph. aureus* was consistently isolated in pure culture from all milk and tissue samples obtained from inoculated ewes.



Fig. (1): Cut surface of the udder of ewes showed suppurative hemorrhagic areas six days post inoculation with *Staph. aureus* (Macroscopic lesion).

The CMT increased 2-3 scores within 24 h post inoculation in all ewes. The cut surface of the inoculated udder halves revealed extensive areas of hemorrhage and accumulations of pus (suppurative exudate) (Fig. 1).

Histopathological examination:

The main lesion in the udder characterized by interstitial fibrosis infiltrated with polymorphic and mononuclear cells (Fig. 2).

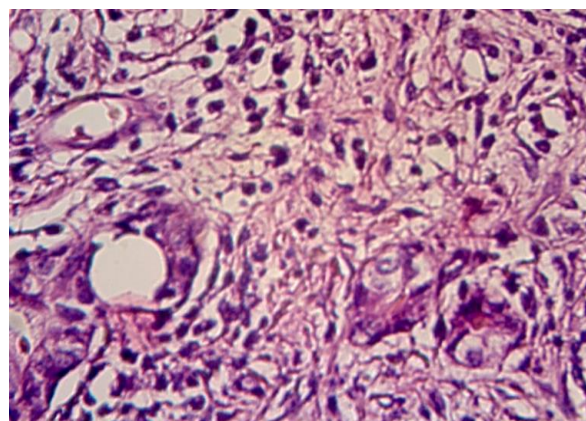


Fig. (2): Interstitial fibrosis infiltrated with polymorphic and mononuclear cells (H&E stain X40).

In other section, necrosis of epithelial cells of milk ducts in addition to suppurative reaction in the wall of necrotic milk ducts (Fig. 3).

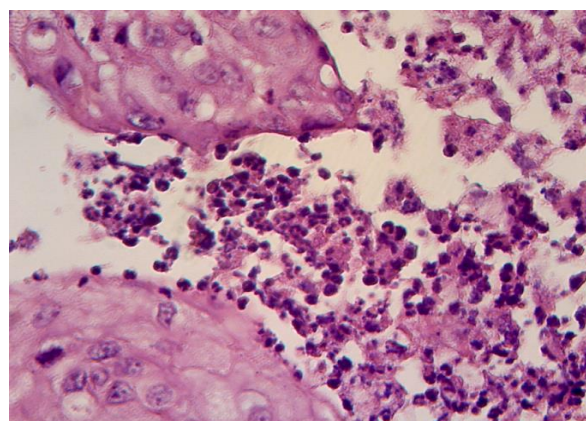


Fig. (3): Show suppuration and necrosis in the wall of milk ducts (H&E stain X40).

As well as PMNs and MNs in the lumen of collected milk ducts (Fig. 4), in addition inflammatory cells infiltration between desquamated acini (Fig. 5), PMN and MNs infiltration were seen in necrotic epidermis and dermal area of the skin (Fig. 6). vacuolar degeneration of acini with inflammatory cells infiltration as well as calcification (Fig. 7)

and in other section large abscess were surrounded by a thick compact fibrous connective tissue (Fig. 8) as well as PMNs

and mononuclear cells in fibrosis of interstitial tissues (Fig. 9).

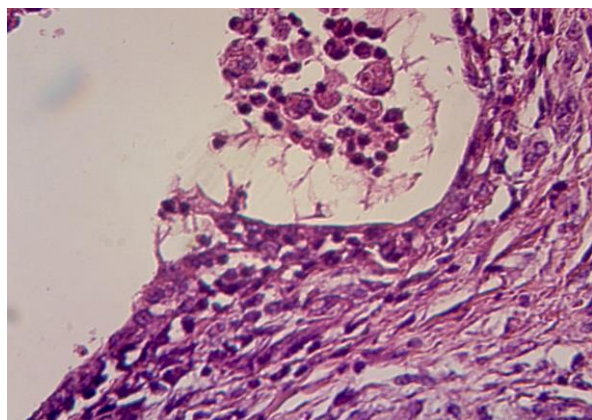


Fig. (4): Shows PMNs and mononuclear cells in the lumen of collected milk ducts (H&E stain X40).

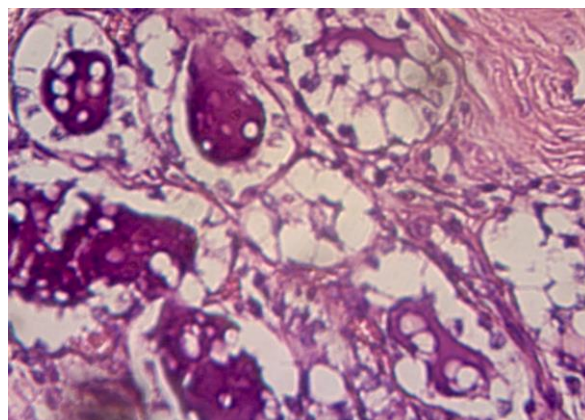


Fig.(5): Show fibrosis of interstitial tissues. The PMNs and mononuclear cells between desquamated acini (H&E stain X40).

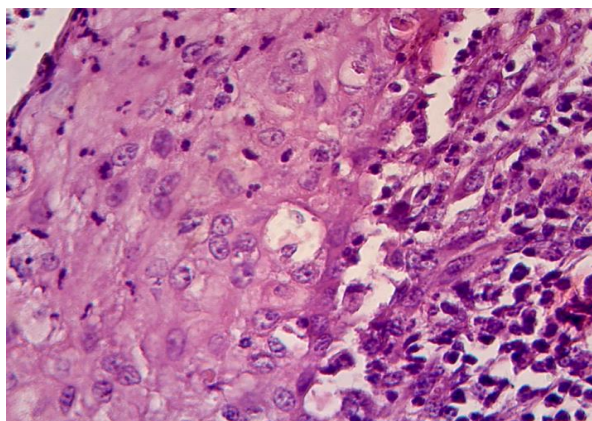


Fig. (6): Shows PMNs and mononuclear cells in the epidermis and dermal layer of the udder (H&E stain X40).

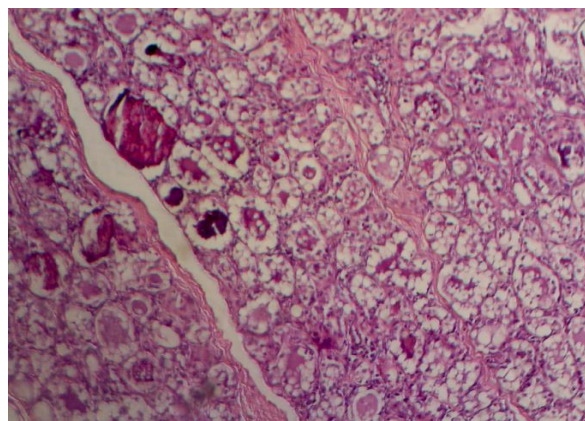


Fig. (7): Shows vacuolar degeneration of acini with inflammatory cells infiltration as well as calcification (H&E stain X10).

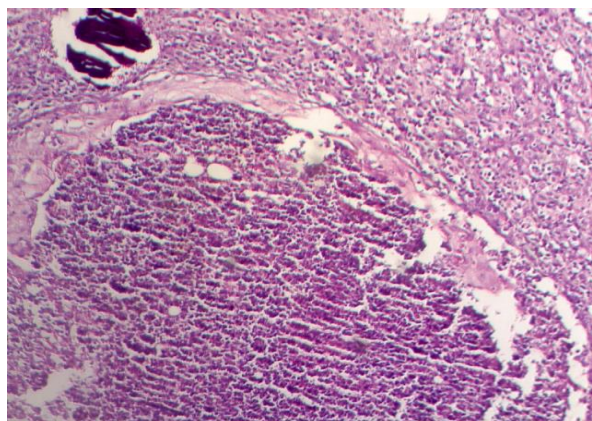


Fig. (8): Shows large abscess surrounded by a thick compact fibrous capsule in the interstitial tissues (H&E stain X10).

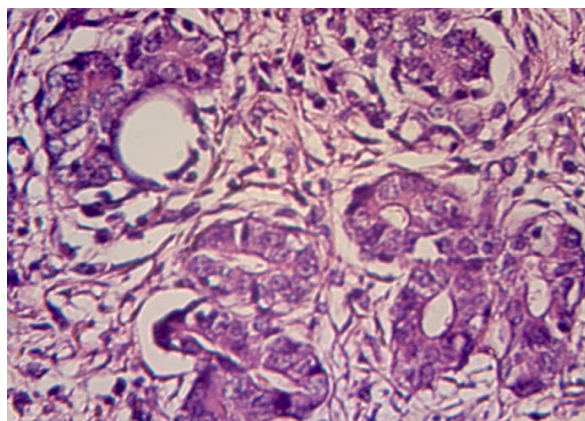


Fig. (9): Shows PMNs and mononuclear cells in fibrosis of interstitial tissues (H&E stain X40).

Discussion

Mastitis is an inflammation of the mammary gland including either the secretory cells or the connective tissue or both, it is accompanied by physical, chemical and commonly microbiological changes in milk and pathological changes in mammary gland, the severity of the inflammation is dependent on the etiological agent and the host response to it. Several microbial pathogens have been associated with mastitis in ewes (18). *Staph. aureus* is recognized as the most common udder pathogens that cause either clinical or sub-clinical mammary gland infections in ewes (19). One of the most important factors accounting for *Staph. aureus* pathogenicity is its ability for slime production (20). Numerous studies reported isolation of a slime producing *Staph. aureus* from severe clinical mastitis in ewes (9,10). In the present study the clinical and histopathological changes were investigated in the mammary gland of Awassi ewes inoculated with a slime producing *Staph. aureus*. Slime layer surrounding the *Staph. aureus* strains help in adherence and colonization of these microorganisms on the mammary gland epithelium (21). The ewes in our study showed signs of subclinical mastitis 24 hours post inoculation as detected by CMT. The CMT carried out as a useful screening test for milk somatic cell count (SCC) to detect subclinical mastitis (15). CMT score is positively correlated with SCC, the number of microorganisms and infection status in lactating animals (22). Mastitis was associated with an influx of somatic cells, primarily PMNs, which could play an important role in breakdown of the blood-milk barrier and damage mammary epithelium by releasing reactive oxygen intermediates and proteolytic enzymes (23). During the infection of the mammary glands, the tissue damage can initially be caused by bacteria and their products, some bacteria produce toxins that damage the milk producing tissue, whereas other bacteria are able to invade and multiply within the epithelial cells before causing cell death (4). Forty eight hours post inoculation, the clinical examination revealed the progression of the signs to include systemic involvement

such as fever, depression, loss of appetite and decrease in milk production. Similar clinical signs were reported in naturally occurring and experimentally induced mastitis in ewes (19, 24). Once necrosis of the mammary stromal and parenchymal tissues has begun, there is a reduction in secretory function and potential decrease in milk production that may be irreversible. *Staph. aureus* produces toxins that damage the cell membranes, directly destroy milk producing tissue and induce necrosis in bovine mammary glands (25). The histopathological changes obtained during the present study revealed that the inoculated ewes mammary glands with slime producing *Staph. aureus* characterized by massive infiltration of inflammatory cells with fibrosis of interstitial tissues and large abscess surrounded by a thick compact fibrous capsule. The infiltration of inflammatory cells to the mammary gland tissue is due to the inflammation induced by *Staph. aureus* intramammary infection, similar evidences were reported suggesting that *Staph. aureus* mastitis was characterized by infiltration of PMN in the mammary gland tissue (26, 27). The PMNs act by engulfing and destroying the invading bacteria via oxygen-dependent and oxygen-independent systems (26). The histopathological lesions of the present study were compatible with that previously reported in animals suffering from staphylococcal mastitis (4, 25). The histopathological changes in experimentally induced *Staphylococcus* mastitis in the rabbits revealed desquamation of epithelium, PMN cells infiltration and necrosis in the mammary gland at 48 hrs. post inoculation (28). The slime-producing *Staph. aureus* strains have a higher capacity of colonizing the mammary gland than its non-slime-producing variants (29, 30). Adhesion to mucosal surfaces may be a significant step in establishing persistent infections (12). To establish intra-mammary infection, *Staph. aureus* has to overcome several host defense mechanisms such as phagocytosis, recruitment of PMN, etc., the presence of slime was responsible for a decreased phagocytic ingestion and overall killing (21).

We concluded that the slime producing *Staph. aureus* isolate is highly pathogenic for Awassi ewes, and induced severe acute mastitis with the challenge dose of 7×10^2 CFU/ml within 24 hrs. Good attention and management practices are require

preventing the occurrence of mastitis.

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