Zand Molecular Microbiology
Volume 1 Number 1 (August 2025) 6 - 14
DOI: https://doi.org/10.22034/zmm.2025.724155

Original Article

Assessment the Role of ESBL Genes and Biofilm Production in Antibiotic-Resistant *Staphylococcus aureus* Clinical Isolates

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Received: *June 2025,* ***Accepted:*** *August 2025*

Abstract

Background & Objectives: *Staphylococcus aureus*, a significant pathogen in both healthcare and community settings, is known for its ability to cause a wide range of infections. This study aimed to investigate the antibiotic resistance profiles and the presence of key β-lactamase genes in *Staphylococcus aureus* isolates from clinical samples.

Materials & Methods: A total of 60 *Staphylococcus aureus* isolates were recovered from 200 clinical samples, which included both wound and nasal swabs. Their resistance to antibiotics was analyzed using disk diffusion testing. Biofilm production was quantitatively measured through a modified colorimetric microtiter plate method. Additionally, polymerase chain reaction (PCR) was utilized to detect the presence of the *blaTEM, blaCTX*, and *blaHSV* genes.

Results: High resistance was found against Erythromycin, Tetracycline, and Clindamycin, while Cotrimoxazole, Ampicillin, Doxycycline, Ciprofloxacin, and Vancomycin showed the highest sensitivity. The study further assessed biofilm production, finding that a larger proportion of Methicillin-resistant *Staphylococcus aureus* (MRSA) isolates exhibited strong biofilm formation, which may contribute to their persistence and resistance in clinical settings. Among β-lactamase genes, *blaHSV* gene was the most prevalent (61.66%), followed by *blaTEM* (38.33%) and *blaCTX* (36.66%).

Conclusion: These findings highlight the high prevalence of Methicillin-resistant *Staphylococcus aureus* in the studied clinical samples, the significant role of biofilm formation in resistance, and the potential challenges in treating infections due to β-lactamase genes. The study emphasizes the need for enhanced infection control protocols and the development of novel therapeutic strategies to manage resistant *Staphylococcus aureus* infections effectively.

Keywords: *Staphylococcus aureus*, β-lactamase enzymes Biofilm, Multidrug resistance.

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Introduction

Staphylococcus aureus (*S. aureus*), a pathogen acquired in hospitals and communities, is responsible for a wide range of diseases, including skin and soft tissue infections, endocarditis, and severe pneumonia (1). Its pathogenicity is linked to various enzymes and toxins, such as enterotoxins, exfoliative toxin, toxic shock syndrome toxin, and Panton-Valentine leucocidin (2). This bacterium possesses an astonishing ability to adapt to different antibiotics; and the rise of multi-drug resistant (MDR) strains of *S. aureus* poses a significant threat to public health. Methicillin-resistant *S. aureus* (MRSA) differs from methicillin-susceptible *S. aureus* (MSSA) in several aspects (3). The detection of methicillin resistance in *S. aureus* initially occurred in Europe during the 1960s, merely one year after the introduction of methicillin. Currently, MRSA isolates are not only prevalent in hospitals worldwide but also in communities, exhibiting resistance to multiple antibiotics. Clinical infections primarily occur among patients in hospital intensive care units, nursing homes, and other long-term care facilities. However, MRSAs are increasingly emerging as significant pathogens in community-acquired infections as well (4). MRSA has been linked to higher rates of antibiotic exposure, leading to increased selection pressure for resistance. This is due to the limited treatment options for MRSA infections, as it is resistant to methicillin and other beta-lactam antibiotics. Furthermore, MRSA infections often result in prolonged hospital stays compared to MSSA infections. The management of MRSA infections can be more challenging, requiring specialized antibiotics and medical interventions (3,5). Efforts to prevent and manage MRSA infections are crucial to minimize the impact on patients, reduce healthcare-associated transmission, and preserve the effectiveness of antibiotics. Methicillin-resistant *S. aureus* strains have the capacity to thrive in the presence of methicillin, oxacillin, and nafcillin. Antimicrobial resistance poses a significant challenge to the healthcare sector in Iran (6,7). The formation of biofilms by *Staphylococcus* strains has been known for years as the main cause of infections caused by biofilms of *S. epidermidis* and *S. aureus* (8). Treatment of infections caused by biofilm production is often difficult because biofilm matrix and phenotypic properties of bacteria cause resistance to host immune response and the function of antimicrobial drugs. Medical instruments are at risk of being colonized by *S. aureus*. The ability to form biofilms on the surfaces of medical devices such as catheters and artificial heart valves varies in *S. aureus* and *S. epidermidis* strains (9). The objectives of this study were determination of antibiotic resistance pattern, biofilm formation and frequency of *blaTEM, blaCTX* and *blaSHV* genes in clinical *S. aureus* isolates.

Materials and Methods

A) Isolation of Staphylococcus aureus: In this cross-sectional descriptive study, a total of 200 clinical specimens were collected during a four-month period from January to April 2024 from Motahari and Shariati hospitals in Tehran, Iran. Specimens, including wound and nasal swabs, were collected using sterile cotton swabs and immediately placed into sterile transport tubes containing Stuart transport medium. They were transported to the laboratory within 2 hours at 4°C to maintain sample integrity and were then streaked onto Mannitol Salt Agar for the isolation of

Staphylococcus aureus. The plates were incubated at 37°C for 24 hours under aerobic conditions. Colonies displaying characteristic golden-yellow pigmentation and mannitol fermentation (yellow zones around colonies) were presumptively identified as *S. aureus*. Obtained colonies were further streaked onto Blood agar to observe hemolytic patterns and ensure pure culture isolation. Plates were incubated at 37°C for 24 hours under aerobic conditions. Beta-hemolysis was recorded as a confirmatory trait for *S. aureus*. Microscopic identification was done by gram staining (10). A total of 200 clinical samples submitted to the microbiology laboratory underwent biochemical and bacteriological testing.

B) Antimicrobial susceptibility test: To determine antimicrobial resistance, isolated strains were subjected to disk diffusion assays on Mueller-Hinton agar following CLSI guidelines. A bacterial suspension was prepared by adjusting the turbidity to 0.5 McFarland standard using sterile saline, and then inoculated evenly onto Mueller-Hinton agar plates using a sterile swab (11). The antibiotic disks (Padtan Teb. Co., Iran) were used included, Rifampin, Clindamycin, Ampicillin (10 µg), Vancomycin (30 µg), Cotrimoxazole (25 µg), Ciprofloxacin (5 µg), Erythromycin (15 µg), Cephalexin (30 µg), Doxycycline (30 µg), Oxacillin (1 µg) and Tetracycline (30 µg). Following a 24-hour the incubation period at 37°C, the inhibition zone surrounding each disk was measured and recorded. Results were reported as susceptible (S), intermediate (I), or resistant (R).

C) Biofilm formation: Biofilm formation was quantitatively evaluated using a modified colorimetric microtiter plate assay (12). An overnight culture of bacteria was adjusted to the turbidity of a 0.5 McFarland standard and then diluted 1:100 in 200 µL of nutrient broth (Merck, Germany). The diluted suspensions were transferred to sterile flat-bottomed 96-well polystyrene microplates (JET Biofil, Guangzhou, China) and incubated for 24 h at 37 °C. Subsequently, the wells were washed three times with sterile phosphate-buffered saline (pH 7.1), and the adherent biofilms were fixed with 99% methanol for 15 minutes. After fixation, the biofilms were stained with 0.1% crystal violet (Sigma, USA) for 5 minutes at room temperature. Following staining, the wells were rinsed with water and air-dried. The biofilms in each well were distained with 95% ethanol for 30 minutes, and the optical density (OD) of the distained solution was measured at 570 nm using a microtiter plate reader (Dena, Iran). All experiments were conducted in triplicate and repeated three times for accuracy. A cut-off value (ODc) was determined as three standard deviations (SD) above the mean OD of the negative control: ODC = average OD of negative control + (3 × SD of negative control). Based on the OD values obtained, the isolates were categorized into four groups:

1. OD ≤ OD control (ODc) = Negative,
2. ODc < OD ≤ 2ODc = Weak positive,
3. 2ODc < OD ≤ 4ODc = Positive and
4. 4OD < ODc = Strong positive (12).

D) Detection of blaTEM, blaCTX, and blaHSV genes: Sixty *Staphylococcus aureus* isolates were sub-cultured onto nutrient broth and incubated at 37°C for 24 hours for subsequent molecular analysis. DNA extraction was done by commercial kit (Karmania Pars Gene, Iran) based on instruction. The prevalence of *blaTEM*, *blaCTX*, and *blaHSV* genes was assessed in *S. aureus* isolates. Polymerase chain reaction (PCR) amplification was carried out in a total reaction volume of 25 µL, comprising 1 µL of target DNA, 12.5 µL of

2X Master Mix (CinaGen, Iran), 1 µL of each forward and reverse primer (20 pM), and 9.5 µL of double-distilled water. Gene-specific primers were utilized to detect *blaTEM*, *blaCTX*, and *blaHSV*, as listed in Table 1 (13). The thermal cycling conditions for all target genes included an initial denaturation step at 95 ºC for 5 minutes, followed by 40 cycles of denaturation at 95 ºC for 40 seconds, annealing at 56 ºC for 1 minute, and extension at 72 ºC for 2 minutes, with a final extension at 72 ºC for 5 minutes. The PCR-amplified products were analyzed via gel electrophoresis on a 1.5% agarose gel in TBE buffer, incorporating ethidium bromide staining. A 10 µL aliquot of the final reaction mixture was loaded onto the gel, and the DNA bands were visualized under UV illumination. To estimate the molecular sizes of the amplified fragments, a 50 bp DNA ladder (Sinaclone, Iran) was used as a reference (13).

Table 1. List of genes and primers for PCR.

Genes	Primer sequence (5' to 3')	Product size (bp)
blaTEM	F: TCGGGGAAATGTGCGCG R: TGCTTAATCAGTGAGGCACC	972
blaCTX	F: ACGCTGTTGTTAGGAAGTG R: TTGAGGCTGGGTGAAGT	857
blaHSV	F: GGGTTATTCTTATTTGTCGC R: TTAGCGTTGCCAGTGCTC	615

Results

Out of 200 samples, 60 isolates of *S. aureus* were successfully identified and isolated. These isolates were obtained from various sample sources, with the highest prevalence observed in skin wound samples (55%) and nasal swabs (45%).

A) Antibiotic resistance pattern: Among the 60 *Staphylococcus aureus* isolates, antibiotic susceptibility testing revealed variable resistance profiles across 11 antibiotics (Fig 1). High resistance rates (≥30%) were observed for Erythromycin (55%), Tetracycline (38.33%), and Clindamycin (35%), indicating these antibiotics may be less effective against clinical *S. aureus* infections in this region. In contrast, lower resistance rates (<25%) were recorded for Cotrimoxazole (13.33%), Ampicillin (16.67%), Ciprofloxacin (23.33%), and Doxycycline (18.33%), suggesting these agents retain higher effectiveness. Rifampin (13.33%), Oxacillin (18.33%), and Cephalexin (18.33%) demonstrated moderate resistance levels. Vancomycin showed a relatively low resistance of 10%, supporting its continued utility for serious infections. Notably, based on resistance and intermediate susceptibility to Oxacillin, 63.33% of the isolates were categorized as Methicillin-resistant *S. aureus* (MRSA), while the remaining 36.67% were identified as Methicillin-susceptible *S. aureus* (MSSA). This high prevalence of MRSA underscores the need for stringent infection control measures and informed selection of antibiotics.

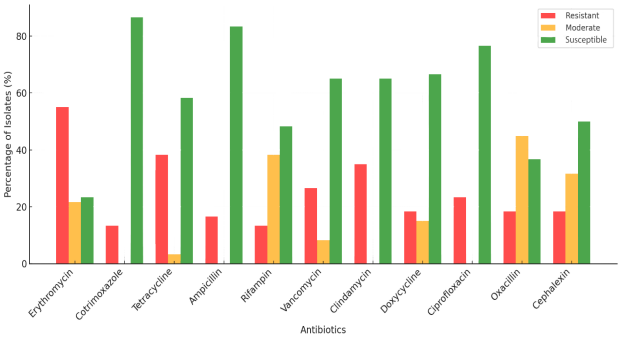

Fig 1. The susceptibly pattern of 60 *S. aureus* isolates to 11 antibiotics.

B) Assessment of biofilm formation ability: According to Fig 2, 60% of MRSA isolates exhibited strong biofilm formation, while only 10% of MSSA isolates showed strong biofilm activity. Notably, 45% of MSSA strains showed no biofilm production, compared to

only 5% of MRSA strains, reinforcing the link between methicillin resistance and biofilm-forming capability. In contrast, MSSA strains generally exhibited weaker biofilm production compared to MRSA. A significant number of MSSA isolates failed to form biofilms, and only a small fraction of MSSA strains displayed strong biofilm formation (Fig 2).

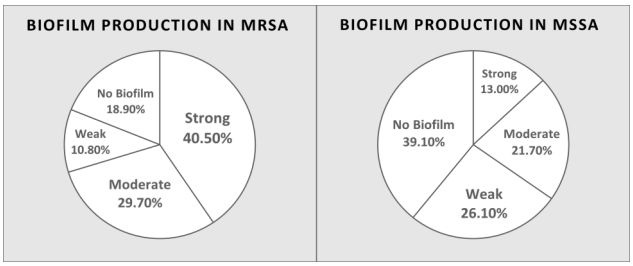

Fig 2. Biofilm production in MRSA and MSSA isolates.

C) The presence of bla TEM, bla CTX, bla HSV genes: The occurrence and proportion of virulence genes *(blaTEM, blaCTX,* and *blaHSV*) were evaluated in *S. aureus* strains. The *blaHSV* gene is the most frequently observed, appearing in 61.66% of the isolates. The *blaTEM* gene was identified in 38.33% of isolates, suggesting a moderate prevalence. Similarly, the *blaCTX* gene was present in 36.66% of isolates, exhibiting a comparable occurrence to *blaTEM* (Fig 3 and 4).

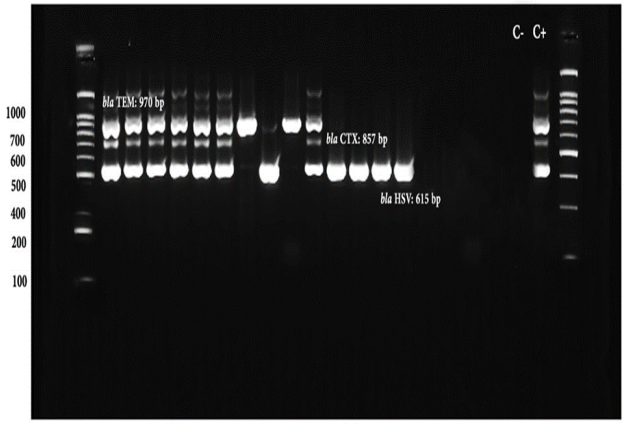

Fig 3. Multiplex PCR product electrophoresis of *blaTEM*, *blaCTX*, *blaHSV* genes in *S. aureus*.

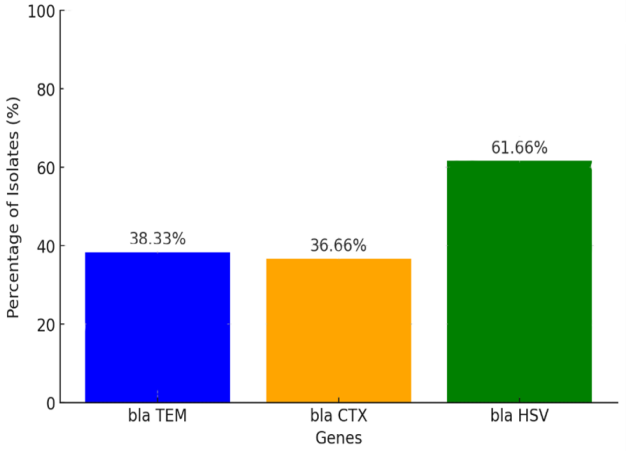

Fig 4. Prevalence of *blaTEM*, *blaCTX*, *blaHSV* genes in *S. aureus*.

Discussion

The analysis demonstrates Cotrimoxazole (86.67% sensitivity), Ampicillin (83.33%), Ciprofloxacin (76.67%), and Doxycycline (81.67%) as the most effective antibiotics against *S. aureus*. Vancomycin showed 73.33% sensitivity, indicating good but not absolute effectiveness. These antibiotics should be prioritized for treatment, particularly in cases where antibiotic susceptibility testing is not readily available. However, Vancomycin with moderate resistance (26.67%) raises concerns, suggesting that while it remains effective, its continued use should be closely monitored to prevent the emergence of Vancomycin-Resistant *S. aureus* (VRSA). Several antibiotics demonstrated high resistance levels, making them less suitable for empirical therapy. Erythromycin (55% resistance), Tetracycline (38.33% resistance), and Clindamycin (35% resistance) exhibited significant resistance rates, limiting their effectiveness. Additionally, Rifampin and Oxacillin showed high moderate resistance levels, indicating the possibility of further resistance development (7,10). The widespread resistance to these antibiotics underscores the necessity of antimicrobial stewardship

programs to ensure their judicious use and prevent further resistance escalation (10,14). A particularly concerning observation is the high proportion of isolates (63.33%) showing resistance or intermediate response to Oxacillin, indicating the prevalence of MRSA in the studied clinical population. The findings of this study align with several recent investigations into the antibiotic resistance patterns of *Staphylococcus aureus*. In a study, MRSA isolates exhibited significant resistance to Penicillin (97.8%), Erythromycin (71.1%), Clindamycin (71.1%), and Tetracycline (32.1%). Notably, MRSA isolates in this study were highly sensitive to Levofloxacin, highlighting the importance of regional surveillance in guiding empirical therapy (14). In a study by Abbasi et al., 36 out of 88 *Staphylococcus aureus* isolates (40.9%) were identified as MRSA, highlighting a notable prevalence that raises clinical concern. These MRSA strains demonstrated resistance not only to methicillin but also to multiple other antibiotics, complicating treatment strategies. Notably, high resistance rates to Gentamicin (83.3%) and Erythromycin (55%) were reported among the MRSA isolates, suggesting limited effectiveness of these agents. On a positive note, no resistance to Vancomycin was detected, reaffirming its continued reliability as a key therapeutic option against MRSA infections (15). Similarly, in over 25% of MRSA isolates were resistant to Ampicillin, Ciprofloxacin, Cotrimoxazole, Erythromycin, Clindamycin, Azithromycin, and Tetracycline. This underscores the growing concern over multidrug-resistant *S. aureus* strains and the necessity for alternative treatment options (16). These studies collectively highlight the critical need for ongoing surveillance of antibiotic resistance patterns in *S. aureus* to inform treatment strategies and mitigate the spread of resistant strains. In addition to the findings in our dataset, several other studies have investigated biofilm production in MRSA and MSSA, offering comparative insights into the biofilm-forming capacity of these two groups of bacteria. Purbowati (2019) found that MRSA and MSSA both had moderate biofilm-forming abilities, although MRSA exhibited a stronger biofilm formation capacity in some isolates. The study highlighted the role of the *icaA* and *icaD* genes in biofilm production, with MRSA strains showing higher expression levels of these genes compared to MSSA isolates, which are aligning with our observation that MRSA exhibited stronger biofilm formation (17). Nuryastuti *et al* (2015). revealed that while MRSA strains had significantly more antibiotic resistance, biofilm formation was predominantly observed in MSSA strains, with all MSSA isolates forming biofilms (100%) in contrast to just 10% of MRSA strains. This study points to variability in biofilm formation that isn't solely tied to methicillin resistance but may also involve other genetic factors (18). McCarthy et al. (2015) discussed the mechanisms behind biofilm formation in MRSA and MSSA, noting that MRSA strains are more likely to use a polysaccharide intercellular adhesin as PIA-independent mechanism for biofilm formation. This mechanism differs from the PIA-dependent pathways seen in MSSA strains (19). The comparison with other studies which supports our findings, MRSA is generally a stronger biofilm producer than MSSA. However, there is variability across studies, with some reporting stronger biofilm formation in MSSA. Differences in study results may stem from the methods used, genetic variability between isolates, and environmental factors influencing

biofilm formation. The presence of β-lactamase genes such as *blaTEM, blaCTX*, and *blaHSV* in *S. aureus* isolates signifies a substantial production of β-lactamases, enzymes that degrade β-lactam antibiotics, rendering them ineffective. Notably, *blaTEM* and *blaCTX* are frequently linked to the production of extended-spectrum β-lactamases (ESBLs), which confer resistance to a wide array of β-lactam antibiotics, including Penicillins and Cephalosporins (20). The high prevalence of the *blaHSV* gene (61.66%) suggests its significant role in β-lactam resistance among these isolates. This prevalence indicates increased resistance to Penicillins and Cephalosporins, potentially compromising the efficacy of standard treatments. Clinically, the presence of these resistance genes raises concerns about potential treatment failures and the necessity for alternative therapies, such as glycopeptides (e.g., Vancomycin) or combination therapies (13,20). Additionally, detection of these genes' points to a higher risk of multi-drug resistant *S. aureus* (MDR-SA) strains, which can complicate treatment protocols in healthcare settings. These studies collectively highlight the critical need for ongoing surveillance of antibiotic resistance patterns and the implementation of effective infection control measures to combat the spread of resistant *S. aureus* strains. In the study by Poorabbas *et al*., 55.2% of *S. aureus* isolates were found to be MRSA and 41% of *Enterobacter* spp. were identified as ESBL producers. These results emphasize the urgent need for robust infection control practices and the careful, responsible use of antibiotics to prevent the further spread of resistant strains (21). Biofilm formation plays a vital role in bacterial survival, antibiotic resistance, and disease-causing potential. In this study, the pronounced biofilm-producing capacity of MRSA may contribute to its increased resistance to antibiotics and prolonged presence in infections. The biofilm acts as a shield, protecting bacteria from immune system attacks and antimicrobial treatments, complicating eradication (17). While MSSA demonstrates both strong and moderate biofilm production in some cases, a significant proportion of strains exhibit no biofilm formation, indicating variability in their biofilm-producing potential, which could influence their pathogenicity. The enhanced biofilm formation in MRSA may be a key factor in its association with persistent and recurrent infections, as biofilms create a protective environment that makes treatment more challenging.

Conclusion

The presence of MRSA strains underscores the need for strict infection control protocols in healthcare environments, which should include regular screening, isolation procedures, and effective antibiotic stewardship. The findings emphasize the considerable impact of β-lactamase-mediated resistance in *S. aureus* isolates. Ongoing monitoring and the development of novel treatment strategies are crucial steps to counteracting the rising resistance levels and preserving the effectiveness of existing antibiotics. The results indicate that MRSA is more prone to forming robust biofilms compared to MSSA, a factor that likely contributes to its enhanced resistance and persistence in clinical infections. A deeper understanding of these differences can inform the creation of improved therapeutic approaches aimed at biofilm-related infections. Further research needs to be directed towards the discovery of new antibiotics that are effective against MRSA, as well as adjunct

therapies that could enhance the efficacy of existing antibiotics. Combination therapies that target both the bacterial cell and biofilm may offer promising results.

Acknowledgments

The authors would like to express their sincere gratitude to the staff of the microbiology laboratories at Motahari and Shariati Hospitals in Tehran for their valuable assistance in sample collection and processing. We also appreciate the support provided by the Islamic Azad University for access to laboratory facilities and research materials essential to this study.

Conflict of interest

The authors declare that there is no conflict of interest regarding the publication of this article.

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