Original Article

Pathological Changes in Experimental Intramammary Infection with Different *Staphylococcus* Species in Mice

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Abstract

Introduction: Mastitis is caused by different *Staphylococcus species*, produce great economic loss to farmers. Present study was conducted to know pathological changes in mice inoculated with *Staphylococcus epidermidis, S. chromogenes, S. haemolyticus* and *S. aureus* isolated from bovine milk. **Materials and Methods:** Mice were inoculated with 50 µl (2x104 cfu organisms) per mammary gland and euthanized at 6, 12, 24, 48, 72 and 96 h. Mammary gland weight, gross and histopathological changes of mammary gland, liver, kidney, spleen, heart, lung and inguinal lymph node were studied. **Results:** Mammary gland weight and percentage of body weight increased at 6 and 96 h in *S. aureus* and *S. haemolyticus* infected mice. Gross changes were observed in mammary gland but not in other organs. Mammary gland revealed gross changes from 24 to 72 h in three Coagulase negative staphylococcal (CNS) species and persisted up to 96 h in S. aureus infected mice. Histopathological changes in mammary glands was severe in *S. aureus* and moderate in CNS species. *S. aureus* infected mice revealed severe damage to alveoli and loss of alveolar architecture at 96 h but three CNS species infection was overcome by host factors which was evident by proliferation of alveolar epithelial cells. No histological changes were observed in kidney, spleen, lung, heart and inguinal lymph nodes. **Conclusions:** *S. aureus* caused severe mastitis in mice when compared to CNS species. Further, it is first report of mice to study CNS mastitis, and in future it can be used as model for CNS mastitis.

Keywords: Mastitis, mice, pathology, *Staphylococcus* species

Introduction

India ranks first in cattle and buffalo population in the world and also ranks first in the world milk production, with total milk production of 132.43 million tonnes during 2013 as per DAHD, India report. Although several diseases affect the milk productivity and economy, mastitis is regarded as one of the costliest diseases confronting the dairy industry. It is estimated that mastitis accounts to about 70% of all avoidable losses incurred during milk production.[1] The direct effects of mastitis include either temporary or permanent loss in milk production, poor milk quality, reduction in milk price, increase in treatment costs, and premature culling. In India, the total annual economic loss due to mastitis was calculated to be 7165.51 crore rupees.[2] Among the bacteria isolated in bovine mastitis, *Staphylococcus* species occupies an important place. Till date, more than 50 staphylococcal species and subspecies have been characterized. Coagulase-negative staphylococcal (CNS) species are the most frequently isolated microorganisms in bovine intramammary infections (IMIs) in many countries. More than ten different CNS species have been isolated from mastitis bovine milk samples in the recent past. The most commonly reported species are *Staphylococcus chromogenes*, *Staphylococcus simulans, Staphylococcus hyicus,* and *Staphylococcus epidermidis*.[3] These bacteria are sometimes referred to as environmental staphs and are frequently isolated from milk samples from herds that have recovered from major mastitis outbreaks.[4] CNS species has traditionally been considered to be minor mastitis pathogens, especially in comparison with major

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pathogens such as *Staphylococcus aureus*, Streptococci, and coliforms.

The study of mastitis in bovines is costly and involves various ethical and social issues, especially, in India and also keeping the bovines in a controlled environment is difficult. Hence, the bovine mastitis is mostly studied in laboratory animal models such as mice, rat, and rabbits. Various studies have been carried out using the animal models with major mastitis-causing pathogens such as *S. aureus, Streptococcus* species, and coliforms. Simojoki *et al*.[5] suggested that studying of host response of CNS species in experimental mastitis model is necessary to understand the host-pathogen interaction. The mouse mastitis model is considered to be very good model to study bovine mastitis compared to other laboratory animals for ease of handling, for ease of keeping them in controlled environment, and less cost. The mouse mastitis model is regarded as straightforward and suitable model for the study of bovine mastitis which provides valuable information about pathogenic mechanisms of variety of organisms involved in the IMIs.[6] Perusal of published reports revealed scanty information is available on experimentally induced mastitis in animal models using coagulase-negative *Staphylococcus* species, especially in mice. Keeping this information in background, the present study was undertaken to know the histopathological changes of three coagulase-negative staphylococcal species, namely, *S. epidermidis, S. chromogenes,* and *S. haemolyticus* with one coagulase-positive *S. aureus* in mice.

Materials and Methods

Bacteria used for inoculation

Three coagulase-negative *Staphylococcal* (CNS) species namely *S. epidermidis, S. chromogenes,* and *Staphylococcus haemolyticus* and one coagulase-positive *S. aureus* species were isolated from milk samples of apparently healthy bovines from dairy farms in Karnataka, and species identification and characterization were carried out by our team as reported earlier.[7,8] The species confirmed and characterized organisms were used for intramammary inoculation in mice.

Experimental animals

The timed pregnant (day 12–15), 168 Swiss albino mice were procured from National Centre for Laboratory Animal Science, National Institute of Nutrition, Hyderabad. The mice were grouped into four groups consisting of 42 mice (36 for bacterial inoculation and 6 for phosphate-buffered saline [PBS] inoculation) with six mice in each time points (six time points) and for four organisms (three CNS and *S. aureus*); a total of 168 mice were used in this study. The mice were housed in individually ventilated cages procured from M/s. Citizen Lab Tech Industries, Ahmedabad, India. The temperature and humidity of the animal room were maintained at 23 ± 3°C and 50–70, respectively. Mice were maintained under standard laboratory hygienic conditions and provided standard laboratory animal pellet feed procured from M/s. Nutripet India Private Limited, Bengaluru, India and reverse osmosis purified water *ad libitum*. The paddy husk was used as bedding material and washed cages were autoclaved at 121°C with 15 pounds per square inch for 15 min before use. The animal experiment was approved by the Institutional Animal Ethics Committee of Veterinary College, Bangalore, with CPCSEA Registration No. 493/01/a/CPCSEA. The experiment was carried out as per the guidelines of Committee for the purpose of Supervision and Experiments on Animals, Animal Welfare Division, and Ministry of Environment, Forests and Climate Change, New Delhi. The organisms, namely, *S. epidermidis, S. chromogenes, S. haemolyticus,* and *S. aureus* isolates were inoculated to 36 mice each on day 7th or 9th of lactation with dose containing 2 × 10^4 cfu of bacterial suspension in PBS (50 µl) per teat of left fourth and fifth (L4, L5) and right fourth and fifth (R4, R5) teats and control mice were inoculated with sterile PBS as reported earlier.[9,10] After 1 h of inoculation, the pups were allowed to suckle the teat to simulate the natural field conditions of bovines in dairy farms. The mice from both bacteria inoculated and PBS control groups were sacrificed at different time intervals at 6, 12, 24, 48, 72, and 96 h, and thorough postmortem examination was carried out.

Animal weight and mammary gland weight

The mice were weighed using weighing balance (Precisa, Switzerland) and recorded the body weight in grams. The mice mammary glands were carefully dissected out using sterile scissors and forceps. The left (L4, L5) and right (R4, R5) mammary glands were separately collected, weighed, and expressed in grams. The mammary gland weight and animal body weights were used to calculate the percentage of mammary gland weight to body weight.

Pathology

The gross changes in the mammary gland and other parenchymatous organs were recorded. Histopathological studies were carried out on representative tissue samples collected during experimental IMI in mice. The mammary gland tissues and parenchymatous organs collected from euthanized mice were immediately fixed in 10% neutral-buffered formalin. After proper fixation, the samples from mammary gland and parenchymatous organs were processed by routine paraffin embedding technique. Sections of 4–5 µ thickness were cut using rotary microtome with disposable blades. These sections were then stained with routine hematoxylin and eosin method[11] and observed under bright field microscope (Nikon, Japan).

Statistical analysis

The data obtained were analyzed using Statistical Analysis System (SAS) software version 9.3 (SAS India limited, Mumbai, India)[12] using one-way analysis of variance method[13] and obtained the significant difference between different groups and time points. The results were expressed as the mean ± standard error (SE) with significant difference at $P < 0.05$ and confidence interval at 95% level.

Results

Clinical signs and mice mammary gland weight

The clinical signs of mastitis were not apparent at 6, 12, 24, 48, 72, and 96 h after intramammary inoculation of three CNS species and *S. aureus* in mice. The mean ± SE mammary gland weight and percentage of body weight at different time points after IMI is presented in Table 1. The mammary gland weight showed significant ($P < 0.05$) increase in *S. haemolyticus* and *S. aureus* after 6 h of IMI when compared to the PBS control. At 12 h, mammary gland weight of *S. haemolyticus* inoculated mice revealed significant ($P < 0.05$) decrease when compared to PBS control. The percentage of mammary gland to body weight showed significant ($P < 0.05$) increase in *S. aureus*-infected mice when compared to PBS control at 6 and 96 h after IMI. At 96 h, the *S. epidermidis* and *S. chromogenes* showed significant ($P < 0.05$) increase in percentage of mammary gland weight when compared to other groups.

Gross pathology

S. epidermidis, S. chromogenes, and *S. haemolyticus* inoculated mice mammary gland showed the development of inflammatory changes at 24 h, which progressed in severity by 48 and 72 h and gradually reduced at 96 h after IMI, and lesions observed were swelling and congestion of mammary glands and were firm to palpate when compared to PBS group. *S. aureus* group showed gross lesions at 12 h onward, and severity of lesions progressed up to 96 h. Upon incision thick, slightly discolored milk oozed out from the infected mice mammary glands. No appreciable gross lesions could be seen in liver, kidney, spleen, heart, lung, and inguinal lymph nodes in mice at different time points.

Histopathology

The mammary glands in mice inoculated with sterile PBS as a control did not reveal any histological changes at 6, 12, 24, 48, 72, and 96 h [Figure 1A(a-f)]. The microscopic appearance of the mammary gland was normal in which several lobules showed secretory acini and were distended with eosinophilic secretory material in the alveolar lumen. Mammary gland of mice infected with three CNS species and *S. aureus* showed no observable histopathological lesions at 6 h after IMI [Figure 1B(a), C(a) and D(a)] except congestion of blood vessels in *S. aureus*-infected mice. The alveoli showed normal architecture and hyperemia and congestion of blood vessels which was more pronounced in *S. aureus*-infected mice mammary gland tissues [Figure 1E(a)]. The *S. epidermidis* showed histopathological lesions at 12 h with mild changes [Figure 1B(b)] and were evident from 24 h after IMI in mice. The lesions at 24 h included thinning of alveolar wall and loss of connective tissue with enlarged lobules, moderate degree of hyperemia with perilobular and interlobular edema. The lumen of acini was filled with varying amount of eosinophilic secretory material along with desquamated epithelial cells [Figure 1B(c)]. At 48 h, moderate infiltration of inflammatory cells, mainly neutrophils in the interalveolar space and vacuolated epithelial cells [Figure 1B(d)]. The alveolar lumen showed denuded and desquamated epithelial cells and infiltration of inflammatory cells, mainly mononuclear cells at 72 h [Figure 1B(e)]. The mammary gland revealed proliferation of alveolar epithelial cells with an increase in alveolar structure and congestion of blood vessels at 96 h after IMI [Figure 1B(f)]. The *S. chromogenes* showed mild changes in the mammary gland tissue which included congestion of blood vessels at 12 h after IMI in mice [Figure 1C(b)]. At 24 h, mild infiltration of polymorphonuclear (PMN) cells [Figure 1C(c)] and progressed to severe infiltration of mononuclear cells in the interalveolar space at 48 h after IMI [Figure 1C(d)]. The alveoli coalesced to form large alveoli with loss of connective tissue was observed at 72 h after IMI [Figure 1C(e)]. The alveoli showed proliferation of epithelial cells and congestion of blood vessels in the interalveolar space with edema at 96 h after IMI [Figure 1C(f)]. Mice infected with *S. haemolyticus* showed severe congestion and edema in the interalveolar space at 12 h after IMI [Figure 1D(b)]. The alveoli coalesced to form dilated large alveoli with the formation of large alveolar lumen at 24 h after IMI [Figure 1D(c)]. The mammary gland revealed

Table 1: The mice mammary gland weight (g) and percentage of body weight after intramammary infection with three coagulase-negative staphylococcal species and *Staphylococcus aureus*

Time points	Mammary gland weight (g)					Mammary gland weight (percentage of body weight)				
	PBS control	*S. epidermidis*	*S. chromogenes*	*S. haemolyticus*	*S. aureus*	PBS control	*S. epidermidis*	*S. chromogenes*	*S. haemolyticus*	*S. aureus*
6 h	0.34±0.07^{c}	0.38±0.03^{b,c}	0.45±0.05^{b,c}	0.57±0.09^{a,b}	0.71±0.01^{a}	1.25±0.21^{b}	1.51±0.08^{b}	1.60±0.15^{b}	1.76±0.29^{a,b}	2.32±0.10^{a}
12 h	0.54±0.06^{a}	0.50±0.04^{a}	0.41±0.01^{a,b}	0.24±0.05^{b}	0.57±0.09^{a}	1.89±0.17^{a}	1.61±0.20^{a}	1.53±0.14^{a}	0.85±0.09^{b}	2.03±0.21^{a}
24 h	0.48±0.13	0.53±0.07	0.58±0.02	0.72±0.12	0.73±0.12	1.71±0.26	1.83±0.11	2.08±0.04	2.36±0.25	2.12±0.22
48 h	0.60±0.09	0.45±0.05	0.42±0.12	0.53±0.13	0.77±0.07	1.93±0.31	1.87±0.09	1.54±0.38	1.81±0.27	2.48±0.11
72 h	0.33±0.11	0.63±0.07	0.45±0.22	0.50±0.12	0.53±0.02	1.28±0.34	2.16±0.27	1.59±0.58	1.95±0.38	1.87±0.04
96 h	0.24±0.04^{b}	0.61±0.04^{a}	0.60±0.03^{a}	0.48±0.05^{a}	0.59±0.07^{a}	1.05±0.05^{c}	1.99±0.12^{a}	1.97±0.09^{a}	1.46±0.17^{b}	1.91±0.10^{a}
Mean	0.40±0.04	0.51±0.03	0.49±0.04	0.51±0.05	0.65±0.03	1.47±0.12	1.81±0.08	1.74±0.11	1.70±0.14	2.12±0.07
CI	0.32-0.48	0.45-0.57	0.41-0.57	0.41-0.61	0.59-0.71	1.24-1.70	1.65-1.97	1.53-1.95	1.43-1.97	1.98-2.26

Values expressed (g) as mean±SE. ^{a,b,c}Means with same superscript within the row do not differ significantly ($P>0.05$). CI: Confidence interval at 95 percent level, SE: Standard error, PBS: Phosphate buffered saline, *S. epidermidis*: *Staphylococcus epidermidis*, *S. chromogenes*: *Staphylococcus chromogenes*, *S. haemolyticus*: *Staphylococcus haemolyticus, S. aureus*: *Staphylococcus aureus*

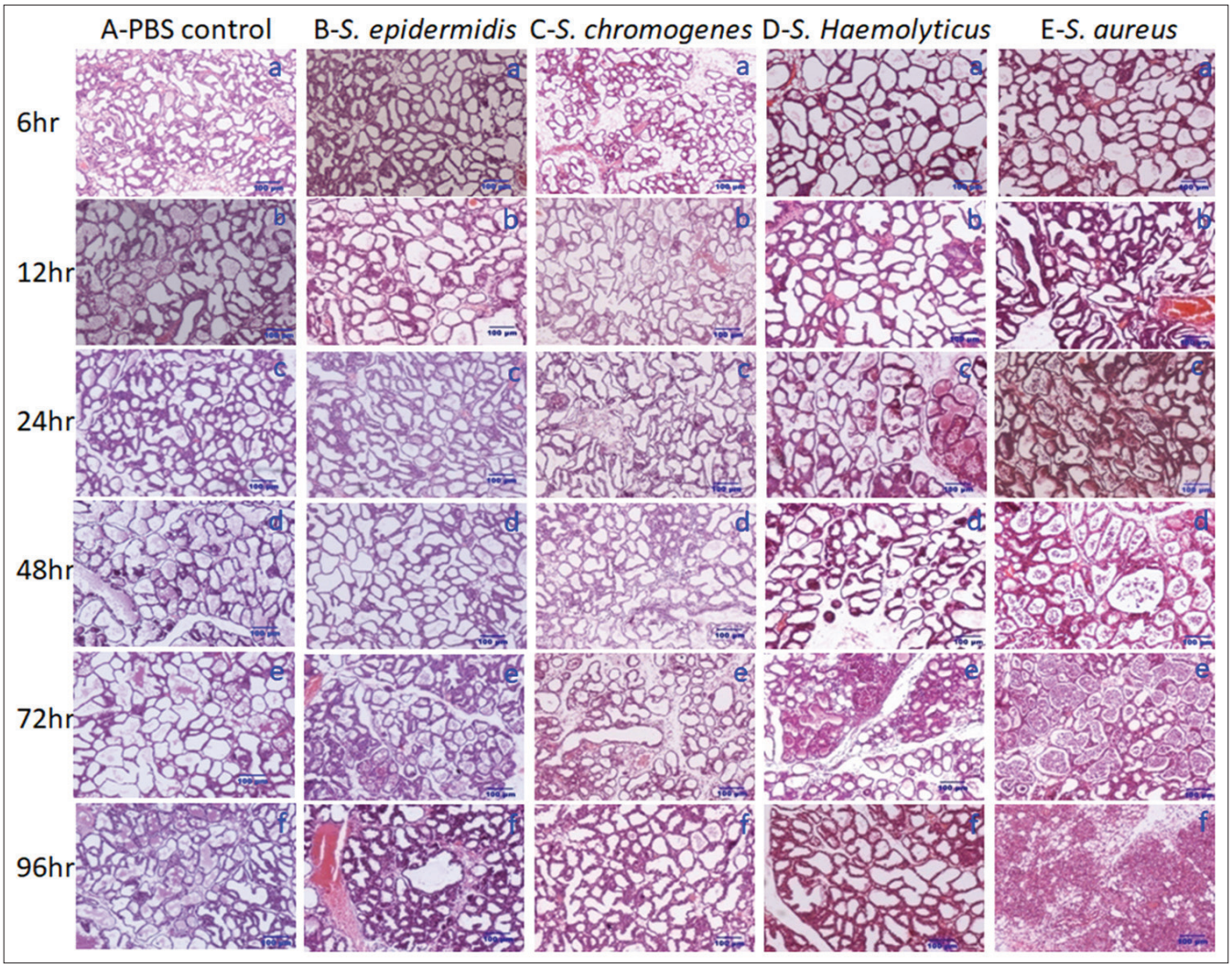

Figure 1: Histopathological changes observed at different time points in mice after intramammary infection with phosphate-buffered saline (A [a-f]), *Staphylococcus epidermidis* (B [a-f]), *Staphylococcus chromogenes* (C [a-f]), *Staphylococcus haemolyticus* (D [a-f]), and *Staphylococcus aureus* (E [a-f]), H and E, Scale bar = 100 μm

thinning of alveolar layer and infiltration of inflammatory cells in the interalveolar and perialveolar space at 48 h after IMI [Figure 1D(d)]. At 72 h after infection, loss of alveolar architecture with alveolar damage and severe infiltration of mononuclear cells were observed [Figure 1D(e)]. The severity of lesions progressed in time course manner and the lesions were more profound at 72 h after IMI in mice. However, the lesions reverted back to normal architecture with proliferation of alveolar epithelial cells at 96 h after IMI [Figure 1D(f)]. The *S. aureus*-infected mice at 12 h showed severe congestion of blood vessels with mild infiltration of neutrophils in the alveolar lumen and interalveolar and perialveolar spaces. The alveolar epithelial cells revealed mild degree of vacuolations with loss of cytoplasm [Figure 1E(b)]. The mammary glands showed increased alveolar lumen size due to flattening of epithelial cells and fusion of adjacent alveolar structures and moderate infiltration of inflammatory cells mainly neutrophils in the alveolar lumen at 24 h after IMI [Figure 1E(c)]. At 48 h, there was severe infiltration of inflammatory cells mainly of neutrophils containing bacteria and mononuclear cells in the alveolar lumen with vacuolar changes of alveolar epithelial cells [Figure 1E(d)]. The blood vessels showed congestion. The alveolar lumen size was increased with thinning of alveoli, contained inflammatory cells and cellular debris with loss of secretory activity. The mammary gland revealed severe infiltration of inflammatory cells occupying the entire alveolar lumen and loss of alveolar architecture. The alveolar epithelial cells showed vacuolar degeneration and loss of epithelial cells by necrosis at 72 h after IMI [Figure 1E(e)]. At 96 h, there was loss of architecture of alveoli, and hemorrhages were observed. The mammary gland tissue was filled with inflammatory cells mostly mononuclear cells and few fat cells were observed [Figure 1E(f)]. The kidney, spleen, lung, heart, and inguinal lymph nodes showed no observable histopathological changes after IMI with three CNS species and *S. aureus*. Liver of *S. aureus*-infected mice showed vacuolar degeneration, condensation of nuclei of hepatocytes, and congestion of blood vessels at 24 and 48 h after IMI.

Discussion

There were no clinical signs reported in mice infected with different isolates of *Staphylococcus* species in the present study. Leitner *et al*.[14] reported no mortality or morbidity in Swiss albino mice inoculated with *S. chromogenes* at the dose of 5×10^9 cfu/mice, which concurred with the present study. However, various researchers have reported occurrence of acute mastitis[15] and rise in body temperature, dullness, and depression at 24 h postinfection.[16] The absence of clinical signs in this study might possibly be due to low dose of organisms as compared to other reported studies in which the dose used was above 10^5 cfu. In the present study, the mammary gland weight showed significant increase in *S. haemolyticus* and *S. aureus* at 6 h after IMI. The increase in the mammary gland weight ranged from two to three folds when compared to the PBS control mice. The increased mammary gland weight may be due to the inflammatory processes, congestion and infiltration of inflammatory cells. Reid *et al*.[17] reported a significant increase in mammary gland weight at 12 h after inoculation with 10^{10} *S. aureus* by intramammary route in mice. In this study, the increase in mammary gland weight occurred at 6 h and not at 12 h as reported previously[18] which may be due to variation in the organism and the dose of inoculation. In the present study, the gross lesions were not observed in liver, kidney, spleen, lung, heart, and inguinal lymph nodes at different time points. However, Azeemulla[16] reported gross lesions restricted to liver, lung, and heart after IMI with 10^5, 10^6, and 10^7 cfu of two strains of *S. aureus* in rabbits. The reason for the same might be due to low dose of organisms (2×10^4) inoculated in mice. Thus, the dose and species of the organisms play an important role in the establishment of infection and generation of lesions in natural infections even in dairy cattle.

The severity of histopathological lesions varied between the three CNS species and *S. aureus* in mice model of mastitis in the present study. The histological changes observed in this study were supported by the gross lesions in the *S. aureus*-infected mice mammary gland. The results concurred with the previous report of Trinidad *et al*.,[19] who reported that quarters infected with CNS species in dairy cows, the histopathologic changes were not as marked as in quarter infected with *S. aureus* mastitis. However, Benites *et al*.[20] observed no difference in histopathologic changes between *S. aureus* and CNS species infected quarters in lactating dairy cows culled due to mastitis. The coagulase produced by *S. aureus* helps in conversion of fibrinogen to fibrin which forms a capsule and protects it from phagocytosis. Thus, the organism multiplies and causes the IMI. However, the three CNS species could not resist phagocytosis and eventually got cleared from mammary gland through phagocytosis by neutrophils at later stages after intramammary inoculation. The results of the present study clearly indicated that there is mammary tissue damage in the mice infected with three CNS species and *S. aureus*. This observation was further confirmed with the average bacterial viable count from the mouse milk reported earlier.[10] The severity of lesions and infiltration of neutrophils and mononuclear cells observed in the histopathology in *S. aureus*-infected mice was in agreement with the increased somatic cell counts at 96 h as reported earlier.[9] The mammary tissue damage has been shown to be induced by either apoptosis or necrosis, and both host and bacterial factors contribute to epithelial tissue damage. During infection of mammary glands, the tissue damage can be initially caused by bacteria and their products. Mastitis is characterized by an influx of somatic cells, primarily neutrophils into the mammary gland which leads to break down of blood-milk barrier; damage to the alveolar epithelium worsens. PMN neutrophils can harm the mammary tissue by releasing reactive oxygen intermediates and proteolytic enzymes.[21] The oxidative stress can damage all types of biomolecules such as DNA, proteins, lipids, and carbohydrates which perpetuates tissue injury. The PMN cells have primary, secondary, and tertiary granules which contain bactericidal peptides, proteins, and enzymes such as elastase, protease, and myeloperoxidases which are released into the extracellular environment and cause tissue destruction during mastitis.[22] The mammary tissue damage could also be caused by the proteinases and collagenolytic enzymes which degrade the extracellular matrix compounds.[23] The tissue damage was induced either by apoptosis or necrosis in the mammary epithelial cells indirectly through induction of proteases or pro-inflammatory cytokines.[21] Peptidoglycan fraction of cell wall of bacteria is involved in hypersensitivity reaction of the mammary gland. Further, Cucarella *et al*.[24] stated that pathogenesis of *S. aureus* is attributed to the combined effect of extracellular factors and toxins, together with the invasive properties of the organisms such as adherence, biofilm formation, and resistant to phagocytosis. *S. aureus* could trigger white blood cells and epithelial cells in the mammary gland to secrete cytokines which can bring about tissue damage by recruiting PMN cells that function as phagocytes at the site of infection. The cytokines also promote a wide variety of function of PMN cells including adhesion, surface receptor expression, free radical production, and release of lysosomal constituents.[22] The levels of cytokines increase during *S. aureus* mastitis could also induce apoptosis in the bovine mammary epithelial cells.[25] The kidney, spleen, lung, heart, and inguinal lymph nodes showed no observable histopathological changes after IMI with three CNS species and *S. aureus*. Liver of *S. aureus*-infected mice showed vacuolar degeneration, condensation of nuclei of hepatocytes, and congestion of blood vessels at 24 and 48 h after IMI. The lesions observed in the liver of mice infected with *S. aureus* could be due to the systemic spread of organisms and their exotoxins as well as the exopolysaccharide of bacterial cell wall and was in agreement with the previous report.[16] This also indicated the severity of the infection caused by *S. aureus* than the other three organisms in the mice.

Conclusions

In the present study, successfully induced mastitis using three coagulase-negative staphylococcal species and *S. aureus* in

mice. Histopathological changes in mammary gland indicated that the *S. aureus* caused severe mastitis when compared to three CNS species in mice. CNS species are less pathogenic when compared to *S. aureus* evident by histopathological changes, but still, it is considered as emerging mastitis pathogens and very important in subclinical mastitis in dairy cattle.

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Conflicts of interest

There are no conflicts of interest.

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