Original Article

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# DETECTION OF BIOFILM FORMATION AMONG METHICILLIN RESISTANT *Staphylococcus aureus* FROM SUBCLINICAL BOVINE MASTITIS

**Muhammad Asif Zahoor[1], Abu Baker Siddique[1], Sultan Ali[2], Mohsin Khurshid[1], Rizwan Aslam[2] and Zeeshan Nawaz[1,*]**

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## ABSTRACT

Mastitis is a condition responsible for causing severe economic losses to dairy industry and a public health threat due to reduced milk production, massive antibiotic usage and deteriorated milk quality. Among the diverse bacterial and physical etiologies of mastitis, *Staphylococcus aureus* remains the most significant. The primary aim of this study is to detect methicillin-resistant *Staphylococcus aureus* (MRSA) in mastitic milk samples and to assess their biofilm-forming ability. Total 368 milk samples were collected from buffaloes targeting various locations and screened for subclinical mastitis using California Mastitis Test. The positive samples were subjected to identification and confirmation of *Staph aureus* using standard microbiological methods and polymerase chain reaction. The Kirby-Bauer method was used to determine the multidrug-resistance profile and methicillin-resistant status of *Staphylococcus aureus* isolates, followed by confirmation through amplification of the *mecA* gene. The biofilm forming potential of all isolates was done using Microtiter Plate Method (MTP). The results showed that 42.40% (156/368) of milk samples were positive for subclinical mastitis and among them *Staph aureus* was detected in 71 isolates. On the basis of Kirby-Bauer test and *MecA* gene (n=26) isolates were confirmed as MRSA. It was observed that all the MRSA isolates exhibited varying levels of biofilm production, with 65.38%, 26.92%, and 7.69% demonstrating strong, moderate, and weak biofilm formation, respectively while the 82.22% of MSSA isolates showed biofilm production. The findings indicate a notable presence of MRSA in bovine mastitis and biofilm formation is more pronounced in MRSA compared to MSSA isolates. The control of biofilm forming MRSA in mastitis requires strict milking hygiene, early detection of infection, judicious antibiotic use and implementation of herbal preparations targeting biofilm disruption to limit infection spread.

**Keywords:** *Bubalus bubalis*, buffaloes, bovine, subclinical mastitis, MRSA, biofilm, Pakistan

## INTRODUCTION

Mastitis is one of the most severe illnesses observed in milking animals which causes a global loss of >32 billion US dollars due to

[1]Institute of Microbiology, Government College University Faisalabad, Pakistan, *E-mail: zeeshannawaz@gcuf.edu.pk

[2]Institute of Microbiology, University of Agriculture, Faisalabad, Pakistan

antibiotic therapy and reduced milk production, condemnation of milk with antibiotic residues and high culling rate of affected animals. Mastitis is associated with considerable alterations in milk chemical composition with a significant decrease in milk synthesis and changes in cell permeability (Kumar *et al*., 2010). According to a recent field survey, mastitis is ranked number one disease of the dairy animals in Pakistan (Salman *et al*., 2023). There are more than 135 bacterial species known to contribute to the development of mastitis (Xia *et al*., 2025) such as *Streptococcus agalactiae*, *Escherichia coli*, *Streptococcus dysgalactiae, Streptococcus uberis*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa* but the most prominent and important is *Staphylococcus aureus* (Cobirka *et al*., 2020), while the minor causes includes physical trauma and mechanical injuries to the mammary glands (Ali *et al*., 2021).

From the past many years, *S. aureus* has been considered as leading reason of mastitis in dairy animals and foodborne illnesses which significantly impact the economy and a threat for public health (Ateba *et al*., 2010). Many food-associated outbreaks have been documented with dairy products as key source of transmission (Verma *et al*., 2023). It produces several virulence factors that protects this organism from immune system of host and enable pathogen adaptation to the host immune system and favors the establishment of intramammary infection including surface binding proteins, enterotoxins, superantigens, and leukocidins (Rossi *et al*., 2021).

*S. aureus* also has the ability to procure antibiotic resistance with methicillin-resistant *S. aureus* (MRSA) being a prevalent type globally (Preziuso *et al*., 2024). The misuse and widespread use of antibiotics for the treatment and prevention of bovine mastitis leads to development and emergence of antibiotic-resistant strains (Oliver and Murinda, 2012). The presence of MRSA in the milk of dairy animals affected by mastitis raises important public health concerns, stemming from the pathogen's resistance to treatment and its potential to spread to humans (Song *et al*., 2016). MRSA strains harbor a modified penicillin-binding protein, having a low affinity to most of the beta-lactam antibiotic drugs. So, resistance to methicillin is mediated by *mecA* gene, located in the staphylococcal cassette chromosome (SCC) component (Hou *et al*., 2023).

Another mechanism which shrinks the effectiveness of treatment for mastitis cases is the production of biofilm. This phenomenon reduces susceptibility of *Staph. aureus* to various antibiotics by decreasing diffusion of antibiotics inside biofilm matrix and becoming resistant to high concentrations of antimicrobials. The production of biofilms promotes adhesion and colonization of bacteria to mammary gland tissue, evasion from adverse/harsh conditions and phagocytosis as well as prolonged infection (Silva *et al*., 2022).

The zoonotic potential, biofilm formation, antimicrobial resistance of *Staph aureus* and its frequent occurrence in bovine mastitis cases emphasizes the need for its comprehensive surveillance and management. So, keeping in consideration the above-mentioned facts, the present study was designed to isolate and characterize MRSA strains from milk samples of buffaloes having subclinical mastitis followed by detection of their biofilm forming activity.

## MATERIALS AND METHODS

### Study design

A cross-sectional study design was

undertaken from July 2024 to June 2025 to estimate the frequency of biofilm forming methicillin-resistant *Staphylococcus aureus* from milk of buffaloes having sub-clinical mastitis.

**Sample size and sampling technique**

The sample size was estimated following the method previously described by (Thrusfield, 2007) keeping an expected prevalence of 40%, absolute precision 5% with 95% confidence interval for *Staphylococcus aureus* in milk (Shahid *et al*., 2023). Accordingly, (n=368) samples of milk were collected. A non-probability convenience sampling procedure was adopted during the study for collection of milk samples.

**Sample collection**

A total of (n=368) raw milk samples from lactating buffaloes were collected from various dairy farms and small holders. A prior consent was obtained after clarifying them the study objectives. Briefly, the quarters of animals were washed with warm water having some sanitizing solution and dried thoroughly. After discarding the first two streams of milk, 10 to 15 ml of milk was collected in sterile plastic screw capped containers aseptically followed by labeling with date, location and identification number. The samples were screened for bovine mastitis using California Mastitis Test (CMT) Kit (Portland, ME, USA) as described by (Javed *et al*., 2024). After screening samples were immediately shifted to Food Microbiology Laboratory in temperature-controlled boxes for further processing.

**Isolation and identification of *Staphylococcus aureus***

The milk samples were spread on mannitol salt agar (MSA) and then incubated at 37°C overnight. Following purity plating, size, shape, margins, pigmentation, elevation, and opacity were observed macroscopically. Gram staining was employed to differentiate Gram-positive cocci followed by performance of catalase test to differentiate Gram-positive *Staphylococci* from *Streptococci*. Afterwards, a coagulase test was also performed for the differentiation of *S. aureus* from other coagulase-negative *Staphylococci* (Javed *et al*., 2024). For the final confirmation of *S. aureus* isolates, the *nuc* gene was subsequently amplified. The DNA of isolates was extracted using Favor Prep Genomic DNA Extraction Kit and amplification of *nuc* gene was done by polymerase chain reaction (PCR). The amplified PCR products were envisioned using a UV transilluminator on a 1.5% agarose gel stained with ethidium bromide. The preparation of reaction mixture, primers used and thermal profile was adopted as previously described by (Taqveem *et al*., 2024) and mentioned in (Table 1).

**Antimicrobial susceptibility profiling**

The Kirby-Bauer disk diffusion method was employed using Mueller-Hinton agar (MHA) plates to perform antimicrobial susceptibility test. The fresh *S. aureus* colonies were mixed in normal saline to adjust the turbidity equals to 0.5 McFarland standards. This freshly prepared bacterial suspension was evenly overlaid on the surface of media plates using a sterile cotton swab and antibiotics were then aseptically settled to media plates which include cefoxitin 30 µg, vancomycin 30 µg, ciprofloxacin 5 µg, trimethoprim-sulfamethoxazole 25 µg, erythromycin 15 µg, gentamicin 10 µg, tetracycline 10 µg, oxacillin 1 µg, chloramphenicol 30 µg and linezolid 30 µg. The results were recorded according to the guidelines devised by Clinical Laboratory Standards Institute

(CLSI, 2024) (Shahid *et al*., 2023).

**Amplification of *mecA* Gene**

The *mecA* gene was amplified using primers and thermal conditions previously described by (Osman *et al*., 2016). The amplicons were envisioned using a UV transilluminator on a 1.5% ethidium bromide-stained agarose gel. A 100 bp DNA marker was used as a reference for estimating the size of the amplified products (Abdulhaq *et al*., 2020).

**Detection of Biofilm by Microtiter Plate (MTP) method**

All the S*taph aureus* isolates were grown overnight at 37°C as pure cultures followed by adjustment and dilution with tryptic soy broth (100 folds) using 0.5 MacFarland standard tube. Aliquots (250 µL) of the isolate suspensions were dispensed into a flat-bottom 96-well polystyrene microtiter plates and incubation at 37°C for 22 to 24 h were facilitated. Following incubation, each well of the plate was washed couple of times using 250 µL of sterile saline and then shaken to remove weakly adherent or non-adherent cells. The plates were then heated dried for fixation of biofilms. The wells were then stained with 250 µL of dye (0.1% crystal violet) solution for 12 to 20 minutes. After washing and drying of plates, the adherent cells coupled with crystal violet dye in every well were dissolved in 250 µL of 50% acetone. The wells which were not inoculated and containing only sterile tryptic soy broth were considered as negative controls. The optical density (OD) of each well was measured at 594 nm using ELISA reader apparatus (BioRad, USA) as described by (Stepanovic *et al*., 2000).

**Statistical analysis**

Each experiment was performed in triplicate. The results were expressed as the mean value using Minitab software (USA) and differences were considered statistically significant when ($P<0.05$) is obtained (Zafar *et al*., 2025).

## RESULTS AND DISCUSSIONS

**Prevalence of *S. aureus* subclinical bovine mastitis**

The frequency of subclinical mastitis screened by CMT among lactating buffaloes was found 156/368 (42.40%). Out of these, *Staphylococcus aureus* was detected (45.51%) on the basis of Gram staining and biochemical tests. Furthermore, all isolates were detected positive for the *nuc* gene. Consequently, both the phenotypic and molecular analysis yielded concordant results (Figure 1A).

**Antimicrobial susceptibility profiles of *S. aureus* isolates**

The antimicrobial susceptibility was performed on confirmed *S. aureus* isolates and results depicted (100%) susceptibility to vancomycin and linezolid, (97.18%) to gentamicin and (95.77%) to sulfamethoxazole/ trimethoprim. The drugs ciprofloxacin and chloramphenicol were found 92.95% susceptible. Furthermore, a noteworthy resistance was observed 64.78% for tetracycline and 46.47% for erythromycin while 36.61% for cefoxitin and oxacillin. It was recorded that the *Staph aureus* isolates showing resistance to cefoxitin were also found resistant to oxacillin as shown in (Table 2). Consequently, among the total 71 isolates, 26 (36.61%) found methicillin resistant (MRSA), while 45 (63.39%) exhibited

susceptibility toward methicillin (MSSA). The isolates resistant to methicillin also harbored the *mecA* gene; thus confirmed as MRSA (Figure 1B).

**Prevalence of biofilm production among MRSA and MSSA isolates**

The biofilm formation of the *S. aureus* isolates was detected using microtiter plate method which is considered as most reliable technique for phenotypic detection of biofilm production. The results of study showed that all the 26 MRSA isolates showed variable degrees of biofilm production presenting 65.38%, 26.92% and 7.69% as strong, moderate and weak biofilm formation respectively. Among the methicillin susceptible 45 *S. aureus* isolates, 20 (44.44%) isolates showed strong biofilm production followed by 11 (24.44%) as moderate followed by 06 (13.33%) as weak while 08 (17.77%) showed no biofilm forming potential as shown in (Table 3).

Bovine mastitis remains a substantial problem for milk producers worldwide. It is one of the most common diseases of dairy industry responsible for severe economic losses affecting animal welfare, milk yield, farm profitability and posing potential public health risks through the transmission of antimicrobial-resistance. *Staphylococcus aureus* is recognized as one of the most prevalent and persistent pathogens responsible for both clinical and subclinical mastitis. The organism's ability to adhere to and invade mammary epithelial cells, form biofilms, and evade the host immune response contributes to chronic and recurrent infections. Additionally, the toxin production and antibiotic resistance further worsen the scenario posing a public health threat.

In this conducted research, total 368 milk samples were collected and tested for subclinical mastitis using California mastitis test and it was found that 156 (42.4%) milk samples were positive for subclinical mastitis which is very close to the results of 38% and 40.42% in Pakistan (Ali *et al*., 2021; Ahmad *et al*., 2021) and 44.13% in Tukiye (Arikan *et al*., 2024). Similar results 45% were also recorded by (AbdelKhalek *et al*., 2019) in Kuwait and 44% by (Algammal *et al*., 2020) in Egypt. This slight variation in the results of different studies can be attributed to the breed of animals, lactation stage, number of parity and hygienic management practices. Among the 156 subclinical mastitis samples, 45.5% were positive for presence of *Staphylococcus aureus* which is in line with the findings of 40% by (Shahid *et al*., 2023) and 42.5% by (Shah *et al*., 2025) in Pakistan, 42.31% by (Kaki *et al*., 2019) in Algeria, 37.6% by (AbdelKhalek *et al*., 2019) in Kuwait. The findings of (Ali *et al*., 2021; Ahmed *et al*., 2021) from Pakistan also favor the fact that *Staphylococcus aureus* is the most abundantly found bacteria among mastitis milk samples. The poor handling of animals, unhygienic practices and contamination of milking equipment and the hands of milking staff are responsible for increased prevalence of subclinical mastitis caused by *S. aureus* in dairy animals.

The drug susceptibility test conducted on *S. aureus* isolates showed 90 to 100% susceptibility to vancomycin, linezolid, gentamicin, ciprofloxacin, sulfamethoxazole/ trimethoprim and chloramphenicol. Similar findings were achieved by (Zihad *et al*., 2021); (Shahid *et al*., 2023); (Batool *et al*., 2025). In the present study 64.78% isolates were found resistant to tetracycline which coincides with the results of (Foster, 2017) with 59.5% and (Shahid *et al*., 2023) with 60%. The increased level of tetracycline resistance is due to the production of ribosomal protection protein produced by *S. aureus*, which competitively binds with tetracycline. Similarly,

46.47% isolates showed resistance to erythromycin which is in agreement with (Kumar *et al*., 2025) from India and (Shahid *et al*., 2025) from Pakistan. *The Staphylococcus aureus* commonly used an acquired erythromycin resistance mechanism through methylation of the 23S rRNA, preventing effective drug-ribosome interaction.

The findings of current study showed that 36.61% (26/71) *Staph aureus* isolates were resistant to oxacillin and cefoxitin (MRSA) while the remaining 45 were susceptible to this drug (MSSA). In Pakistan, very familiar results were achieved by (Shahid *et al*., 2023) with 30% and (Aqib *et al*., 2017) with 34% prevalence of MRSA. Additionally, the findings of (Kaur *et al*., 2021) in India and (Tesfaye *et al*., 2021) in Ethiopia showed 34.5% occurrence of methicillin resistant *Staph aureus*. In *Staphylococcus aureus*, methicillin

Table 1. Nucleotide sequences of primers used for PCR amplification.

| Name of Primer | Primer sequence | Product size | References |
|---|---|---|---|
| *nuc*-F | 5-GCGATTGATGGTGATACGGTT-3 | 270bp | Taqveem *et al*., 2024 |
| *nuc*-R | 5-AGCCAAGCCTTGACGAACTAAAGC-3 | | |
| *mec*A-F | 5-AAAATCGATGGTAAAGGTTGGC-3 | 533bp | Shahid *et al*., 2023 |
| *mec*A-R | 5-ATCTGTACTGGGTTAATC-3 | | |

Table 2. Antibiotic susceptibility profiling of *Staphylococcus aereus* isolates.

| Name of antibiotic | Susceptibility pattern | |
|---|---|---|
| | Sensitive | Resistant |
| Vancomycin | 71 (100%) | 00 (00%) |
| Linezolid | 71 (100%) | 00 (00%) |
| Gentamicin | 69 (97.18%) | 02 (02.82%) |
| Sulfamethoxazole/Trimethoprim | 68 (95.77%) | 03 (04.23%) |
| Ciprofloxacin | 66 (92.95%) | 05 (07.05%) |
| Chloramphenicol | 66 (92.95%) | 05 (07.05%) |
| Cefoxitin | 45 (63.39%) | 26 (36.61%) |
| Oxacillin | 45 (63.39%) | 26 (36.61%) |
| Erythromycin | 38 (53.53%) | 33 (46.47%) |
| Tetracycline | 25 (35.22%) | 46 (64.78%) |

Table 3. Biofilm formation in MRSA and MSSA isolates.

| Isolates | Strong biofilm | Moderate biofilm | Weak biofilm | None | Total positive |
|---|---|---|---|---|---|
| MRSA | 17 (65.38%) | 07 (26.92%) | 02 (7.69%) | 00 (0.0%) | 26 (100%) |
| MSSA | 20 (44.44%) | 11 (24.44%) | 06 (13.33%) | 08 (17.77%) | 37 (82.22%) |
| Total | 37 (52.11%) | 21 (29.57%) | 09 (12.67%) | 04 (5.63%) | 67 (94.36%) |

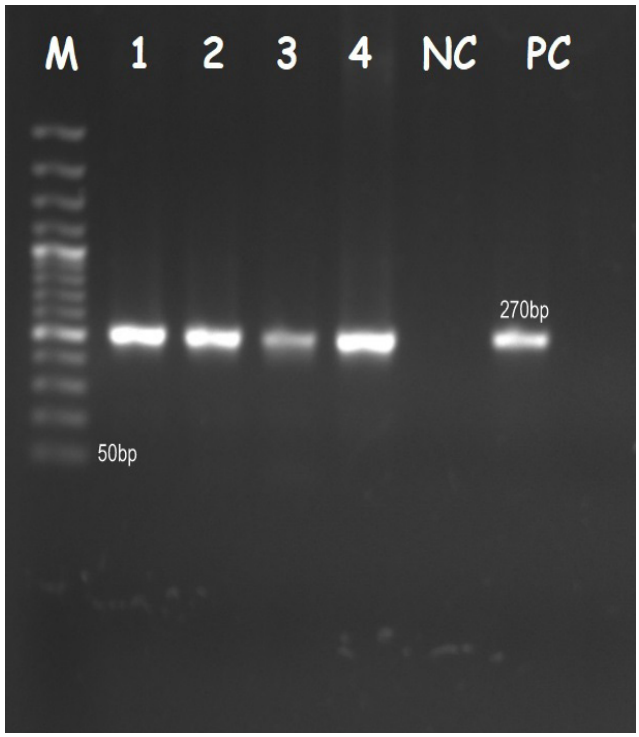


Figure 1A. Agarose gel electrophoresis displaying PCR product of *nuc* gene of *Staphylococcus aureus* having 280 bp band. M: 50bp Marker, 1 to 4: Positive-samples, PC: Positive- control, NC: Negative-control.

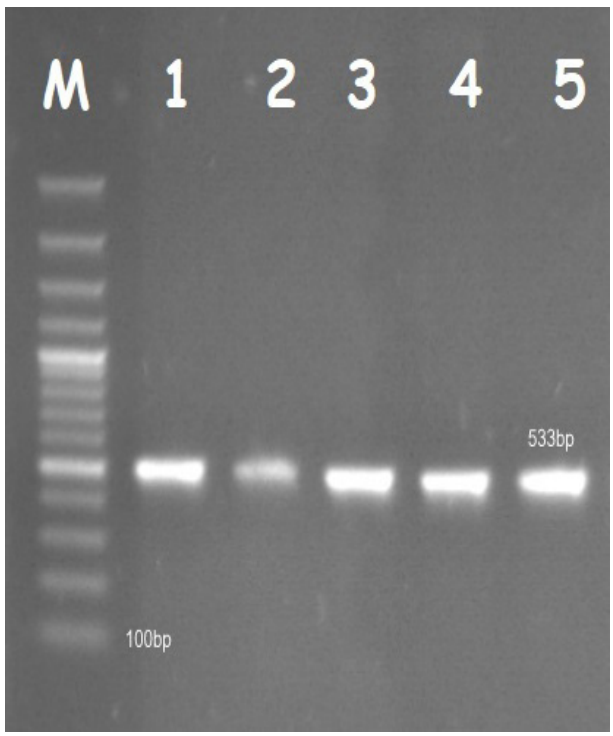


Figure 1B. *mec*A gene of *Staphylococcus aureus* having 533 bp band. M: 100bp Marker, 1 to 5: Positive-samples.

resistance arises from the acquisition of the mecA gene, which encodes the low-affinity penicillin-binding protein PBP2a and thereby confers resistance to virtually all β-lactam antibiotics. In the presence of *β*-lactamase enzymes, methicillin remains stable and functional in treating *S. aureus* infection but not against MRSA (Eid *et al*., 2019). The excessive use of antibiotics, including incorrect dosages, incomplete treatment, and use without sensitivity testing are the key factors responsible for emergence of antibiotic resistance and its spread to other organisms.

The biofilm forming ability of the *Staph aureus* isolates were detected using the microtiter plate assay and it was found that all the MRSA isolates showed variable degree of biofilm formation with 65.38% as strong and 26.92% as moderate biofilm producers. The results of (Verma *et al*., 2023) from India, (Ibraheim *et al*., 2023) from Iraq and (Kang *et al*., 2024) from Korea align closely with the findings of our study while the results of (Chowdhry *et al*., 2025) showed higher frequency (50%) of moderate biofilm producers. Biofilm-embedded *S. aureus* cells exhibit increased tolerance to β-lactam antibiotics compared to their planktonic counterparts. Biofilm formation acts as an additional resistance mechanism that synergizes with methicillin resistance to promote multidrug tolerance in *S. aureus.*

The increasing percentage of biofilm-forming MRSA among subclinical mastitis cases underscores the dual challenge posed by antimicrobial resistance and biofilm-associated tolerance, reinforcing the importance of improved surveillance and herd management practices. The strong association between biofilm formation and methicillin resistance in *S. aureus* highlights the multifactorial nature of its persistence and pathogenicity, emphasizing the need for therapeutic strategies that target both mechanisms simultaneously.

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