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Research Article

Journal of Veterinary and Animal Sciences
ISSN (Print): 0971-0701, (Online): 2582-0605
https://doi.org/10.51966/jvas.2025.56.1.93-102

Pathological changes and biofilm formation in bovine *Staphylococcus aureus* mastitis tissues[#]

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Citation: Sreepriya T. R., K. Kuppusamy, Nair S. R., K. S. Prasanna, I. S. Sajitha and R. Anoopraj. 2024. Pathological changes in mammary gland and its association with biofilm forming *Staphylococcus aureus* in mastitis milk of cattle. *J. Vet. Anim. Sci.* **56** (1):93-102

Received: 29.09.2024 Accepted: 05.12.2024 Published: 31.03.2025

Abstract

Mastitis, with its complex pathogenicity and multi-aetiological factors, is a major threat to the cattle population, causing major production losses. The present study was conducted in representative tissue samples from the udder, teat and tissue swabs taken from mammary glands of thirty-two cattle with red swollen udders brought to slaughterhouses and from animals brought for post-mortem examination. The tissues having prominent gross pathological changes, such as congestion, inflamed appearance and tissue thickening were selected for the study. The tissue swabs were subjected to microbial culture examination by streaking on Brain heart infusion agar (BHIA) initially and then on Mannitol salt agar (MSA). Of these, the colonies isolated from 41 tissue samples were mannitol fermenters and identified as *Staphylococcus aureus* (*S. aureus*). These colonies were preserved and subjected to genotypic confirmation by polymerase chain reaction (PCR) using 16SrRNA for genus identification and 23SrRNA for species identification. Histopathological examination of *S. aureus* infected tissues revealed various lesions. These included mononuclear inflammatory cell infiltration indicating a chronic infection, alveolar epithelial hyperplasia, cytoplasmic vacuolation, eosinophilic secretions within the lumens, degeneration of the alveolar epithelial membrane, presence of corpora amylacea in the alveoli and moderate to severe thickening of connective tissue. Histopathology grading showed that *S. aureus* infected tissues exhibited Grade 3 and Grade 2 inflammation, characterised by a significant infiltration of mononuclear cells, particularly lymphocytes. The 41 isolates were further subjected to PCR for screening for the presence of biofilm formation genes *icaA, icaD, bap* and *eno*. The percentage of isolates with the studied biofilm-forming genes were as follows- *icaA* (63.41 per cent), *icaD* (58.53 per cent), *eno* (70.73 per cent) and *bap* (12.2 per cent). The presence of *eno* gene was seen in most of the isolates while that of *bap* gene was the least. The alcian blue staining technique demonstrated the presence of biofilms in the infected tissue, revealing them as light blue filaments within the interstitial spaces of the mammary gland.

Keywords: Mastitis, *Staphylococcus aureus,* biofilm, *icaA, icaD, bap, eno*

Bovine mastitis has been a persistent concern among livestock farmers for ages. *Staphylococcus aureus (S. aureus)* is a ubiquitous pathogen which causes mammary gland infection in cattle. It is the most common gram-positive pathogen associated with both clinical and sub-clinical mastitis (Vasudevan *et al.,* 2003).

[#]*Part of MVSc thesis submitted to Kerala Veterinary and Animal Sciences University, Pookode, Wayanad, Kerala*

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The health of the mammary gland is crucial in determining an animal's production status, as mastitis often leads to significant tissue damage, both gross and microscopic, that impairs its function. Common changes, such as fibrosis, inflammation and swelling, as well as microscopic alterations like inflammatory cell infiltration and epithelial necrosis, are key contributors to production loss. (Trinidad *et al.,* 1990; Nandakumar, 1998;). Benites *et al.* (2002) also observed inflammatory and reparative responses in 44.6 per cent of mammary glands from slaughtered dairy cows, highlighting the importance of maintaining udder health to manage and prevent mastitis.

Staphylococcus aureus is a ubiquitous contagious pathogen which induces clinical and subclinical mastitis in cattle (Easaw *et al.,* 2021). This organism has the ability to produce biofilms that contribute to its antibiotic resistance (Taponen and Pyorola, 2009). According to (Donlan *et al.,* 2002), biofilms are clusters of surface-associated microbial colonies enclosed in a polymeric substance matrix. These biofilms enable the survival of organisms in turbulent environments, confer resistance to antibiotics and disinfectants, and aid evasion of phagocytosis (Melchior *et al.,* 2006). According to Abdelraheem *et al.* (2020), biofilm acts as an important mechanism to employ resistance against antibiotics. Previous records of the presence of biofilm formation genes such as intercellular adhesion (*icaA, icab, icaD*), gene encoding laminin binding protein (*eno*), biofilm-associated protein (*bap*) etc. have been reported from bovine mastitis milk isolates (Vasudevan *et al.,* 2003; Darwish and Asfour, 2013; Vijayakumar and Jose, 2021).

The presence of biofilm forming genes in *S. aureus* isolates obtained from mastitis affected bovine mammary tissue samples that can contribute to antimicrobial resistance has not been studied so far in Kerala. This study focuses on isolation and identification of *S. aureus* from bovine mastitis cases and further screening the isolates for biofilm formation genes *icaA, icaD, eno* and *bap*.

Materials and methods

Sample collection

Representative tissue samples and swabs that collected from all the quarters of 32 animals, exhibiting signs of inflammation in the mammary gland formed the coheart of the study. Samples were collected from all the quarters. Animals brought to slaughter houses in Thrissur and Thiruvananthapuram district and the Department of Veterinary Pathology for post mortem examination formed the units of study.

Histopathological study

The representative samples of udder tissues and teats were collected in 10 per cent neutral buffered formalin for histopathological study. Formalin-fixed, paraffin-embedded tissue sections of 4-5 µm thickness were subjected to Haematoxylin and Eosin staining (Suvarna *et al*., 2019). The histopathological grading of all the collected mammary glands and the teat tissues was done based on based on the inflammatory response (Restucci *et al.,* 2019), while the data from samples from which *S. aureus* was isolated has been used in this article. The obtained results were reported as the average number of inflammatory cells counted across 20 selected fields in each region. Four infiltration grades (G) were defined: G0 (absent, fewer than 20 inflammatory cells); G1 (low, 20–40 inflammatory cells); G2 (moderate, 40–60 inflammatory cells); and G3 (severe, more than 60 inflammatory cells).

To demonstrate the biofilm formation, a special staining technique using alcian blue was done in representative tissue samples (Harrington *et al.,* 2020).

Bacterial isolation

Isolation of bacteria was attempted by direct streaking of the tissue swabs collected from four quarters of 32 animals (128 swabs) onto brain heart infusion agar (BHIA) followed by incubation of the plates at 37°C for 24 h. Plates were examined after 12 and 24 hours of incubation. The isolates were identified based on morphology and cultural characteristics. The microscopic morphology of the colonies was examined after Gram's staining. Gram-positive bacteria were stained purple-blue colour and Gram-negative took up the pink colour of the counter stain. The gram-positive cocci were streaked on mannitol salt agar (MSA). The colony characteristics of the culture were observed after incubation at 37 ºC for 24-48 h. Most strains of coagulase-positive staphylococci produce yellow-colored colonies and others produce small red colonies in MSA.

Extraction of Bacterial DNA by Heat Lysis Method (snap chill method)

The DNA extraction was performed using a modified version of the snap-chill method as described by Junior *et al.* (2016). In summary, the culture isolates, presumptively identified as *S. aureus*, were cultured overnight in BHI broth and centrifuged at 10,000 rpm for 10 minutes at 4°C. The resulting bacterial pellets were resuspended in 200 µL of TE buffer (10 mM Tris-HCl, 1 mM EDTA) and boiled for 15 minutes. After boiling, the microfuge tubes were immediately placed in ice flakes for 30 minutes and then again centrifuged. The supernatant, containing the DNA, was transferred to a clean tube and stored at –20°C.

Polymerase Chain Reaction (PCR)

All the isolates presumptively identified as *S. aureus* obtained in the study were subjected to genotypic characterisation by amplification of 16SrRNA, and 23SrRNA genes for molecular confirmation of

Table 1. Details of primers used for genotypic characterisation of *S. aureus*

Organism	Genes	Primer sequence	Amplicon Size (bp)	Reference
Staphylococcus spp.	*16S rRNA*	F: AACTCTGTTATTAGGGAAGAA CA	756 bp	Ciftici *et al*. (2009)
		R: CCACCTTCCTCCGGT TTG TCA CC		
S. aureus	23S rRNA	F: GGACGACATTAGACGAATCA	1318 bp	El–Razik *et al*. (2010)
		R: CGGGCACCTATTTTCTATCT		

F: Forward primer, R: Reverse primer

Table 2. Details of primers used for genotypic characterisation of biofilmformation in *Staphylococcus aureus*

Biofilm formationfactors	Genes	Primer sequence	Amplicon size (bp)	References
Biofilm associated protein	*bap*	F: CCCTATATCGAAGGTGTAGAATTGCAC	971bp	Cucarella *et al*. (2001)
		R: GCTGTTGAAGTTAATACTGTACCTGC		
Intercellular adhesion	*icaA*	F: ACACTTGCTGGCGCAGTCAA	188 bp	Notcovich *et al.* (2018)
		R: TCTGGAACCAACATCCAACA		
Intercellular adhesion	*icaD*	F: AAACGTAAGAGAGGTGG	381 bp	Vasudevan *et al.* (2003)
		R: GGCAATATGATCAAGATAC		
Gene encoding laminin binding protein	*eno*	F: ACGTGCAGCAGCTGACT	301 bp	Kot *et al.* (2018)
		R: CAACAGCATTCTTCAGTACCTTC		

F: Forward primer, R: Reverse primer

Table 3. PCR protocols for the amplification of genes

Gene	Initial Denaturation	Denaturation	Annealing	Extension	Final Extension	Number of cycles
16SrRna	94°C, 5 min	94°C, 45 sec	60°C, 45 sec	72°C, 90 sec	72°C, 10 min	30
23SrRna	94°C, 5 min	94°C, 45 sec	58.6°C, 45 sec	72°C, 90 sec	72°C, 10min	30
icaA	95°C, 5 min	94°C, 1 min	57°C, 30 sec	72°C, 45 sec	72°C, 7 min	35
icaD	94°C, 5 min	92°C, 45 sec	49.4°C, 45 sec	72°C, 1 min	72°C, 7 min	30
eno	94°C, 5 min	94°C, 1 min	56°C, 1 min	72°C, 1 min	72°C, 10 min	35

After completion of PCR reaction, amplified products were subjected to submarine agarose gel electrophoresis and analysed on 1.2 per cent agarose gel stained with ethidium bromide, visualised under Ultraviolet transilluminator and photographed using Gel doc apparatus (Bio-Rad).

Staphylococcus spp. and *S. aureus* respectively. From this, the positive isolates were screened for the presence of four selected biofilm formation genes- *icaA, icaD, bap, and eno*. The reagents and chemicals used for the PCR were Emerald Amp GT PCR master mix (2X) (Takara Bio Inc) forward and reverse primer set (100nM/mL, Sigma Aldrich) and sterile nuclease-free water. The primers used for the study are mentioned in Table 1 and Table 2. All the primers were reconstituted in sterile nuclease-free water to a final concentration of 10 pmol/µL and stored at -20°C. The PCR was performed in 12.5 µL reaction volume using 200 µL PCR tubes (Table 3). The tubes containing the mixture were spun briefly and placed in the T100 Thermal Cycler (BIO-RAD, USA).

Results and discussion

Gross lesions

The gross pathological lesions observed in the mastitis quarters during the study included noticeably enlarged mammary gland tissues with areas of redness. On palpation, these tissues were firm and hard. Upon dissection, some glands exuded milk, while others, particularly in mastitis-affected animals with severe inflammation, released straw-coloured or clear fluid. In several cases, the supra-mammary lymph nodes were significantly enlarged, highlighting the severity of the inflammatory response in the affected areas (Fig. 1 to Fig. 4). Similar findings have been reported in other studies investigating the gross pathology of bovine mastitis in both cattle and buffaloes (Biju, 1996; Lalitha Kunjamma, 1976; Nandakumar, 1998; Restucci *et al.,* 2019).

Bacterial isolation and identification

Upon initial inoculation of swabs from 128 mastitic tissue quarters onto Brain Heart Infusion Agar (BHIA), the following results were obtained (Table 4).

Table 4- Details of bacterial isolation and identification

Method of identification	Characteristics	No. of isolates	Total no. of isolates
BHI agar culture	Isolates showing growth	110	128
	Isolates without growth	18	
Gram's staining	Gram positive cocci	102	110
	Gram negative bacilli	8	
Mannitol salt agar culture	Mannitol fermenters	41	102
	Mannitol non fermenters	71	

The yellow colonies on MSA were identified as *S. aureus* (Fig. 5) and DNA from those were subjected to PCR targeting 16SrRNA gene for the identification of *Staphylococcus spp*. All 41 isolates produced amplicons of 756 bp, confirming the presence of 16SrRNA (Fig. 6). These isolates were then subjected to *S. aureus* specific PCR targeting the 23SrRNA gene. All these isolates tested positive for 23SrRNA, producing a PCR product of 1318 bp (Fig. 7), and were thus genotypically confirmed as *S. aureus*. In this study, we recorded 23 cases affected with *S. aureus* mastitis out of the 32 cases included for sample collection. Similar results were observed in a study involving 22 cows, where 20 isolates of *S. aureus* were identified, with multiple quarter infections present within individual animals (Piccinini *et al.,* 2012). In another study by Hussain *et al.* (2012), *Staphylococcus spp*. was identified in 41 cases out of 100 animals brought for slaughter.

Histopathological lesions in S. aureus infected tissue

Lesions in bovine mammary tissue infected with *S. aureus* demonstrated a complex interplay of inflammatory and degenerative processes. The infiltration of inflammatory cells, notably lymphocytes, a few neutrophils (Fig. 8), and plasma cells (Fig. 9), underscored the chronicity of the infection, as these immune cells localise around ducts, alveoli, and blood vessels, occasionally extending into the lumens. These observations aligned with earlier findings by Trinidad *et al.* (1990), who reported similar inflammatory cell infiltration in heifers with *S. aureus* mastitis. Further studies, such as the mouse model experiment by Chinchali and Kaliwal(2014), revealed consistent patterns of leukocyte infiltration, epithelial damage, and secretions within the alveoli. As the severity of the infection increased, the immune response intensified, correlating with increased leukocyte infiltration and epithelial hyperplasia (Fig. 10), which indicated tissue remodelling efforts. The degenerative changes observed, such as cytoplasmic vacuolation of alveolar epithelial cells (Fig. 11), basement membrane disruption, and the presence of corpora amylacea (Fig. 12), highlighted the chronicity of the infection. Additionally, hyperaemia, haemorrhage (Fig. 13), and in severe cases, keratinisation of the teat epithelium (Fig. 14) further reflected the sustained irritation and injury. The findings of Gogoi-Tiwari *et al.* (2017) on *S. aureus*-induced lesions in a mice model mirrored those observed in bovine cases, demonstrating the similarity in immune responses across species. The spectrum of inflammatory severity among bovine samples, ranging from mild to severe (Grades 1-3), correlated with the duration of infection and extent of tissue damage, emphasising the role of both immune and bacterial factors in shaping the disease course.

Histopathological grading

Six out of the twelve cases with *S. aureus* infection alone had high infiltration of mononuclear cells resulting in Grade 3 inflammation (Fig. 15). The infiltration grade was similar in each quarter of the individual mammary gland. Five animals, had a moderate infiltration of inflammatory cells, indicating a Grade 2 inflammation (Fig.16), while one animal had minimal or non-severe infiltration of inflammatory cells with Grade 1 inflammation (Fig. 17). The details of the histopathology grades are given in the table below (Table-5). Similar studies done previously recorded Grade 3 inflammation in all the *S. aureus* affected mammary tissues (Chinchali and Kaliwal, 2014; Gogoi-Tiwari *et al.,* 2017).

Table 5. Details of histopathology grades of *S. aureus* infected tissues

Sl. No.	**Organisms**	**No. of cases with Grade 1 inflammation**	**No. of cases with Grade 2 inflammation**	**No. of cases with Grade 3 inflammation**	**Total number of cases**
1	*Staphylococcus aureus* alone from all four quarters	1	5	6	12
2	Mixed *S. aureus* & other *Staphylococcus spp.* from multiple quarters of single animal	1	3	7	11

Biofilm formation

The template DNA from all 41 *S. aureus* isolates was subjected to genotypic characterisation for biofilm formation genes, including *icaD, icaA, eno* and *bap*, using PCR. Out of the 41 *S. aureus* isolates, 26 (63.41 per cent) contained the intercellular adhesion gene *icaA*, producing amplicons of 188 bp (Fig. 18). Similar results were obtained in a study by Vijayakumar and Jose (2021), where there was 63.63 per cent *icaA* genes in *S. aureus* positive isolates from bovine mastitis milk sample. Similarly, a meta-analysis investigating the frequency of adhesion and biofilm-related genes in *Staphylococcus aureus* isolates from bovine mastitis milk reported that the prevalence of the *icaA* gene was 69.4 per cent, indicating its widespread occurrence among these isolates (Sharifi *et al.,* 2024). Twenty-four (58.53 per cent) *S. aureus* isolates were detected with amplicons of 381 bp for the intercellular adhesion *icaD* gene (Fig. 19). Suvajdžić *et al.* (2017) also reported a similar finding, with 65.9 per cent of *S. aureus* isolates testing positive for the *icaD* gene. Among the 41 *S. aureus* isolates, 29 isolates (70.73 per cent) tested positive for the gene encoding laminin-binding protein (*eno*), which resulted in amplicons of 301 bp (Fig. 20). *Staphylococcus aureus* from at least one quarter of all the 23 animals had the presence of *eno* gene. Similar results were obtained in the study conducted by Darwish and Asfour (2013), where *eno* genes were observed in 75 per cent of *S. aureus* isolates from bovine mastitis milk. In a study by Singh *et al.* (2023), the prevalence of the *eno* gene in *S. aureus-positive* bovine mastitis isolates was reported to be 82.6 per cent. The biofilm associated protein (*bap)* gene was detected in five (12.2 per cent) isolates of *S. aureus* with an amplicon size of 971 bp (Fig. 21). A study by Felipe *et al.* (2017) reported that the *bap* gene was present in 11 per cent of biofilm-forming *S. aureus* isolates. The *bap* gene, which encodes the biofilm-associated protein, was not uniformly distributed among *S. aureus* isolates. Studies indicate that its presence was generally limited to a minority of strains, particularly those isolated from bovine mastitis in certain geographical areas (Vautor *et al.,* 2008). One key reason for its low prevalence was phase-variable expression, where *bap* expression could be switched off due to regulatory changes without altering the gene itself, leading to a temporary loss of function (Tormo *et al.,* 2007). Additionally, extracellular proteases in *S. aureus*, which degrade surface proteins including *bap*, could inhibit biofilm formation and contribute to the reduced functional effects of the bap gene in some strains (Martí *et al.,* 2010). Furthermore, the presence of *bap* could interfere with the functions of other adhesins, such as MSCRAMMs (microbial surface components recognising adhesive matrix molecules), potentially reducing bacterial adherence and initial colonisation, which might be a selective disadvantage in certain environments (Cucarella *et al.,* 2002). The details of the presence of biofilm-forming genes in *S. aureus* isolates are given in the following table (Table 6).

Biofilm demonstration in tissues

Representative histological sections of tissue quarters positive for biofilm-forming genes were stained using the alcian blue technique, which is capable of staining the mucopolysaccharide components of biofilm. The stained tissues revealed biofilms as filamentous sheets or film-like structures adhered to the connective tissue and epithelium (Fig. 22, Fig. 23). In cases with extensive biofilm formation, the filamentous biofilms were also observed within the interstitium of the mammary glands (Fig. 24). These findings were consistent with a study by Harrington *et al.* (2020), which used the same staining method to identify biofilm in pig lung bronchiolar tissue. This study recorded that biofilm formation was observed in cases with higher grades of inflammation and extensive tissue damage which would have resulted in loss of tissue function. Moreover, the biofilm formation also indicates the persistence of infection resisting the action of antibiotics. Similarly, Gogoi-Tiwari *et al.* (2017) observed that *S. aureus* strains with strong biofilm production led to severe mammary tissue damage, marked by Grade 3 histopathological changes, as observed in the present study.

Conclusion

This study concluded that *S. aureus* infections in the bovine mammary gland could lead to severe gross and microscopic pathological lesions. Histopathological grading revealed that tissues infected by *S. aureus* displayed mostly Grade 3 and Grade 2 inflammation, characterised by a high infiltration of mononuclear inflammatory cells, particularly lymphocytes, indicating a predominantly chronic reaction rather than an acute one. Additionally, the study identified *S. aureus*'s biofilm-forming ability in tissue samples, a feature previously demonstrated mainly in milk samples, with the *eno* gene being the most frequently observed. The presence of biofilms in infected tissues was effectively visualised using the alcian blue

Table 6. Details of the presence of biofilm-forming genes in *S. aureus* isolated from tissue samples

Gene (a)	No. of isolates from tissue samples showing biofilm-forming genes (n=41isolates) (b)	Percentage (c) (c=(b*100/41)
icaA	26	63.41 %
icaD	24	58.53 %
bap	5	12.19%
eno	29	70.73%

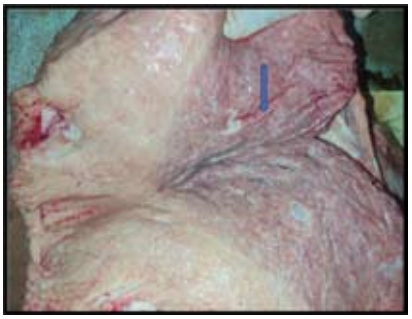

Fig. 1: Mammary gland- Congestion in forequarters (blue arrow)

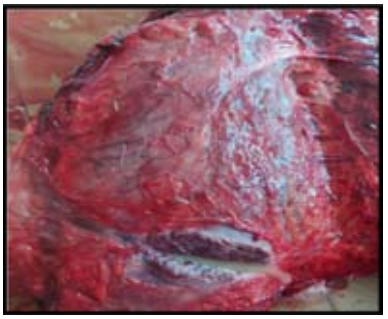

Fig. 2: Mammary gland- Hyperaemic and swollen mammary gland with oozing out of milk (blue arrow)

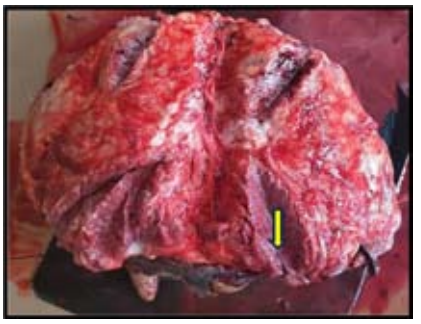

Fig. 3: Mammary gland- Hyperaemic and swollen mammary gland with oozing out of transudate (blue arrow)

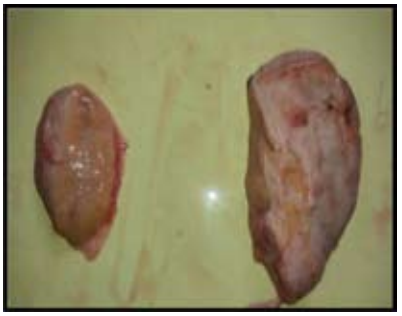

Fig. 4: Lymph node-Enlarged & edematous right supramammary lymph node and normal left node

Fig. 5: Growth of *S. aureus* in MSA plate as yellow colonies

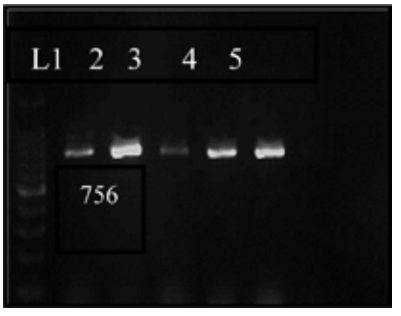

Fig. 6: Agarose gel electrophoresis of 16srRNA specific PCR *Staphylococcus* spp. (756 bp)

Lane 1 → L1 – 100 bp ladder
Lane 2 → Positive control
Lane 3 to Lane 6 → Positive samples
Lane 7 → Negative control

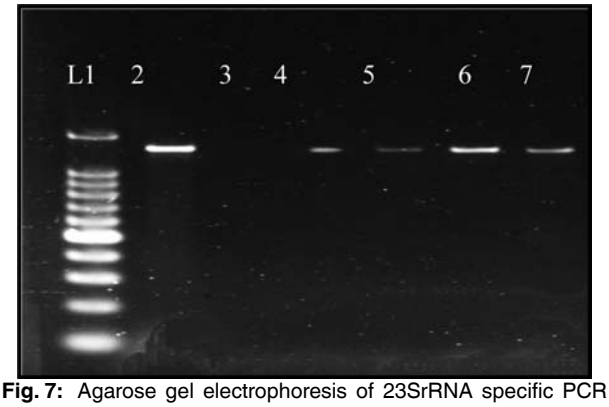

Fig. 7: Agarose gel electrophoresis of 23SrRNA specific PCR *Staphylococcus aureus* (1318 bp)

Lane 1 → L1 – 100 bp ladder
Lane 2 → Positive control
Lane 3 → Negative control
Lane 4 to Lane 7 → Positive samples

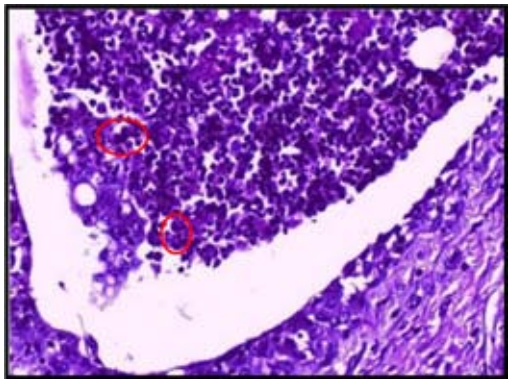

Fig. 8: Mammary gland- Infiltration of lymphocytes and neutrophils (red circle)- H&E 400x

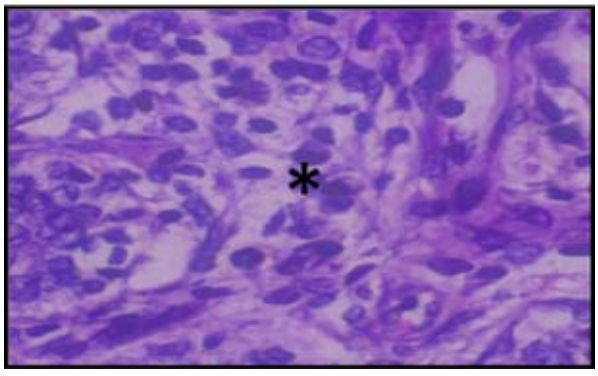

Fig. 9: Mammary gland- Infiltration of lymphocytes and plasma cells (asterisk)- H&E 400x

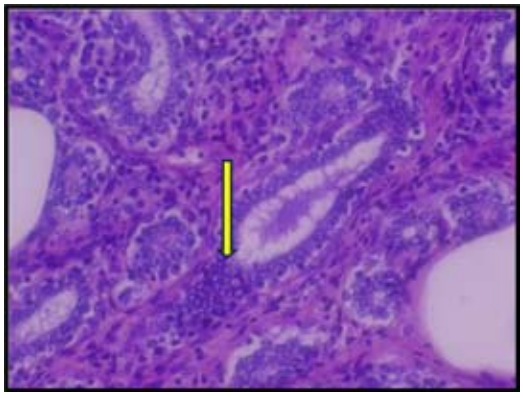

Fig. 10: Mammary gland- Interlobular ductal epithelial cell hyperplasia (yellow arrow) - H&E 400x

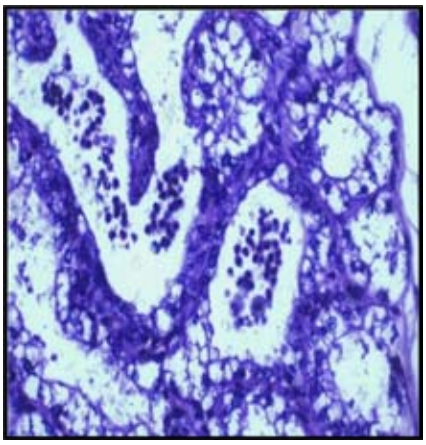

Fig. 11: Mammary gland- Infiltration of lymphocytes into alveolar and ductal lumen- H&E 400x

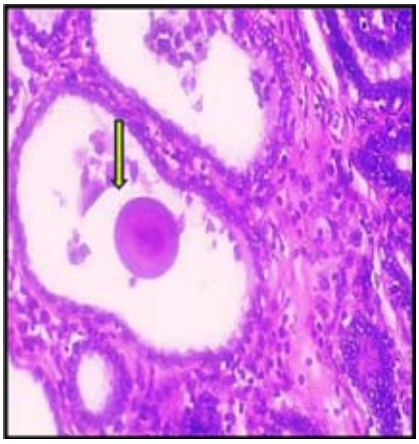

Fig. 12: Mammary gland- Eosinophilic corpora amylacea inside ductal lumen (yellow arrow)- H&E 400x

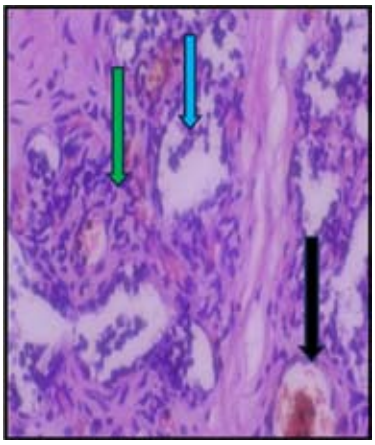

Fig. 13: Mammary gland- Congestion (black arrow), haemorrhage (green arrow)- infiltration of lymphocytes into alveoli, ducts & surrounding stroma (blue arrow)- H&E 400x

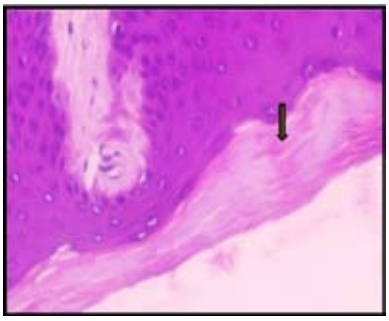

Fig. 14: Teat- Hyper keratinised teat epithelium (yellow arrow)- H&E 400x

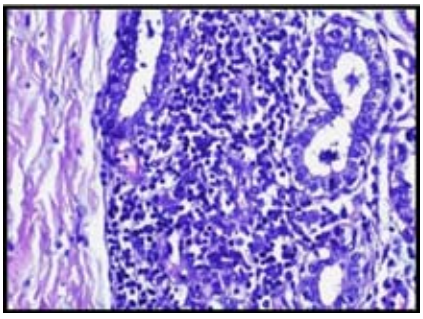

Fig. 15: Mammary gland- Grade 3 inflammation with severe infiltration into the interalveolar space – H&E 400x

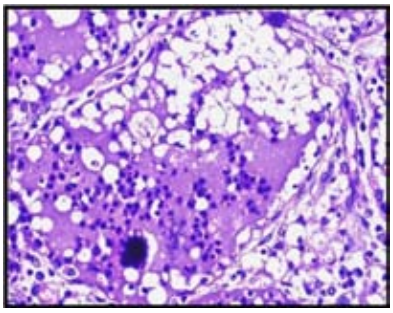

Fig. 16: Mammary gland- Grade 2 inflammation with moderate infiltration of inflammatory cells into the ductal lumen - H&E 400x

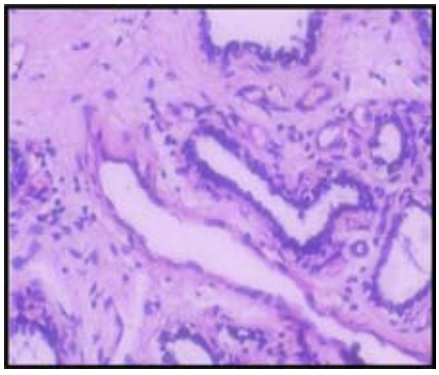

Fig. 17: Mammary gland- Grade 1 inflammation with minimum infiltration of inflammatory cells into the interstitium - H&E 400x

Fig. 19: Agarose geL electrophoresis of *icaD* specific PCR for intracellular adhesion (381 bp)

Lane 1 → L1 – 100 bp ladder
Lane 2 → Negative control
Lane 3 → Positive control
Lane 4 to Lane 6 → Positive samples

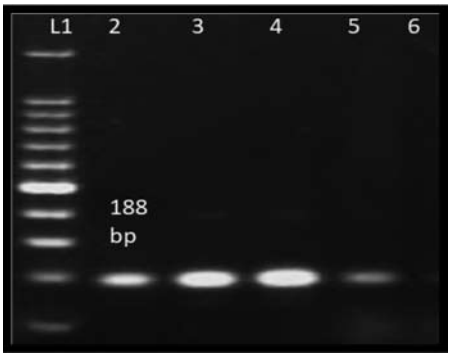

Fig. 18: Agarose gel electrophoresis of *icaA* specific PCR for intracellular adhesion (188 bp)

Lane 1 → L1 – 100 bp ladder
Lane 2 → Positive control
Lane 3to Lane 5 → Positive samples
Lane 6 → Negative control

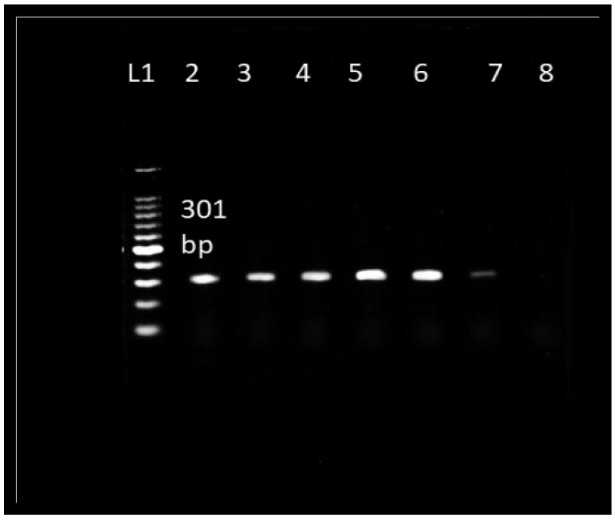

Fig. 20: Agarose gel electrophoresis of *eno specific* PCR for enolase encoding laminin binding protein (301 bp)
Lane 1 → L1 – 100 bp ladder
Lane 2 → Positive control
Lane 3 to Lane 7 → Positive samples
Lane 8 → Negative control

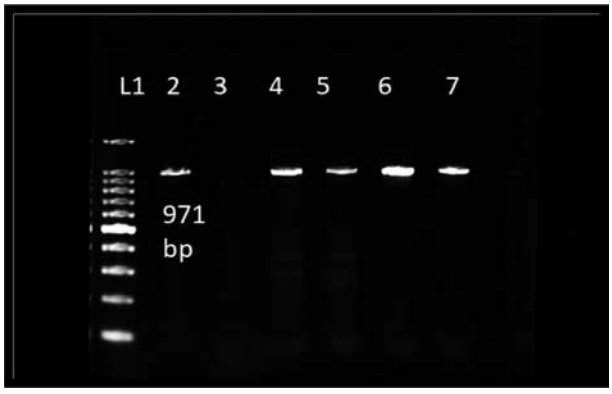

Fig. 21: Agarose gel electrophoresis of *bap* specific PCR for biofilm associated protein (971 bp)
Lane 1 → L1 – 100 bp ladder
Lane 2 → Positive control
Lane 3 → Negative control
Lane 4 to Lane 7 → Positive samples
Lane 8 → Negative samples

staining technique, where biofilms appeared as light bluish filaments within the mammary gland interstitium. Thus, it can be concluded that understanding biofilm formation in *S. aureus* associated with bovine mastitis is crucial, as it is a primary cause of antibiotic resistance and treatment failures leading to persistent infections. From this study, it could be identified that the production loss resulted from chronic infections including mastitis resulted in the culling of the animals. These findings can be utilised to develop and implement strategies that disrupt *S. aureus* biofilm formation through proper management practices which is necessary to eliminate the persistence of infections in herds.

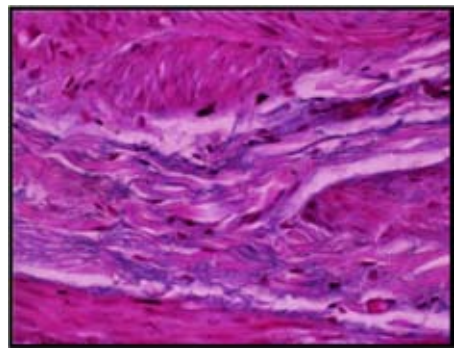

Fig. 22: Mammary gland- Presence of moderate biofilm formation in between connective tissue as filamentous structures- *S. aureus* infected tissue sample - Alcian blue stain 400x

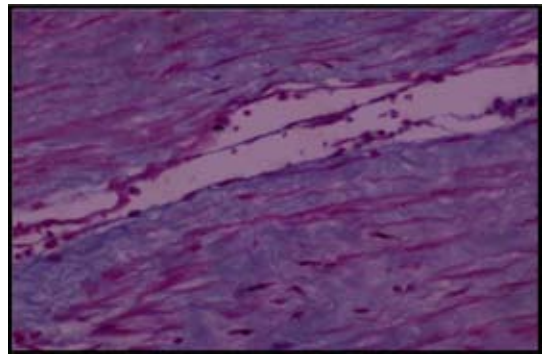

Fig. 23: Mammary gland- Presence of strong biofilm formation in between connective tissue as filamentous structures- *S. aureus* infected tissue sample - Alcian blue stain 400x

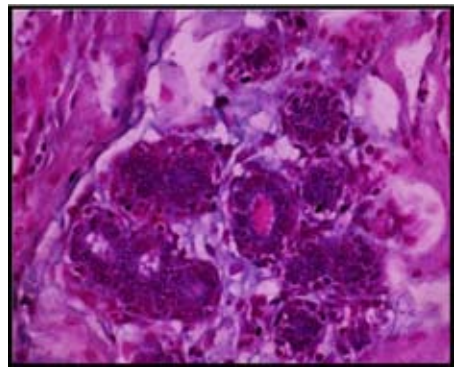

Fig. 24: Mammary gland- Presence of strong biofilm formation filamentous structures in inter-alveolar interstitium- *S. aureus* infected tissue sample - Alcian blue stain 400x

Acknowledgements

The authors are thankful to the Dean, College of Veterinary and Animal Sciences, Mannuthy for providing the facilities necessary to carry out the study.

Conflicts of interest

The authors declare that they have no conflict of interest.

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