Journal of Animal Research: v.13 n.06, p. 947-955. December 2023

DOI: 10.30954/2277-940X.06.2023.11

Biofilm-Forming and Resistance to Antimicrobials Potential of *Staphylococcus aureus* Isolated from Bovine Mastitis Milk

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Received: 10 Aug., 2023 **Revised:** 29 Sept., 2023 **Accepted:** 18 Oct., 2023

ABSTRACT

Mastitis in dairy cows is a condition that is seen as being economically significant. The major pathogen in infectious bovine mastitis is known to be *Staphylococcus aureus (S. aureus)*. The production of biofilms is a rising concern in human and animal health. Because of the minimal association between standard *in vitro* antimicrobial susceptibility to antimicrobial therapy efficacy, it was hypothesized that biofilm could play a significant role in the establishment of chronic *S. aureus* bovine mastitis. This research aims to isolate and characterize *S. aureus* strains from mastitic milk of bovine animals to evaluate biofilm formation by *in vitro* phenotypic tests like Congo red agar (CRA) and Microtitre plate (MTA) assay and molecular detection used to find specific biofilm-forming genes *icaA* (intercellular adhesion gene A) and *icaD* (intercellular adhesion gene D) and antimicrobial susceptibility testing. Traditional microbiology was used to isolate and identify *S. aureus* species, and the biofilm-forming genes (*icaA* and *icaD*) were detected by polymerase chain reaction. A total of 60 mastitis milk samples were subjected to culture and isolation, out of which, 14 isolates were identified as *S aureus*. A sensitivity test for antimicrobial properties was performed on all the isolates *in vitro* using 16 antimicrobial agents. All isolates developed biofilms, with 9/14 (64%) strongly biofilm-forming, 3/14 (21%) moderately biofilm-forming, and 2/14 (14%) weakly biofilm-forming. The research demonstrated AMR, invasiveness, and biofilm formation in *S. aureus* isolated from bovine mastitis. This feature adds to the difficulties of current antibiotic therapy.

HIGHLIGHTS

- Isolation and characterization of *S. aureus* strains isolated from mastitic bovine animals.
- Assessment of the biofilm-forming and antimicrobial resistance ability of *Staphylococcus aureus*.

Keywords: *Staphylococcus aureus*, bovine mastitis, biofilm, antibiotic resistance

Bovine Mastitis is a major disease that affects the dairy business, lowering animal health and milk quality and, as a result, farmers' income (Wang *et al*., 2018; Zhou *et al*., 2018). Mastitis is defined as mammary gland inflammation characterised by physical, chemical, and microbiological alterations in milk as well as pathological abnormalities in the mammary tissue (Khan and Muhammad, 2005). Mastitis has also been linked to decreased reproductive efficiency in dairy cows (Wadhwa *et al*., 2003). Seasonal variation has a clear impact on the occurrence of cow mastitis and the microorganisms associated with it (Ranjan *et al.,* 2011). Mastitis prevalence is growing in both cattle and buffaloes, posing a considerable challenge to field veterinarians and researchers (Awale *et al.,* 2012). Mastitis is a complex disease caused by infectious and non-infectious causes. Mastitis of an infectious origin is well recognised to occur more commonly than those of non-infectious origin. Bacterial pathogens are frequently important in the pathophysiology of mastitis among

How to cite this article: Puvvala, B., Sinha, E., Behera, S., Sarkar, R., Sriharsha, Y., Manimaran N., Khan S. and Qureshi, S. (2023). Biofilm-Forming and Resistance to Antimicrobials Potential of *Staphylococcus aureus* Isolated from Bovine Mastitis Milk. *J. Anim. Res.,* **13**(06): 947-955.

Source of Support: None; **Conflict of Interest:** None

infectious causes. S*taphylococcus aureus* is the leading cause of clinical mastitis and high somatic cell counts in many cases, lowering the quality of milk submitted to processing plants (Petrovski *et al*., 2009). *S. aureus* pathogenicity in the bovine udder is not fully understood. Sometimes, chronic or recurring *S. aureus* infections can't be treated because the bacteria have developed virulence mechanisms that make them resistant to antimicrobials and the host's immune response. Antibiotics are the most frequent treatment for mastitis in dairy cattle, and it is typically delivered by intramuscular and intramammary methods (Abdi *et al*., 2021). With only a 10–30% cure rate, the treatment has, however, lost some of its effectiveness (Gomes and Henrique, 2016). Antimicrobial resistance in bacteria (AMR) causes the antimicrobials to lose some of their effectiveness in treating mastitis (Jamali *et al.,* 2015; Sasidharan *et al.,* 2011). Microorganisms use biofilm formation as a way to protect themselves and increase their virulence. (Melchior *et al*., 2006). The biofilm matrix is mostly composed of water and macromolecules such as polysaccharides, proteins, DNA, lipids, mineral salts, and various cell lysis products. Poly-N-acetylglucosamine polysaccharide (PNAG), also known as polysaccharide intercellular adhesin (PIA), is a key component of biofilm formation and is expressed by genes in the *ica* ADBC locus. The transcription of the *ica* locus and synthesis of PNAG can be influenced by various external and environmental factors (Jefferson *et al*., 2004). The *icaA* and *icaD* genes are essential for *S. aureus* biofilm formation (Vasudevan *et al.,* 2003).

According to reports, *S. aureus* biofilm-forming strains are the culprit in certain episodes of mastitis (Szweda *et al*., 2012). In a self-generated extracellular polymeric substance (EPS) matrix microorganism cells stick to every possible surface, whether it is alive or not, to form a biofilm, an association of microbes. There are numerous processes involved in creating a biofilm, commencing with adhesion to a surface, followed by the occurrence of a microcolony that results in the assembly of a three-dimensional structure, and finally maturation and separation. In order to survive in extremely dynamic ecosystems with sporadic food depletion, resistance to broad-spectrum antibiotics, disinfectants, and radically distinct constitution changes, microorganisms aggregate for a variety of advantageous reasons (Nadell *et al*., 2009). Additionally, biofilms can shield the bacterial community from antimicrobial substances, which could hinder the effectiveness of antimicrobial therapy (Melchior *et al*., 2006). Despite an *in vitro* antimicrobial susceptibility profile, treating cows with chronic mastitis caused by *S. aureus* frequently fails (Melchior *et al.,* 2007). The efficacy of antimicrobial therapy is dependent on various aspects, including the animal's age, immunological health, the presence of other illnesses, the route of antibiotic administration, and bacteria that have the ability to form biofilms. (Mestorino and Errecalde, 2012). Because of these reasons, an antibiotic agent may not reach the site of infection at its required effective concentration, the Minimal Inhibitory Concentration (MIC), and may only be present at subinhibitory concentrations (subMIC) (Amini *et al.,* 2009). A biofilm's significance in the development of cow mastitis and its connection to antibiotic resistance should be noted. In this study, *S aureus* strains from mastitic bovine animals will be isolated and characterized in order to assess the formation of biofilms, certain tests can be conducted such as Congo red agar (CRA) and microtitre test (MTA) assays, along with molecular detection to identify specific biofilm-forming genes *(icaA* and *icaD*) and antimicrobial susceptibility testing.

MATERIALS AND METHODS

Isolation and identification

In brief, 1 ml of milk sample was centrifuged at 6000 rpm for 5 minutes in microcentrifuge tubes, and the sediment was streaked onto Sheep Blood Agar and Mueller-Hinton Agar plates. Gram staining was used to stain the cultures after they had been incubated at 37º C for 24-48 h. A series of biochemical reactions included catalase and oxidase production, nitrate reduction, oxidation-fermentation test, sugar fermentation (lactose, maltose, mannitol, mannose, sucrose, and trehalose), ability to produce coagulase and urease, and Voges-Proskauer test to identify and differentiate staphylococci from other cocci. Apart from other regular biochemical tests, coagulase-positive staphylococci and coagulase-negative staphylococci were distinguished by colony pigments.

Species confirmation by PCR

DNA Isolation and Polymerase Chain Reaction

The DNA was obtained using the boiling and chilling

method described by Arora *et al.* (2006), and 100 µl broth culture was centrifuged in a microcentrifuge tube for 5 min at 2,348 × g. The supernatant was removed, and the pellet was resuspended in 100 µl of nuclease-free water before being placed in a boiling water bath for 10 min. Following heat treatment, the cell lysate was immediately chilled at -20° C for 4h before being centrifuged at 2,348 × g for 5 min. The DNA-containing supernatant was aliquoted into a sterile tube and kept at -20 °C until further use. The PCR was carried out for the detection of *S. aureus* by the method described by Braksta *et al*. (1992). The PCR amplification was carried out according to the following program. Initial denaturation at 95 ºC for 10 min followed by 36 cycles of denaturation at 94 ºC for 1 min, annealing at 55º C for 30 sec and extension at 72 ºC for 1. 5 min, and final extension at 72 ºC for 5 min. The amplified products were visualized by electrophoresis on 1% agarose gel for 90 min and the gel was stained with 0. 5% gel red.

Table 1: Species-specific primer sequence of *Staphylococcus aureus*

Gene	Primer sequence (5'-3')	Size (bp)	References
nuc gene	5'-GCGATTGATGGTGATA CGGT-3'	270	Brakstad *et al*. (1992)
	5'AGCCAAGCCTTGACGA ACTAAAGC-3'		

Antimicrobial susceptibility testing

The antibiogram assay was performed by standard disc diffusion method using sixteen standard antimicrobial agents (Amoxicillin @ 10 µg, Ampicillin @10 µg, Amoxicillin-Clavulanic acid @ 30 µg, Ceftriaxone @ 30 µg, Cephotaxime @ 30 µg, Ciprofloxacin @ 5 µg, Chloramphenicol @ 30 µg, Cloxacillin @ 5 µg, Co-trimoxazole @ 25 µg, Enrofloxacin @ 10 µg, Gentamicin @ 10 µg, Methicillin @ 5 µg, Oxacillin @ 1 µg, Penicillin G @ 10 µg, Streptomycin @ 10 µg and Tetracycline @ 30 µg) and the resistance was assessed according to Clinical Laboratory Standards.

Biofilm detection methods

Micro titre plate method

The gold-standard technique for detecting biofilms is the quantitative test as described by Christensen *et al.* (1995). Isolated organisms from freshly prepared agar plates were inoculated in a 10 mL trypticase soy broth solution containing 1% glucose and broths were incubated at 37° C for 24 h. Later, fresh medium was diluted 1:100 with the cultures. 200 µl of the diluted cultures were placed in each of the 96 wells of sterile, flat-bottomed polystyrene tissue culture plates (Sigma-Aldrich). The inoculated sterile broth was kept as a negative control wells. The plates were incubated for 24 h at 37°C. Following incubation, each well's contents were taken out using light tapping. Four times, 0. 2 mL of phosphate-buffered saline (pH 7. 2) was used to wash the wells. It eliminated microorganisms that were floating around. The bacterial biofilm that had grown on the wells was preserved with 2% sodium acetate and stained with 0. 1% crystal violet. Deionized water was used to remove any excess discolouration, and the plates were stored to dry. Using a micro-ELISA auto reader (model 680, Biorad) at a wavelength of 570 nm, the optical density (OD) of stained adherent biofilm was measured. Three times the experiment was carried out in duplicate.

Congo Red Agar Method

The use of Congo Red Agar (CRA) media has been described by Freeman *et al.* (1989) as a straightforward qualitative method to identify the development of biofilms. Brain heart infusion broth (37 g/L), sucrose (50 g/L), agar (1.5%) and Congo Red indicator (8 g/L) were used to make the CRA medium. The first step involved making a concentrated aqueous solution of Congo Red stain, which was then autoclaved at 121°C for 15 min without the other components of the medium. Then, at a temperature of 55 °C, it was added to the autoclaved brain heart infusion agar with sucrose. Five CRA plates were infected with test organisms and then incubated aerobically for 24 h at 37ºC. A biofilm was produced by the black colonies with a dry, crystalline quality consistency indicating biofilm production.

Analysis of the *icaA* and *icaD* genes to determine biofilm development

All isolates were evaluated for biofilm production and submitted to PCR for detection of the *icaA* and *icaD* genes.

For amplification of the *icaA* gene, the PCR mixture was performed in 30 cycles consisting of denaturation at 92°C for 45 s, annealing at 58°C for 45 s and extension at 72°C for 1 min, followed by a final extension at 72°C for 7 min. The amplification conditions for the *icaD* gene were 30 cycles of denaturation at 92°C for 45 s, annealing at 49°C for 45 s and extension at 72°C for 1 min, followed by a final extension at 72°C for 7 min. The amplified products were visualized by electrophoresis on 1% agarose gel for 90 min and the gel was stained with 0. 5% gel red.

Table 2: Primer used for the detection of Biofilm development by *Staphylococcus aureus*

Gene	Primer sequence (5'-3')	Size (bp)	References
icaA	F: 5′-CCT AAC TAACGA AAG GTA G-3′	1315	Vasudevan *et al.* (2003)
	R:5′-AAG ATA TAG CGA TAA GTGC-3′		
icaD	F: 5′-AAA CGT AAG AGAGGT GG-3′	381	Vasudevan *et al.* (2003)
	R:5′-GGC AAT ATG ATC AAG ATA C-3		

RESULTS

Isolation of microorganisms from milk samples

A total of 60 mastitis milk samples were subjected to culture, biochemical tests and isolation (Fig. 1, 2, 3, 4, 5, and 6) out of which, 14 isolates were identified as *S. aureus*. All isolates were subjected to the Antibiotic sensitivity test, Micro Titre plate method, and Congo Red Agar method to assess biofilm formation, and then subjected to PCR to detect the *icaA* and *icaD* genes.

Antimicrobial susceptibility profile

All the isolates were subjected to *in vitro* antimicrobial sensitivity test using 16 antimicrobial agents. Out of 14 *S. aureus* (11) was sensitive to ciprofloxacin, (11) to cloxacillin and tetracycline, (8) were sensitive to ceftriaxone, (9) to enrofloxacin and gentamicin and (12) were sensitive to chloramphenicol. Among them nine *S. aureus* were resistance to amoxicillin/Clavulanic acid, (13) to methicillin, (11) to penicillin G, (9) to co-trimoxazole, (8) to oxacillin and streptomycin, and (10) ampicillin and amoxicillin (Fig. 7)

Table 3: Resistance pattern of biofilm-producing *S. aureus*

Antimicrobial agent	Biofilm-producing *S. aureus* (%)
Methicillin	92. 82
Penicillin G	78. 57
Ampicillin and Amoxicillin	71. 42
Amoxicillin/Clavulanic acid	64. 28
Co-trimoxazole	64. 28
Oxacillin and Streptomycin	57. 14

Micro Titre Plate and Congo Red Agar techniques were used to screen the isolates for biofilm development.

All isolates developed biofilms, with 9/14 (64%) strongly biofilm-forming, 3/14 (21%) moderately biofilm-forming, and 2/14 (14%) weakly biofilm-forming (Fig. 8 & 9).

Table 4: Detection of Biofilm Formation in Tissue Culture Plate Method

No. of isolates (14)	Biofilm formation	Tissue Culture Plate	Congo Red Agar
14	Strong	9/14 (64%)	2/4(14%)
14	Moderate	3/14 (21%)	4/14(28%)
14	Weak	2/14 (14%)	8/14(57%)

PCR assay for *icaA* and *icaD* genes to detect biofilm formation

Simplex PCR assays were used to assess the presence of *icaA* (intercellular adhesion gene A) and *icaD* (intercellular adhesion gene D) in two biofilm-related genes. Agarose gel electrophoresis of amplified PCR products was carried out along with negative control and molecular size marker (100 bp ladder). Two biofilm-related genes *icaA* and *icaD* could detect in *S. aureus* isolates under study. In *S. aureus* isolates the positive rates of *icaA* and *icaD* and the combination of both *icaA* and *icaD* genes were 71.42%, 92. 85%, and 42.85% respectively. The presence of the *icaD* gene was high versus that of the *icaA* gene (Fig. 10, 11 & 12).

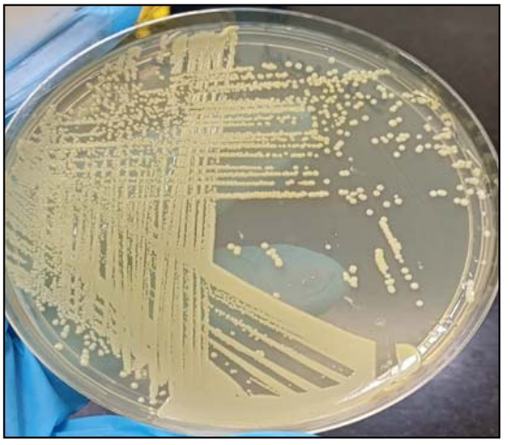

Fig. 1: Growth of *S. aureus* on Nutrient agar

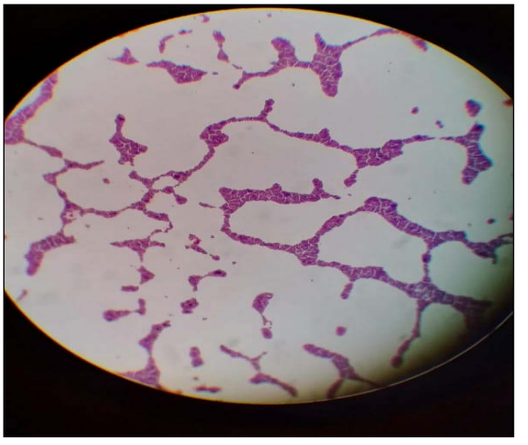

Fig. 2: Photomicrograph of *S. aureus* (Gram's stain x1000)

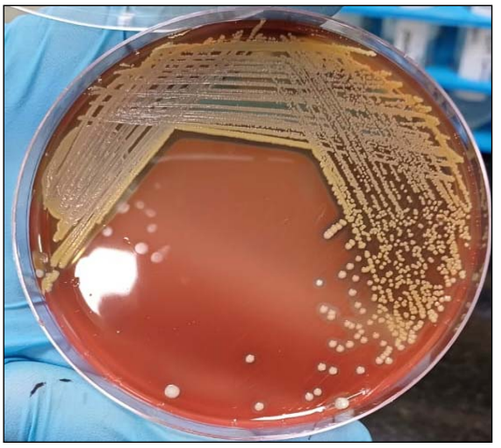

Fig. 3: Haemolytic colonies of *S. aureus* on Blood agar

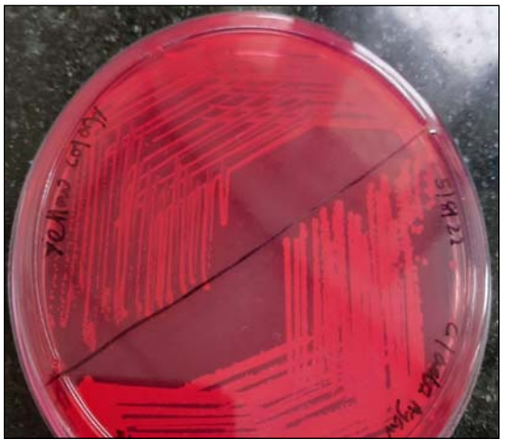

Fig. 4: Growth of *S. aureus* on CLED agar

Fig. 5: Positive reaction of *S. aureus* for Catalase test

Fig. 6: Negative reaction of *S. aureus* for Oxidase test

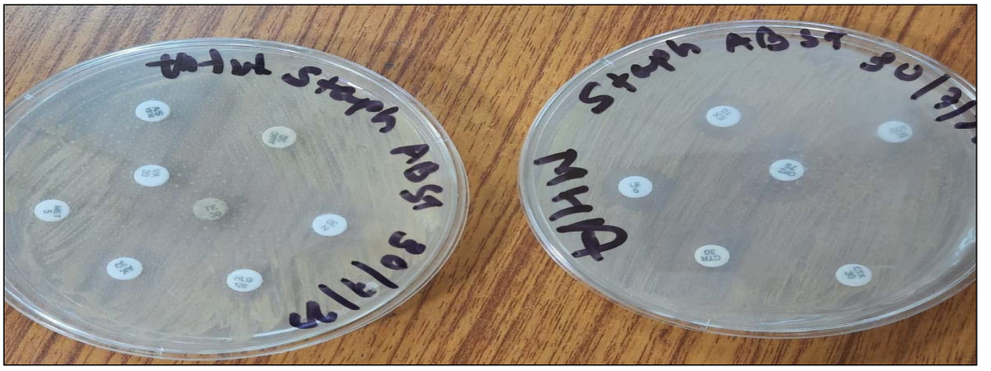

Fig. 7: Antibiotic resistance pattern of *S. aureus* on MHA

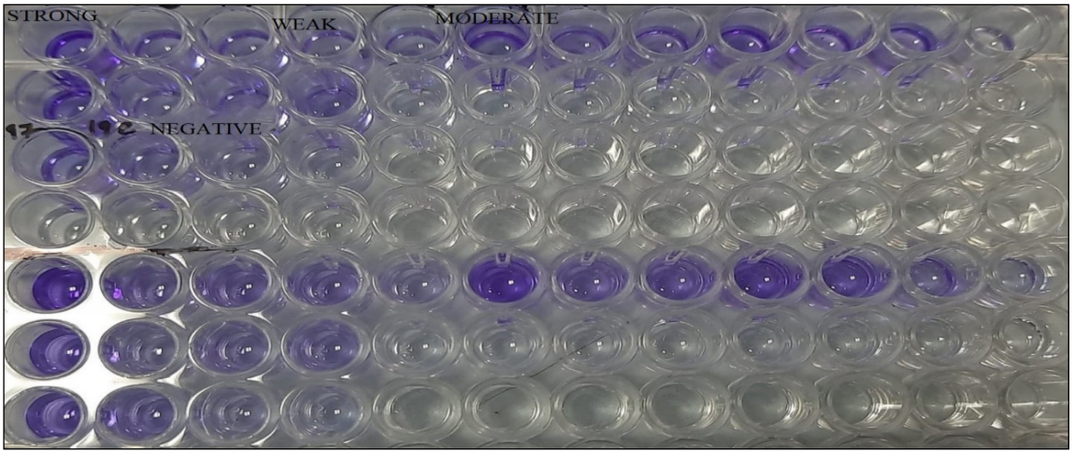

Fig. 8: Screening of biofilm-producing *S. aureus* by Micro titre Plate method: Strong, Moderate and Weak slime producers were differentiated with crystal violet staining

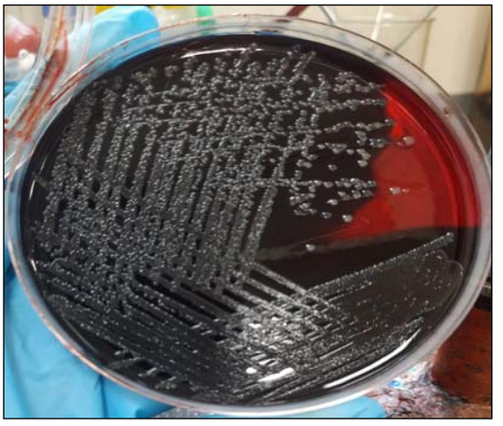

Fig. 9: Growth of *S. aureus* on Congo red agar medium

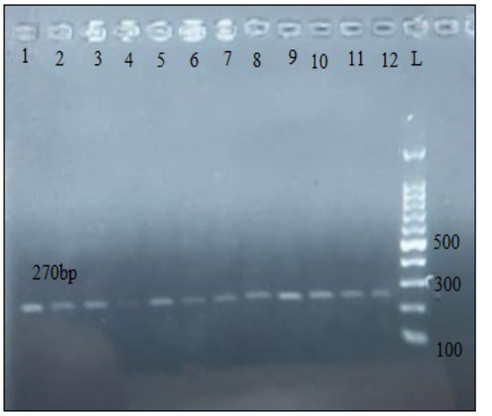

Fig. 10: PCR amplification of *nuc* gene showing 270bp amplicon

Lane 1 -12: Positive isolates; **Lane 13:** Negative control; **Lane L:** 100bp ladder

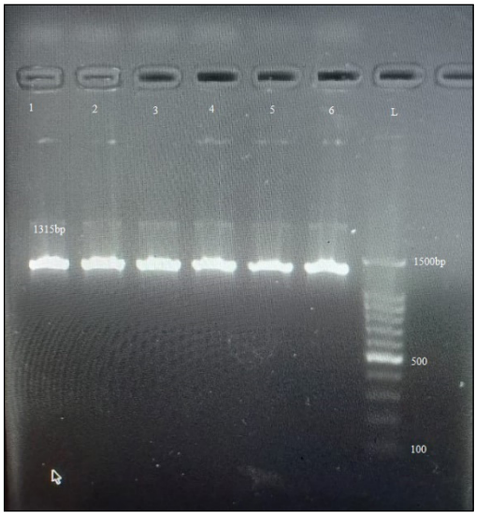

Fig. 11: PCR amplification of *icaA* gene showing 1315 bp amplicons

Lane 1 -6: Positive isolates; **Lane L:** 100bp ladder

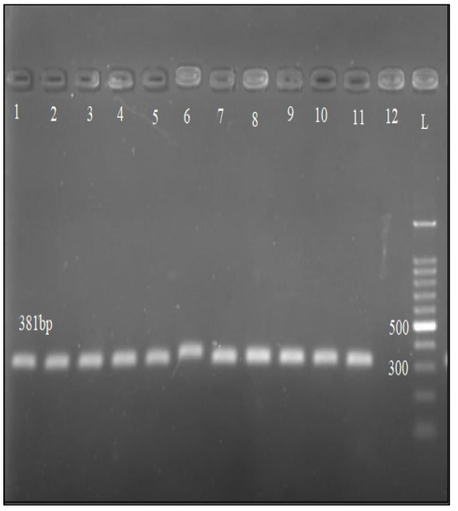

Fig. 12: PCR amplification of *icaD* gene showing 381 bp amplicons

Lane 1 -12: Positive isolates; **Lane L:** 100bp ladder

Table 5: Detection of Biofilm genes in *S. aureus* Isolated from Bovine Mastitis Milk

Targeted genes	No. of isolates harbours	Presence in percentage
icaA	10	71. 42%
icaD	13	92. 85%
icaA and *icaD*	6	42. 85%

DISCUSSION

One of the causes of chronic and persistent infections is the ability of bacteria to create biofilms inside the host, which allows them to adhere to and live in hostile conditions. Many persistent infections are caused by biofilm-producing bacteria, which are extremely challenging to eliminate. Due to the staphylococci's capacity to adhere to the mammary epithelium and the development of numerous layers of cells participating in the biofilm matrix, this virulence factor facilitates staphylococci's persistence in the udder in cases of mastitis (Baselga *et al*., 1993). They demonstrate resistance to antibiotics in a variety of ways, including limited antibiotic uptake by biofilms, slowed growth, and production of resistance genes. There are numerous techniques for detecting biofilm. In this investigation, all isolates were screened using three different approaches for their capacity to produce biofilms. Studies have shown that isolates taken from bovine intramammary infections frequently form biofilms and contain the *ica* genes. Vasudevan *et al*. (2003), 35 *S. aureus* strains obtained from instances of bovine mastitis were examined, and 91. 4% of the isolates produced biofilm on CRA, and 100% tested positive for the *icaA* and *icaD* genes. By using two distinct techniques, Krukowski *et al.* (2008) examined the biofilm generation of 59 *S. aureus* strains that were isolated from 45 cows that had mastitis. The Christensen approach and the CRA method both revealed biofilm formation in 47. 45% and 42. 37% of the strains, respectively. In the present study, although 100% of the *S. aureus* isolates produced biofilms *in vitro* on CRA, the *icaA* gene was detected in 71. 42% of the isolates by PCR, and the *icaD* gene in 92. 85%. Despite having the *ica* locus, biofilm formation may not happen *in vitro* or on inert surfaces because *S. aureus* strains are extremely sensitive to the growth conditions, such as the amount of glucose or glucosamine available for matrix formation, according to Cramton *et al*. (1999). Some accessory genes may also affect how strains behave phenotypically on CRA, resulting in the development of colonies that do not entirely express the *ica* genes (Ciftci *et al*., 2009). Several researchers have reported the isolation of multiresistant pathogens, particularly

S. aureus, that cause bovine mastitis. Moroni *et al*. (2006) conducted a study on commercial cattle herds in Italy and isolated 68 *S. aureus* strains from cases of subclinical mastitis. They discovered a 94% rate of resistance to three or more different antimicrobial drugs. In the MTA method, the number of isolates showing biofilm formation was 64%, and weak biofilm forming was 14%. Regional data from India also revealed that out of 152 isolates examined, the MTA technique classified 53. 9% of them as biofilm producers and 46% as non-biofilm producers (Mathur *et al*., 2006). In the present study, most of the isolated *S. aureus* from bovine mastitis cases were at least one tested antimicrobial agent-resistant. The discovery of resistant *S. aureus* in lactating cows is concerning because these strains may reduce the range of available treatments and result in chronic inflammation that is challenging to cure. The choice of strains resistant to one class of antibiotics can result in cross-resistance to another class, even if various classes of antibiotics are utilized for the treatment and prophylaxis of cow mastitis. Due to the ineffective treatment of the disease, the improper use of antimicrobial drugs might result in multiple drug resistance and losses to rural producers (John *et al*., 2002). In the case of infections with a zoonotic potential, monitoring the evolution of resistant pathogens in animal reservoirs is crucial (Haran *et al*., 2012).

CONCLUSION

The current findings emphasize the value of combining phenotypic and genotypic tests for more precise detection of biofilm formation because several factors can affect how it manifests phenotypically. For bovines, it is necessary to put in place preventative and controlling measures for mastitis. The ability of antibiotic strains to produce biofilm in cattle poses a hazard to public health and animal welfare as well as financial losses for dairy farmers.

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