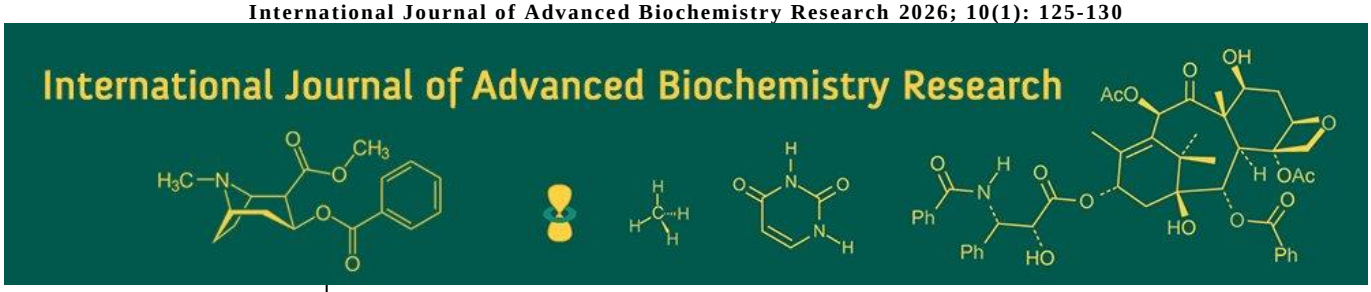

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MRSA and MRCNS: Identification and risk-associated factors in Caprine mastitis

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Abstract
Mastitis is a multi-aetiological disease that causes production losses in goats and is characterized by physical, chemical, pathological, and microbiological alterations in milk and glandular tissue. Total 76 bacterial isolates from mastitic milk were identified based on morphological, cultural, and biochemical characteristics. *Staphylococcus* spp. (39.47%) was the predominant organism (having 33.33% of *S. aureus* and 66.67% of CNS). By using PCR, 33.33% (10/30) of the culturally positive Staphylococci isolates were identified as *S. aureus*. Out of 30 isolates of *Staphylococcus* spp., 80% (24) isolates exhibited biofilm formation by the CRA method. All *S. aureus* (100%) isolates and 70% (14) CNS isolates exhibited biofilm formation by the CRA method.

Keywords: CNS, *Staphylococcus*, subclinical mastitis, biofilm

Introduction
Goats and Sheep are two important livestock species in the Livestock Production System. A large part of the rural landless population and marginal farmers are dependent on them. Goat milk is cheap, wholesome, easily digestible, and nutritious (Panicker, 2015) [34]. After Dairy cattle, Sheep and goats are the most important group of milk-producing animals in both temperate and tropical regions (Devendra and Coop, 1982) [15]. The data by the Food and Agriculture Organization (FAO) of the United Nations estimated the total goat population in 2023 in the world to 1.1 billion, of which India had an estimated 154 million. India accounted for the largest population of Goats in the world, with 14.3% of the world's population (FAO, 2023) [16].
Inflammation of the mammary gland (udder), causing chemical and physical changes in milk produced by goats, is called mastitis (Radostits *et al.*, 2000) [35]. Mastitis causes economic losses in the form of disposal of milk, treatment costs, and reduced milk efficiency in the following lactations. Mastitis is more frequent in goats due to intensive and semi-intensive management practices. It appears in two forms: sub-clinical and clinical, and may be acute or chronic.
Bacterial Etiological agents causing mastitis in goats are: *Staphylococcus aureus*, coagulase-negative *Staphylococci* (CNS), *Escherichia coli*, *Pseudomonas* spp., *Bacillus* spp., *Klebsiella pneumoniae*, *Streptococcus* spp., *Mycoplasma* spp., Methicillin-resistant *S. aureus* (MRSA), *Coliforms*, *Micrococci*, *Corynebacteria*, *Pasteurella* spp., and *Actinomyces* spp. have been reported by Manser (1986) [26]; Kalogridou-Vassiliadou (1991) [23]; Deinhofer (1995) [14]; Mishra *et al.* (1996) [31]; White & Hinckley (1999) [37]; Bergonier *et al.* (2003) [7]; da Silva *et al.* (2004) [12]; Amin *et al.* (2011) [6]; Marogna *et al.* (2012) [28]; Cortimiglia *et al.* (2015) [10]; Kumar *et al.* (2016) [25] and Gabli *et al.* (2019) [18].
Diagnosis of mastitis is carried out based on clinical signs and the history of the herd. Somatic Cell Count (SCC) and California Mastitis Test (CMT) are the most common tests used to diagnose sub-clinical mastitis in dairy goats (Raikwar and Shukla, 2015) [36].

Materials and Methods
A total of 82 milk samples were aseptically collected, out of which 30 samples were from clinical cases and 52 samples were collected for screening of subclinical mastitis (between January, 2021 and July, 2021).

Cultural examination and morphological characteristics of the bacterial isolates:
Milk samples were opened under aseptic conditions and streaked on 5 percent sheep blood agar (BA) using the sterile platinum loop for initial bacterial isolation, and the plates were incubated at 37 °C for 24-48 hours. The plates were checked for bacterial growth after incubation, and the morphological properties of bacterial colonies were reported. Each isolate's pure culture was determined based on morphological, cultural, and biochemical traits.
The smear was formed from an isolated bacterial colony on a clean, grease-free microscopic slide, stained with Gram's staining, and inspected under a microscope for bacterial isolates. Gram-positive cocci grouped irregularly in clusters like bunches of grapes were suspected of being staphylococci (oxidase negative).

Biochemical Characterization of Bacterial Isolates:
The bacterial colonies were identified using the methods described by Cowan *et al.* (2003) [11] and Markey *et al.* (2013) [27].

a) Potassium Hydroxide (KOH) String test
On a clean microscopic slide, a loopful of culture was taken from the blood agar and combined with an equivalent amount of 3 percent potassium hydroxide (KOH). The loop was lifted at many intervals after full mixing to test if the gel was developing. A viscous gel formed in 60 seconds if the bacteria were Gram-negative, but no gel formed in 60 seconds if the bacterium was Gram-positive.

b) Catalase test
A drop of 3 percent hydrogen peroxide was introduced to a loopful of the bacterial colony on a clean microscope slide. A positive reaction was indicated by the generation of the effervescence of oxygen gas within a few seconds.

c) Oxidase test
The test was carried out using standard Oxidase discs (HiMedia). The disc surface was rubbed with a single colony using the platinum loop. The rapid development of blue color in less than 10 seconds was regarded as quite positive.

d) Pigment production on Nutrient agar (NA)
Staphylococcus spp. Pigment synthesis was detected by inoculating them on NA and incubating them at 37 °C for 24 hours. Yellow colonies were regarded as pigment production in staphylococci isolates, while white colonies were regarded as non-pigment production.

e) Detection of Hemolysin activity of Staphylococci and Streptococci on SBA
Staphylococci were grown on 5% defibrinated SBA to detect hemolytic activity. The isolates were streaked on the agar and incubated for 24 hours at 37 °C. Staphylococci were classified as α-hemolytic (complete hemolysis), β-hemolytic (incomplete hemolysis/hot-cold lysis), and γ-hemolytic (no hemolysis).

f) Mannitol Salt Agar (MSA)
Gram-positive cocci isolates were inoculated on MSA and incubated for 24 hours at 37 °C. Mannitol fermentation was defined as the change in color of the media from red to yellow, while mannitol non-fermentation was defined as the change in color from red to pink.

g) Oxidase-Fermentation (O-F) test
The organisms were inoculated in two tubes of O-F basal medium by stabbing with a straight wire. One tube was covered with a layer of soft paraffin. The tubes were incubated at 37 °C for up to 14 days. The results were noted daily.

Biofilm Production by Bacterial Isolates using Congo Red Agar Method:
In Brain Heart Infusion (BHI) agar (HI Media Pvt. Ltd.), sucrose 7 g/l, bacteriological agar powder (HI Media Pvt. Ltd.) 10 g/l, and Congo red 0.8 g/l were used to make the medium. Congo red stain was made as a concentrated aqueous solution that was autoclaved (121 °C for 15 minutes) separately from the other medium constituents and then added when the agar had cooled to 55 °C, as described by Freeman *et al.* (1989) [17]. The medium was inoculated and incubated aerobically at 37 °C for 24 hours. Black colonies with a dry crystalline consistency indicated a positive result. Non-biofilm producers remained pink for the most part; however, there was some darkening in the center of the colonies that gave the appearance of a bull's eye.

PCR-based Identification
Reference Strains
The DNA of standard strains of *S. aureus* (ATCC 6538) of the Department of Veterinary Pharmacology and Toxicology Stored at the Department of Veterinary Microbiology, AAU, was used for the standardization of PCR.

Table 1: Details of primers used for amplification of target genes for the identification of *Staphylococcus* spp. in the PCR reaction:

Name of the target organism	Primer Sequence (5'-3')		Size of amplified products (bp)	References
S. aureus	Sau F	TCAACGATATTCTTCACGACTAA	179	Gillespie & Oliver (2005) [20]
	Sau R	CCAGCTTCGGTACTACTAAAG		
Staphylococcus spp.	*coa* F	ATAGAGATGCTGGTACAGG	polymorphism	Hookey *et al.* (1998) [21]
	coa R	CTTCCGATTGTTCGATGC		
Staphylococcus spp.	*mecA*-F	AAAATCGATGGTAAAGGTTGGC	533	Hsueh *et al.* (1996) [22]
	mecA-R	AGTTCTGCAGTACCGGATTTGC		

Table 2: Steps and conditions of thermal cycling for different primers in PCR:

Primers (forward and Reverse)	Cycling Conditions				
	Initial denaturation	Denaturation	Annealing	Extension	Final Extension
Sau F Sau R (*S. aureus*)	95 °C 3 min	95 °C 15 sec	57 °C 45 sec	72 °C 30 sec	72 °C 10 min
		Repeated for 30 cycles			
*coa*F *coa*R (*Staphylococcus* spp.)	94 °C 45 sec	94 °C 20 sec	57 °C 15 sec	70 °C 15 sec	72 °C 2 min
		Repeated for 30 cycles			
mecA-F *mecA*-R (*Staphylococcus* spp.)	94 °C 5 min	94 °C 30 sec	55 °C 30 sec	72 °C 60 sec	72 °C 5 min
		Repeated for 40 cycles			

Results

Out of the total of 76 cultural isolates, 47.37% (36) were found to be Gram-positive cocci arranged in clusters. Out of which, 83.33% (30/36) isolates were positive for *Staphylococcus* spp. (11 isolates from CM and 19 from SCM) were catalase positive, oxidase negative, and fermentative reaction by O-F test.

Staphylococci were identified as isolates with white to golden yellow color colonies on blood agar and NA (Fig. 1 to 3), Gram-positive cocci in a bunch of grapes-shaped clusters (Fig. 4), negative KOH test, catalase positive, oxidase negative, a fermentative reaction by O-F test, and failure to grow on MacConkey agar. Based on the above characters, 39.47% (30/76) isolates were found positive for staphylococci.

In the present study, 63.33% (19/30) of staphylococci isolates showed fermentation on MSA, while 36.67% (11/30) were mannitol non-fermentative. Around 63.33% (19/30) of staphylococci were reported to produce pigment, while 36.67% (11/30) isolates developed white colonies on NA.

On 5% SBA, 16.67% (5/30) Staphylococci isolates produced α-haemolysin (complete haemolytic), 33.33% (10/30) isolates produced β-haemolysin (partial haemolytic), and 50.00% (15/30) isolates were non-haemolytic.

In this investigation, all the 30 culturally positive Staphylococci isolates were tested for identification of *S. aureus*. Among the isolates, 33.33% (10/30) were positive for *S. aureus*, which generated an expected amplification product of 179bp by PCR, indicating that they were *S. aureus*. Around 6.66% (2/30) isolates from clinical mastitis cases revealed an amplified 850 bp product of the coa gene, and the remaining 28 isolates were considered as CNS. Two isolates from subclinical and one isolate from clinical mastitis, 10% (3/30) cases revealed an amplified 533 bp product of the *mecA* gene (Fig. 5 & 6).

In the study, out of 30 isolates of *Staphylococcus* spp., 80% (24/30) (100% (10/10) *S. aureus* and 70% (14/20) CNS) isolates exhibited biofilm formation by the CRA method. From clinical mastitis, 90.91% (10/11) and 73.68% (14/19) from subclinical mastitis exhibited biofilm formation (Fig. 7 & 8. A higher percentage of biofilm formation in clinical mastitis can be accounted for by severe clinical conditions.

Discussion & Conclusion

Two isolates from subclinical and one isolate from clinical mastitis, 10% (3/30) cases revealed an amplified 533 bp product of the *mecA* gene. PCR-based identification of the *mecA* gene using the same primers used in this study was carried out by Bhanderi *et al.* (2016) [8], Altaf (2019) [5], Ghodasara *et al.* (2019) [19] and Ahasan *et al.* (2020) [2].

MRSA is a ubiquitous pathogen with zoonotic importance. It causes severe conditions in humans like necrotizing pneumonia, necrotizing fasciitis, severe sepsis, and Waterhouse-Friderichsen syndrome (Boucher *et al.*, 2010) [9]. MRSA is present in goats, which might constitute a risk of transmission of MRSA to humans and other animals. MRSA was shown to be responsible for more than half of all suppurative skin infections among patients who reported to emergency departments (EDs) in 11 US metropolitan areas (Moran *et al.*, 2006) [32].

In the present study, out of 30 isolates of *Staphylococcus* spp., 80% (24/30) (100% (10/10) *S. aureus* and 70% (14/20) CNS) isolates exhibited biofilm formation by the CRA method. From clinical mastitis, 90.91% (10/11) and 73.68% (14/19) from subclinical mastitis exhibited biofilm formation. Various researchers like Darwish & Asfour (2013) [13], 70.37%, Melo *et al.* (2013) [29], 80%, Al-iedani (2016) [3], 80.6%, Almeida *et al.* (2017) [4], 100%, Thiran *et al.* (2018), 29.17%, de L. Silva *et al.* (2012) [4], 8.8%, Achek *et al.* (2020), 41.8%, Kotzamanidis *et al.* (2021) [24] 100% and Michael *et al.* (2021) [30] 61.9% also reported biofilm formation by *Staphylococcus* spp.

Biofilm is a slime matrix made up of exopolysaccharides that surrounds numerous layers of cells. Biofilm formation is a key virulence component that contributes to the chronicity of many illnesses. It is also responsible for the growth of multidrug-resistant bacteria, which can lead to treatment failure. The ability of Staphylococci to form biofilms is one of the virulence factors that facilitate Staphylococci adhesion and colonization on the mammary gland epithelium, as well as contributing to pathogen evasion and the difficulty of pathogen eradication, resulting in recurrent or persistent infections (Oliveira *et al.*, 2006; Melo *et al.*, 2013) [33, 29].

Fig 1: Yellow colored colony on NA by *S. aureus*

Fig 2: Yellow colored colony on BA by S. aureus

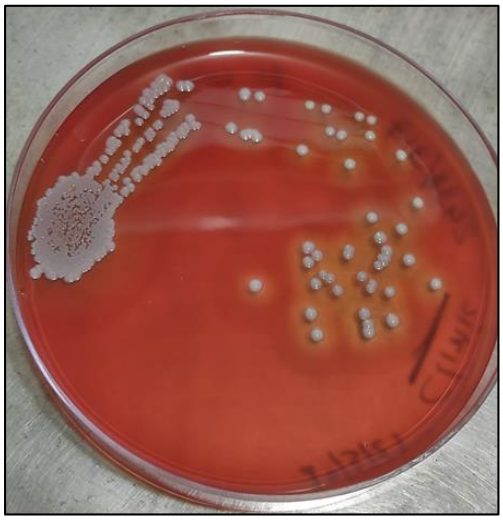

Fig 3: White colored colony on BA by *S. aureus*

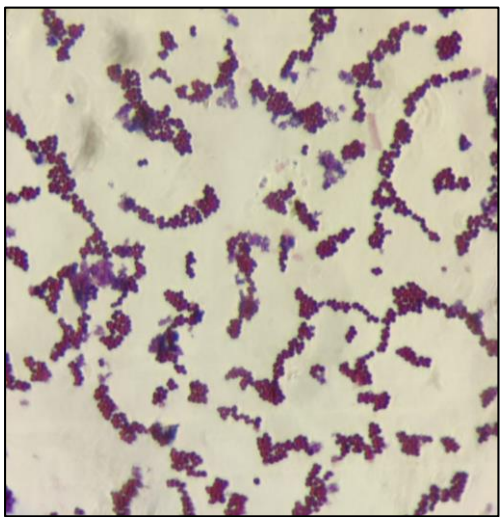

Fig 4: Microscopic morphology of Staphylococci in clusters (100X)

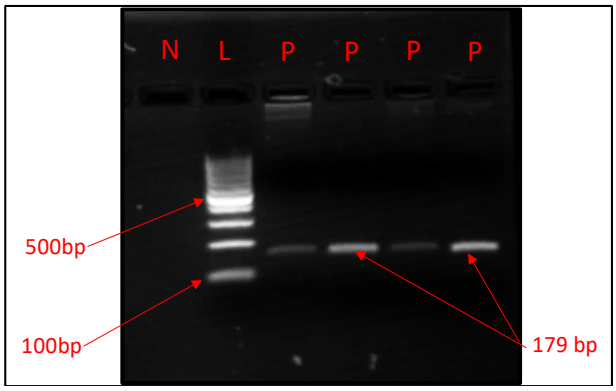

Fig 5: Agarose gel electrophoresis of amplified *S. aureus* PCR product of 179 bp
L: Ladder, P: Positive milk sample, N: Negative milk sample

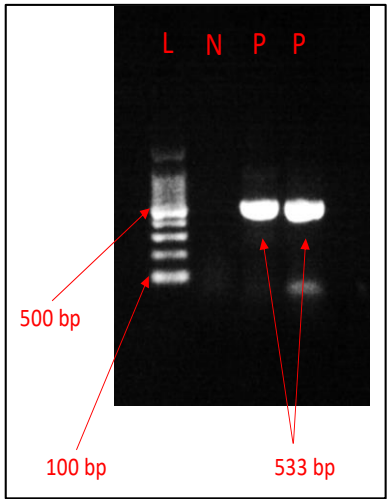

Fig 6: Agarose gel electrophoresis of amplified *mecA* gene PCR product of *S. aureus* of 533 bp
L: Ladder, P: Positive milk sample, N: Negative milk sample

Fig 7: Biofilm formation of *staphylococci* by Congo Red Agar (CRA) (+ = Biofilm producing and- = non-biofilm producing)

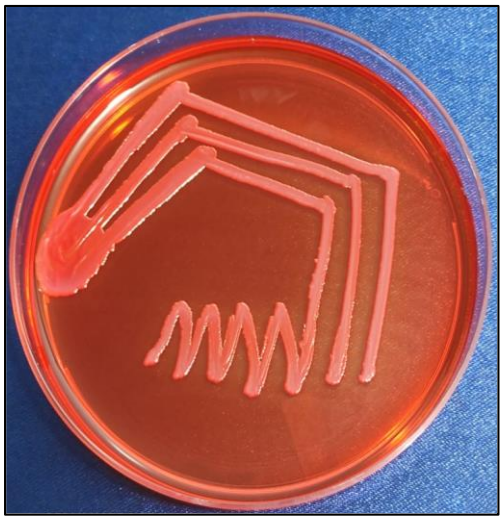

Fig 8: Non-biofilm-producing staphylococci by the CRA method

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