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Immunopathological Responses to the Bovine Mastitis Associated with *Staphylococcus* Species Infection

Agharid A Al-Rasheed*[1], Sana'a S Ahmed[1], Karim A Al-Jashamy[2], Bashiru Garba[3]

[1]Department of Microbiology, College of Veterinary Medicine, University of Tikrit, Tikrit, Iraq, [2]Radiology and Sonar Technology Department, Bilad Alrafidain University College, Iraq, [3]Veterinary Public Health and Preventive Medicine Department, Faculty of Veterinary Medicine, Usmanu Danfodiyo University, Nigeria

***Correspondence:**
agharidalrasheed@tu.edu.iq

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ABSTRACT

Bovine mastitis is a disease that concerns animals' welfare and increases the economic production losses. Bacterial agents such as *Staphylococcus* species are the main causative agent of bovine mastitis. This bacterial agent expresses some inflammatory cytokines that might enhance the cell-mediated, which may promote the pathogenesis of mastitis. The objective of the current study was to investigate the bovine innate immune response circulating levels of pro-inflammatory and anti-inflammatory cytokines. A total of 10 mL of milk specimens were collected randomly from 100 clinically mastitic cows, and another 20 clinically healthy cows were considered as a control group for the California Mastitis test. The microbiological cultures of milk specimens were performed. The interleukins (ILs)that involved IL-4, IL-6, and IL-10 were detected using the ELISA test for the evaluation of the pro-inflammatory bovine mastitis pathophysiology. The results of this study showed that *Staphylococcus aureus* detection was in 31.2% of mastitic milk and 8.7% of non-mastitic milk specimens; and the coagulase-negative *Staphylococcus* was detected in 14.8% and 18.7% in the mastitic and non-mastitic milk specimens, respectively. The IL-6 level was shown significantly higher (P<0.05) in the specimens of mastitic milk (194±12.8 pg/mL) compared to the non-mastitic milk (31±2.9 pg/mL). In conclusion, the elevated level of expression of IL-6 cytokine in the milk of cows with mastitis suggested that IL-6 might be used as a potentially suitable biomarker for early bovine mastitis diagnosis.

Keywords: pathophysiology, bovine mastitis, intra-mammary infection, *Staphylococcus* spp, inflammatory cytokines

INTRODUCTION

Mastitis refers to the inflammation of the intra-mammary gland, which is considered the most economical disease in livestock (1). The disease exerts serious to the well-being of the affected animal resulting in poor productivity due to a reduction in milk yield, with a resultant economic loss to the farmer (2). Other important losses are occurred as a result of the disease including veterinary expenditure, an increased risk factor of other diseases, reduced fertility, increased premature culling rate, with mortality in some severe cases and complications. In the face of global food security challenges, mastitis is viewed as an important disorder of the attainment of food sufficiency in the worldwide economic sustainable development programs, especially in developed countries that rely on many dairy products (3,4).

Among the wide range of microbial species associated with bovine mastitis, bacterial species including

Staphylococcus (*S.*) *aureus* and coagulase-negative *staphylococci* (CNS) group are the most popular pathogenic agents (5). Infection with these organisms causes subclinical and clinical mastitis. CNS is a normal microflora in the skin of animals, and little is known about its virulence. In recent years, the CNS becomes one of the most common causes of bovine mastitis in many countries; hence, it is described as an emerging mastitis pathogen (6,7). On the other hand, *S. aureus* becomes a superbug agent of the dairy udder, causing intra-mammary infection, thereby compromising animal health and economy (8). These bacterial pathogens cause clinical and subclinical disease due to their pathogenicity of producing a wide range of virulence determinants that aid their invasion, attachment, proliferation, and persistence by resisting drugs and escaping the immune response (9-11).

Protection of the host against intra-mammary *Staphylococcus* spp. invasion is largely dependent upon bacterial virulence and skin integrity, which serves as a barrier against the bacteria preventing its entry into the mammary gland. In addition, there are several functional innate phagocytic immune components as leucocytes and the immune system that activate the phagocytizing to destroy the invading pathogens (12, 13). The host response following CNS intra-mammary infection (5) is indicative of mastitis associated with the milk somatic cell count (SCC), which tends to increase proportionately the damaging of the tissue of mammary glands (14). Pathophysiological inflammatory reactions following mastitis have also been associated with systemic response, which is characterized by increased protein concentrations in the acute phase of inflammation as well as other cytokines (15, 16). This study is going to highlight the kinds of literature information on anti-inflammatory cytokines and their role in cow mastitis. The objective of the current study was undertaken to investigate the bovine innate immune response circulating levels of pro-inflammatory and anti-inflammatory cytokines.

MATERIALS AND METHODS

Ethics and Sample Collection

All procedures used in this study were reviewed and approved by The Scientific Committee of the Department of Microbiology, College of Veterinary Medicine, University of Tikrit, Iraq in compliance with the ethical principles of animal welfare.

A total of 10 mL of milk specimens from 100 clinically mastitic cows and another 10 mL of milk specimens from 20 clinically healthy cows were also collected as a control. The specimens were collected from a farm of a local breed of cows in the north of Tikrit province, Iraq from March 2021 to October 2021. In each case, the udder of the lactating cows was first cleaned with a warm and disinfected towel, before the first stream of milk from each quarter was expressed and discarded, and then 10 mL was collected into a sterile container and screened for mastitis.

California Mastitis Test (CMT)

CMT was conducted to detect the presence of subclinical and clinical mastitis infection based on SCC in the milk samples. After discarding the first stream of milk, the next milk was drawn into the shallow cups on the paddle, and an equal amount of the reacting solution was added and then gently rotated in the horizontal plane for 10-30 sec. The result was interpreted as negative when the mixture remains liquid, with no slime or gel formation, while it was interpreted as positive when the mixture thickens immediately and tends to form jelly according to the method of Dingwell et al. (17).

Bacterial Culture and Identification

The bacteriological culturing and identification of milk specimens were achieved according to the guidelines of the National Mastitis Council (18). Briefly, it was about 0.01-mL of milk samples using the special loop to spread the milk sample on a blood agar plate, mannitol salt agar, and nutrient agar (Oxoid, UK) then incubated in the aerobic jar at 37 °C. The phenotypic features of the cultures were examined after 24, and 48 h. The isolates were identified after subjecting to Gram staining, together with biochemical tests including catalase, and coagulase tests for routine identification methods of CNS (14, 19). The positive pure culture of *Staphylococci* was identified and stored at -25 °C in 1 mL of brain heart infusion broth supplemented with 10% glycerin until further analysis.

Identification of Cytokines

The concentrations of interleukin (IL)-4 and IL-10 were determined using the bio-X-Diagnostics enzyme-linked immunosorbent assay (ELISA) kit for Bovine Haptoglobin Assays and IL-6 (antibodies-online GmbH, Germany) based on the instructions of the manufacturer. The assay of quantitative sandwich enzyme immunoassay technique was employed using microplates that have been pre-coated with specific antibodies for IL-4, IL-6, and IL-10, respectively. The milk samples and their standard were pipetted into the wells that had interleukins, which helped the samples binding by the immobilized antibody. This was followed by the removal of unbound substances, and the addition of a biotin-conjugated antibody specific for each of the assayed interleukins. The wells were washed using avidin conjugated horseradish peroxidase (HRP), the wells were washed after incubation then removed of any unbound avidin-enzyme reagent. The substrate solution was added to the wells and incubated for a period until developing its color in the proportion concentration of the interleukins. Concentration and the intensity of the color were measured according to the instructions of the manufacturer.

Statistical Analysis

The data were analyzed using IBM SPSS Statistics (Version 26) predictive analytics software. The statistical analysis was achieved using Student's *t* test, the mean and standard deviation (SD) statistic method, and a $P \leq 0.05$ was considered a significant value.

RESULTS

The results of bacterial cultures showed that *S. aureus* was isolated from milk of mastitic and healthy cows in 31.2% and 8.7% of the milk specimens respectively. Coagulase Negative *Staphylococcus* was isolated from 14.8% and 18.7% of mastitic and healthy cows respectively (Table 1). Also, the growth and morphological features of the bacteria on the selective Mannitol agar showed *S. aureus* producing yellow colonies, whereas the CNS produced small pink or reddish colonies on the blood agar (Figure 1A, B).

With respect to the interleukins concentration in milk specimens, the result indicated that IL-6 was only considerably to be significantly higher ($P<0.05$) in mastitic milk specimens in comparison to the non-mastitic milk specimens, while, both IL-4 and IL-10 concentrations were significantly higher ($P<0.05$) in the non-mastitic milk specimens (Figure 2). However, among the interleukins assayed, the IL-6 level had the highest concentration (194±12.8 pg/mL) among the mastitic milk samples.

Table 1. Number and percentage of *Staphylococcus* species isolated from milk of mastitic and non-mastitic cows

			Staphylococcus aureus		Coagulase-negative *Staphylococcus*		Total	
Cows	**Case**	**Udder quarters**	**Isolates**	**%**	**Isolates**	**%**	**Isolates**	**%**
Mastitic	100	310	97	31.2	46	14.8	143	46
Non-mastitic	20	80	7	8.70	15	18.7	22	27.4
Total	120	390	104	39.9	61	33.5	165	73.4

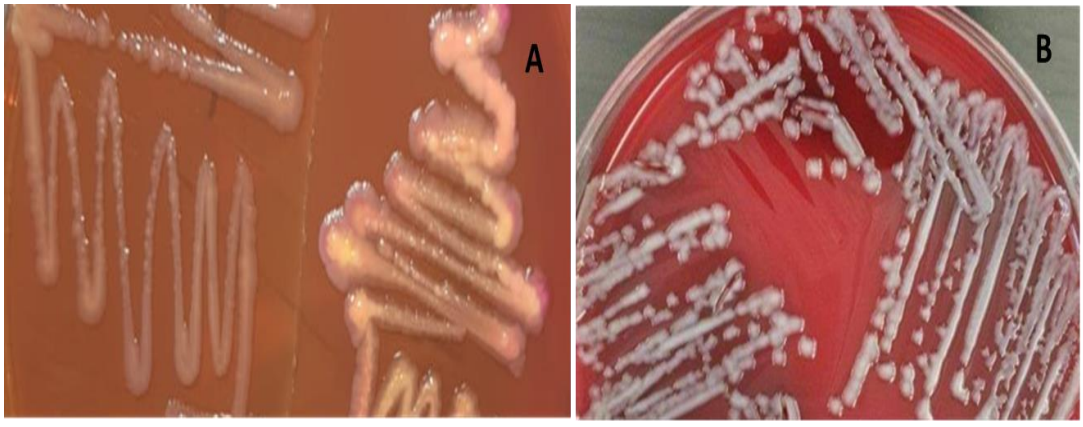

Figure 1. *Staphylococcus* species growth on A: Mannitol salt agar and B: Blood agar

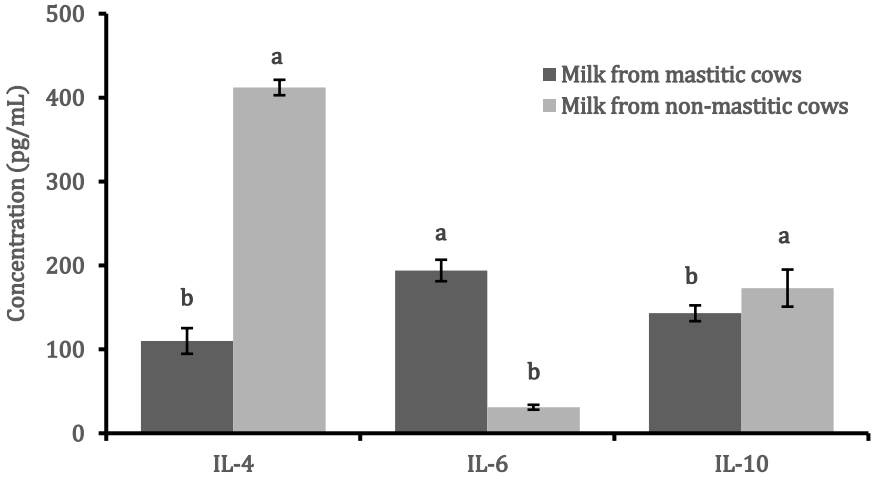

Figure 2. Concentration of Interleukins (IL)-4, -6, and -10 in milk samples collected from mastitic and non-mastitic cows

DISCUSSION

In this study, the highest percentage of bacterial isolation was 46% which may envisage the *Staphylococcus* species existence in contaminated milk with different microorganisms. *S. aureus* and CNC are two of the most common causes of intra-mammary infection resulting in mastitis which may consider as an important public health and economic diseases (4). The investigation of responses in dairy cattle with clinical and subclinical mastitis might cause by pathogenic *Staphylococcus* species most important to avoid endemic mastitis. Traditionally, the diagnosis of clinical mastitis is achieved by organoleptic tests which comprise the physical appearance, consistency, and presence of impurities like blood or pus. These depend on the amount of swelling; the severity of pain and the cow's disposition may indicate the severity of infection. However, direct measurement of the somatic cell count (SCC) level using the CMT is proven to be a suitable milk screening technique capable of determining the severity of the infection depending on the somatic cell counts (20). This is a desirable because although bacterial culture is more accurate test for determining the actual cause of the disease, it cannot define the severity of the infection. In our results, some milk samples that were found to be CMT-positive turned out to have no growth for the *Staphylococcus* species. This can be due to the fact that the mastitis may be caused by other bacterial pathogens than *Staphylococcus* including, *Streptococcus*, *Mycoplasma*, and other Gram-negative bacteria (21). Another possible cause of the non-isolation of the bacteria is by existence of some antibiotic in the cow milk during treatment prior to the collection of the milk samples (22).

In term of the bacterial culture, *S. aureus* was isolated at a rate of 31.2% and 8.7% of mastitic and healthy cows, respectively. Several authors have also reported *S. aureus* as the main cause of mastitis with a detection rate of 21.3% to 62.8% (23). *S. aureus* can cause many diseases in animals including mastitis because of their pathophysiology factors such as catalase, coagulase, and DNase. Inflammatory cytokines such as toll-like receptors, complement proteins, and interleukins are comely present in the inflamed mammary glands and might cause preclinical or clinical bovine mastitis (24, 25). Evaluation of milk revealed that interleukin 6 concentrations in the milk of mastitic cows were higher (194±12.8 pg/mL) than in healthy cows (31±2.9 pg/mL). IL-6 concentrations usually correlate with the levels of inflammation, and it was reported to be the main inducer of hepatic synthesis of acute phase proteins. Research by Sakemi et al. (25) confirmed the presence of high levels of IL-6 is already seen in the first stage of the disease. IL concentrations were 5 times higher in specimens taken from acute mastitic cows than those from control; and this result is close to the result of the current study. Bovine IL-6 showed multifunctional as a cytokine secreted by different immune cells especially T-lymphocytes and macrophages (26). IL-6 has a different range of activities that included the differentiation of B-cell types into Ig-producing cells of lymphocytes and monocytes. Thus, IL-6 might consider a non-specific indicator of various inflammatory states and a potential marker of clinical infection.

In contrast, IL-10 is produced at the late stages and regulates the course of inflammation, while IL-10 might absent or delayed in bacterially contaminated milk (27). Similarly, IL-4 levels in our study were significantly lower in the milk of infected cows compared to normal cows. The significant decrease of IL-4 in milk from mastitic cows in relation to that from a healthy cow is similar at the earlier reports (28). IL-4 produced from T-helper-2, natural killer cell, mast cells, and basophils lymphocytes act as a stimulator to B-cell for proliferation, and functions mainly as a regulator of immunity; however, playing an important role in leukocyte survival under both physiological and pathological states (29, 30).

The outcomes of this investigation imply that cows with mastitis caused by *Staphylococcus* species elicit an immune response in the mammary gland as part of the cow's innate immune mechanisms against the invading pathogen. However, in this study, the considerable increase in the expression of IL-6 cytokine in the milk of cows with mastitis suggested that IL-6 might be used as a suitable biomarker for the diagnosis of mastitis.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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الاستجابات المناعية المرضية لالتهاب الضرع البقري المرتبط بعدوى أنواع المكورات العنقودية

أغاريد الرشيد[١]، سناء سعود أحمد[١]، كريم الجشمعي[٢]، بشيرو غربا [٣]

[١]قسم الأحياء المجهرية، كلية الطب البيطري، جامعة تكريت، تكريت، العراق، [٢]قسم الأشعة وتقنيات السونار، كلية بلاد الرافدين الجامعية، بغداد، العراق، [٣]قسم الصحة والطب الوقائي، كلية الطب البيطري، جامعة عثمانو دانفوديو، نيجيريا

الخلاصة

يعد التهاب الضرع البقري مرضًا يسبب قلقًا لرفاهية الحيوانات وخسائر فادحة في الإنتاج الاقتصادي. العوامل البكتيرية مثل أنواع المكورات العنقودية هي العامل المسبب الرئيسي لالتهاب الضرع البقري. يعبر هذا العامل البكتيري عن بعض السيتوكينات الالتهابية التي قد تعزز التوسط الخلوي، مما قد يعزز التسبب في التهاب الضرع. تم جمع ما مجموعه ١٠ ملم من عينات الحليب من ١٠٠ بقرة مصابة سريريًا، واعتبرت ٢٠ بقرة أخرى صحية سريريًا كمجموعة تحكم لاختبار كاليفورنيا لالتهاب الضرع. أجريت الزروع الميكروبيولوجية لعينات الحليب. تم الكشف عن الإنترلوكينات التي تتضمن IL-4 و IL-6 و IL-10 باستخدام اختبار ELISA لتقييم الفيزيولوجيا المرضية لالتهاب الضرع البقري المؤيد للالتهابات. أوضحت نتائج هذه الدراسة أن الكشف عن المكورات العنقودية الذهبية في ٣١.٢٪ من لبن الضرع و ٨.٧٪ من عينات اللبن غير الخبيث ، و Coagulase Negative *Staphylococcus* تم اكتشافه في ١٤.٨٪ و ١٨.٧٪ في عينات لبن الضرع و non-mastitic على التوالي. أظهر مستوى IL-6 أعلى بكثير في عينات حليب الضرع (194 ± 12.8) مقارنة بصحة الحليب غير الضاري (31 ± 2.9). في الختام ، يشير المستوى العالي من التعبير عن IL-6 cytokine في حليب الأبقار المصابة بالتهاب الضرع إلى أنه يمكن استخدام IL-6 مؤشراً بيولوجياً مناسباً محتملاً لتشخيص التهاب الضرع البقري المبكر.

الكلمات المفتاحية: ا الإمراضية، التهاب الضرع البقري، خمج الضرع، المكورات العنقودية، السايتوكينات الالتهابية