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MOLECULAR INSIGHTS AND ANTIMICROBIAL RESISTANCE PATTERNS OF BIOFILM-PRODUCING STAPHYLOCOCCUS AUREUS IN CLINICAL ISOLATES

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ABSTRACT

Background: Biofilm-forming Staphylococcus aureus complicates treatment by promoting tolerance and resistance to antimicrobials. This study investigated the association between biofilm formation, biofilm-associated genes, and antibiotic susceptibility in clinical S. aureus isolates from Faisalabad hospitals.
Methods: A total of 3300 samples (wound swabs, catheter tips, sputum, and pus) were collected, yielding non-duplicate 1000 S. aureus isolates, identified phenotypically and through nuc-PCR. Biofilm production was assessed using Congo Red Agar and microtiter plate assays, and isolates were tested for ica operon (icaA, icaB, icaC, icaD) and fibronectin-binding genes (fnbA, fnbB) via PCR. Antimicrobial susceptibility was evaluated using modified Kirby–Bauer disk diffusion method (cefoxitin 30 µg, fusidic acid 10 µg, erythromycin 15 µg, ciprofloxacin 5 µg, linezolid 30 µg, trimethoprim–sulfamethoxazole 25 µg, clindamycin 2 µg, levofloxacin 5 µg) following CLSI guidelines; Vancomycin minimum inhibitory concentrations (MICs) were determined by broth microdilution. Multidrug resistance (MDR) was defined as resistance to three or more antimicrobial classes. Selected isolates showing phenotypic resistance were tested for mecA and vanA, and representative amplicons underwent Sanger sequencing.
Results: Among 1000 isolates, 760 (76.0%) were methicillin-resistant, determined by cefoxitin resistance. High resistance rates were observed for cefoxitin (760/1000, 76.0%), ciprofloxacin (750/1000, 75.0%), erythromycin (700/1000, 70.0%), levofloxacin (650/1000, 65.0%), clindamycin (500/1000, 50.0%), and fusidic acid (590/1000, 59.0%). Resistance to trimethoprim–sulfamethoxazole was 400/1000 (40.0%), while linezolid resistance was rare (20/1000, 2.0%).
Vancomycin MIC testing classified 140/1000 (14.0%) isolates as vancomycin-resistant S. aureus (VRSA; MIC ≥16 µg/mL), 300/1000 (30.0%) as vancomycin-intermediate S. aureus (VISA; MIC 4–8 µg/mL), and 560/1000 (56.0%) as vancomycin-susceptible S. aureus (VSSA).
Biofilm phenotyping identified strong producers (n = 550), intermediate producers (n = 150), and non-producers (n = 300). Strong biofilm producers showed significantly higher MDR prevalence (480/550; 87.3%) compared with non-producers (140/300; 46.7%) ($\chi^2 = 32.43$, $p < 0.001$) and a higher rate of vancomycin non-susceptibility (VRSA + VISA: 270/550; 49.1%) than non-producers (60/300; 20.0%) ($p < 0.001$). The presence of icaA and icaD genes was strongly associated with the strong biofilm phenotype ($p < 0.001$). Molecular analysis of 150 resistant isolates detected mecA in 50 (33.3%) and vanA in 45 (30.0%), and sequencing confirmed the identity of the amplified genes.
Conclusions: High MRSA prevalence and significant vancomycin non-susceptibility were found. Strong biofilm formation, particularly in icaA/icaD-positive isolates, associates with MDR and reduced vancomycin susceptibility, highlighting the need for targeted infection control and antimicrobial stewardship.

Keywords: Staphylococcus aureus, MRSA, VRSA, antimicrobial resistance, gene detection.

1. INTRODUCTION

Staphylococcus aureus (S. aureus) remains a significant cause of infections in hospital and community settings, affecting humans and animals. Approximately 30% of individuals are asymptomatic carriers of S. aureus in their normal microbial flora [1, 2]. This opportunistic pathogen causes a spectrum of diseases, including pneumonia, endocarditis, osteomyelitis, meningitis, skin and soft tissue infections, sepsis, food poisoning, and toxic shock syndrome [3].

Horizontal transfer of resistance genes among Staphylococcus species results in resistance to various antibiotic classes, including methicillin, macrolides, lincosamides, aminoglycosides, and their combinations [4] Methicillin resistance is primarily due to the mecA gene, which encodes the altered penicillin-binding protein PBP2a, enabling resistance to β-lactam antibiotics [5].

The escalating resistance to antimicrobials among pathogenic and commensal bacteria has become a pressing concern. Glycopeptide antibiotics, like vancomycin, have been the cornerstone for treating methicillin-resistant Staphylococcus aureus (MRSA) infections [6] However, overuse of these agents has led to vancomycin-resistant S. aureus (VRSA) strains. VRSA strains acquire resistance through genes such as vanA and vanB from Enterococcus species [7].

Increased MRSA prevalence has led to higher vancomycin usage, accelerating the emergence of vancomycin-resistant phenotypes in MRSA populations [8]. Studies have documented MRSA strains from diverse clinical environments, showing the organism's distribution and resistance capabilities [9]. This study aimed to investigate antibiotic resistance profiles of S. aureus strains from clinical samples, identify resistance-conferring genes (mecA, vanA, icaABCD), and evaluate alternative therapeutic agents against MRSA and VRSA isolates [10].

2. MATERIALS AND METHODS

2.1 Sample collection and identification

3300 clinical specimens, comprising wound swabs, catheter tips, sputum, and pus, were obtained from patients in Faisalabad hospitals. The specimens were collected using sterile cotton swabs, placed in labeled screw-capped bottles with patient identification and clinical details, then transported to the microbiology laboratory. Each specimen was inoculated onto Mannitol Salt Agar (MSA) plates and incubated at 37 °C for 18 to 24 hours. Colonies suspected to be Staphylococcus aureus were identified by colony morphology, pigmentation, Gram staining, and standard biochemical tests. From these specimens, 1,000 non-duplicate S. aureus isolates were recovered and selected for further phenotypic and molecular analyses.

2.2 Confirmation of Staphylococcus aureus by PCR

Genomic DNA was extracted from suspected S. aureus colonies using the QIAamp DNA Mini Kit (Qiagen Inc.) according to manufacturer's instructions. DNA concentration and purity were evaluated spectrophotometrically by measuring absorbance at 260 nm and determining A260/A280 ratio using a NanoDrop device (Biotek, USA). The DNA was stored at −20 °C until required. Species verification was performed through PCR amplification of the nuc gene using specific primers. PCR reactions (50 µL total) contained 25 µL PCR master mix (Thermo Scientific, USA), 5 µL template DNA, 1 µL each of forward and reverse primers, and 18 µL PCR-grade water. Thermal cycling began with initial denaturation at 94 °C for 3 minutes, followed by 35 cycles of denaturation at 94 °C for 30 seconds, annealing at 55 °C for 30 seconds, extension at 72 °C for 45 seconds, and final extension at 72 °C for 7 minutes. PCR products were separated by electrophoresis on 1% agarose gel and visualized after ethidium bromide staining.

2.3 Phenotypic detection of biofilm production

Biofilm formation was assessed using both Congo Red Agar (CRA) method and microtiter plate assay. In the CRA method, bacterial strains were cultured on Brain Heart Infusion (BHI) agar with 5% sucrose and Congo Red. The CRA medium comprised BHI (37 g/L), sucrose (50 g/L), agar No. 1 (10 g/L), and Congo Red stain (0.8 g/L). Congo Red solution was added to the medium before plate preparation, and plates were incubated aerobically with bacteria at 37 °C for 24 to 48 hours. Biofilm-producing strains appeared as black colonies with dry, crystalline texture, while non-producers formed red colonies. In the microtiter plate assay, isolates from fresh agar plates were transferred to broth and incubated for 24 hours at 37 °C, then diluted 1:100 in fresh medium. Sterile 96-well tissue culture plates were filled with 0.2 mL of diluted cultures, with broth-only wells serving as controls. After 24-hour incubation at 37 °C, planktonic contents were removed, wells were washed, and adherent cells were fixed and stained with 1% crystal violet. Plates were left at room temperature for 15 minutes, rinsed with water to remove excess stain, and air-dried. The optical density (OD) of each well was measured using an ELISA plate reader (Biotek, USA). An OD cut-off of 0.240 distinguished biofilm producers from non-producers, with strains showing OD < 0.120 considered non-biofilm forming (Nourbaksh & Namvar, 2016).

2.4 Detection of biofilm-associated genes

Genes implicated in biofilm formation were identified using PCR with primers specific to the ica operon (icaA, icaB, icaC, icaD) and fibronectin-binding protein genes (fnbA and fnbB).

2.4.1 Detection of icaA gene

Polymerase chain reaction (PCR) was carried out using an Eppendorf Mastercycler® gradient thermal cycler. The amplification profile for the icaA gene began with an initial denaturation step at 94 °C for 5 minutes, followed by 30 successive cycles of denaturation at 94 °C for 1 minute, primer annealing at 55 °C for 1 minute, and extension at 72

°C for 1 minute. A final elongation step was performed at 72 °C for 10 minutes. PCR amplicons were resolved on 1.4% agarose gels, stained with GelRed, and visualized under UV illumination.

2.4.2 Detection of icaB gene

The icaB gene was amplified under the following conditions: initial denaturation at 94 °C for 5 minutes, followed by 30 cycles consisting of denaturation at 94 °C for 1 minute, annealing at 52 °C for 30 seconds, and extension at 72 °C for 90 seconds. The amplification concluded with a final extension at 72 °C for 10 minutes.

2.4.3 Detection of icaC gene

For icaC, the PCR program started with an initial denaturation step at 94 °C for 5 minutes. This was followed by 30 cycles comprising denaturation at 94 °C for 1 minute, annealing at 55 °C for 30 seconds, and extension at 72 °C for 30 seconds. A final extension was carried out at 72 °C for 10 minutes.

2.4.4 Detection of icaD gene

Amplification of the icaD gene involved an initial denaturation at 94 °C for 5 minutes, then 30 cycles of denaturation at 94 °C for 30 seconds, annealing at 55 °C for 30 seconds, and extension at 72 °C for 1 minute. A final extension step of 10 minutes at 72 °C was included.

2.4.5 Detection of mecA and vanA genes

PCR reactions were carried out in a final volume of 25 µL, containing 12.5 µL of 2× WizPure PCR Master Mix (Wiz Bio Solutions), 10 pmol of each forward and reverse primer, and 25 ng of genomic DNA template. Amplifications were performed in a Bio-Rad T100™ thermal cycler using the following program: an initial denaturation step at 94 °C for 5 minutes; 40 amplification cycles consisting of denaturation at 94 °C for 30 seconds, primer annealing at 57 °C for mecA or 58 °C for vanA for 1 minute, and extension at 72 °C for 1 minute; followed by a final extension at 72 °C for 5 minutes. PCR products were subsequently examined by agarose gel electrophoresis to verify the presence of the expected amplicons. The primer sequences and expected product sizes for all target genes are summarized in Table 1.

Table 1. Primers Used for Detection of Resistance Genes

Target Gene	Primer Direction	Nucleotide Sequence (5′→3′)	Amplicon Size (bp)	Reference
icaA	Forward	TCTCTTGCAGGAGCAATCAA	188	[11]
	Reverse	TCAGGCACTAACATCCAGCA		
icaB	Forward	AGAATCGTGAAGTATAGAAAATT	900	[11]
	Reverse	TCTAATCTTTTTCATGGAATCCGT		
icaC	Forward	ATGGGACGGATTCCATGAAAAAGA	1100	[11]
	Reverse	TAATAAGCATTAATGTTCAATT		
icaD	Forward	ATGGTCAAGCCCAGACAGAG	198	[11]
	Reverse	CGTGTTTTCAACATTTAATGCAA		
mecA	Forward	AGAAGATGGTATGTGGAAGTTAG	583	
	Reverse	ATGTATGTGCGATTGTATTGC		
vanA	Forward	GGCAAGTCAGGTGAAGATG	713	[11]
	Reverse	ATCAAGCGGTCAATCAGTTC		

2.5 Antibiogram analysis of biofilm-producing Staphylococcus aureus

Antibiogram analysis of biofilm-producing Staphylococcus aureus was performed using the modified Kirby–Bauer disk diffusion method, with results interpreted per CLSI (2024) guidelines. Antibiotics tested included cefoxitin (30 µg), fusidic acid (10 µg), erythromycin (15 µg), ciprofloxacin (5 µg), linezolid (30 µg), trimethoprim–sulfamethoxazole (25 µg), clindamycin (2 µg), and levofloxacin (5 µg). Isolates were classified as multidrug resistant (MDR) if resistant to three or more antibiotic classes. Vancomycin minimum inhibitory concentrations (MICs) were determined using broth micro dilution with Mueller–Hinton broth and antibiotic stock solutions, following CLSI (2024) guidelines.

2.6 Statistical analysis

Statistical analysis used Minitab statistical software (Minitab 13.1, Minitab Inc., USA). Descriptive statistics were used to summarize frequencies and proportions, while inferential statistical tests (chi-square test) were applied to evaluate differences between groups. A p-value of < 0.05 was considered statistically significant.

3. RESULTS

Isolates (n=1000) were analyzed, and S. aureus isolates were obtained and examined (refer to Methods). Biofilm phenotype was assessed using microtiter plate assay, with OD thresholds specified in Methods. For antibiogram

comparison, we focused on isolates identified as strong biofilm producers (OD ≥ 0.240; n = 550) versus non-producers (OD < 0.120; n = 300). Intermediate/weak producers (n = 150) were omitted from the comparison below to highlight antibiogram results as outlined by the synopsis methodology.

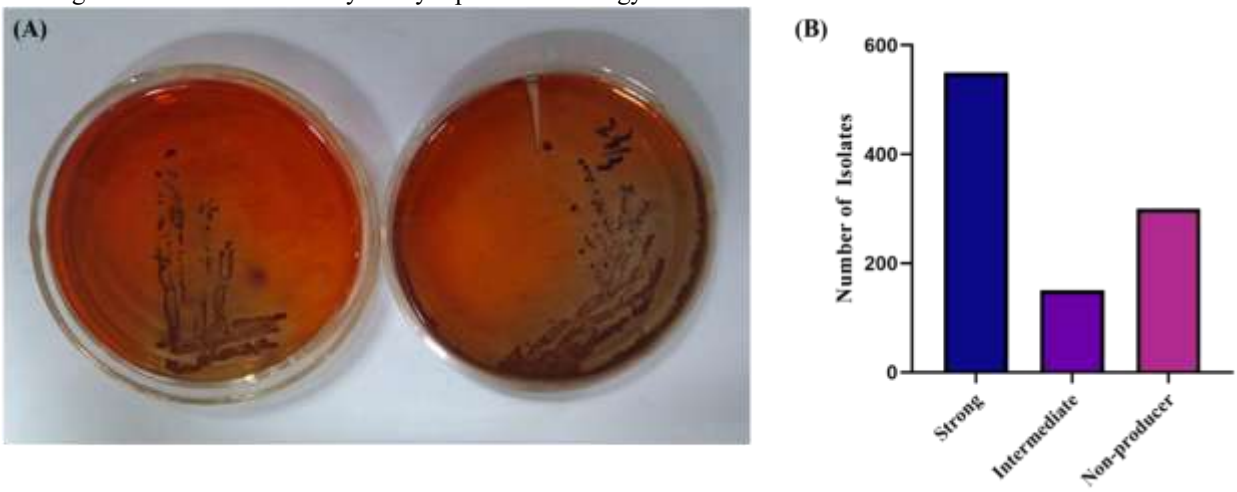

Figure 1. Phenotypic detection of biofilm formation in Staphylococcus aureus using Congo Red Agar (CRA). Biofilm-producing isolates formed black colonies with a dry crystalline morphology, while non-biofilm-producing isolates formed smooth red colonies after incubation at 37 °C for 24–48 h.

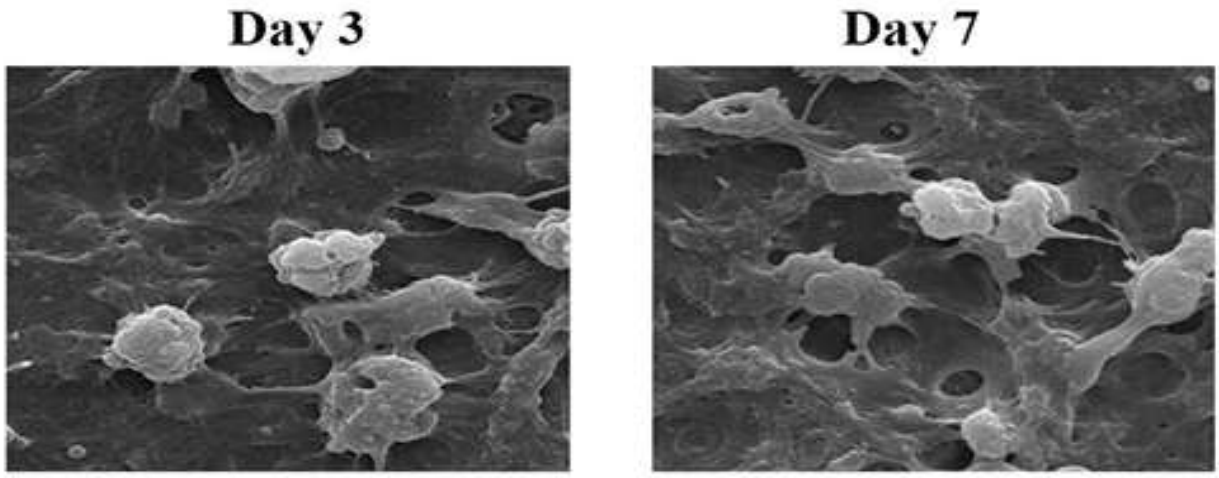

Figure 2. Microscopic observation of biofilm formation in Staphylococcus aureus using silver staining. Biofilm structures were visualized under light microscopy at 400× magnification, demonstrating dense bacterial aggregation and extracellular matrix formation on the surface.

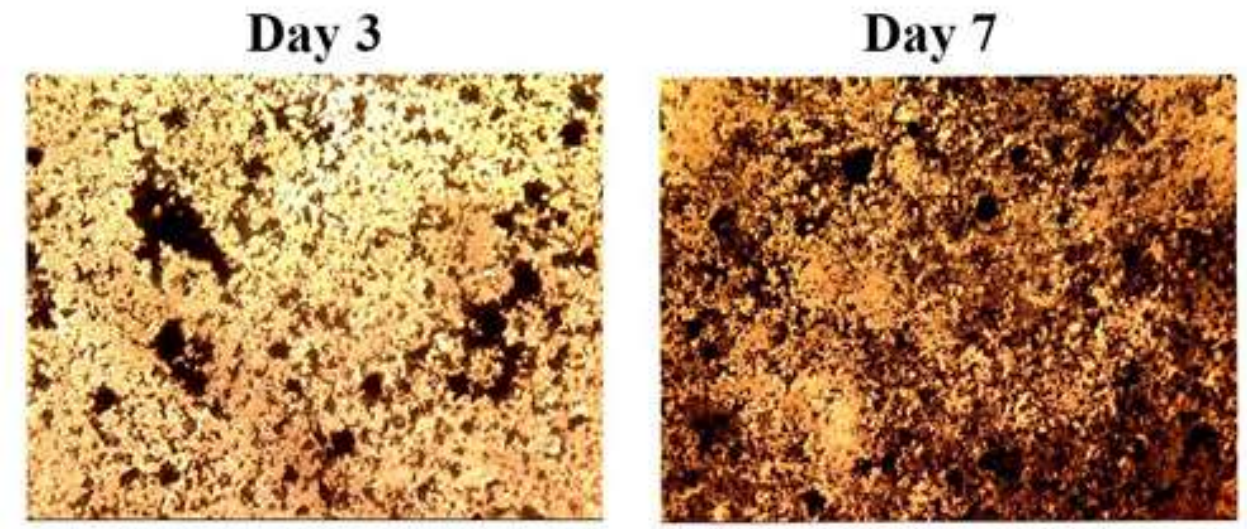

Figure 3. Scanning electron microscopy (SEM) visualization of Staphylococcus aureus biofilm architecture. SEM micrographs (8000× magnification) reveal clustered bacterial cells embedded within an extracellular polymeric matrix characteristic of mature biofilm structures.

3.1 Antimicrobial susceptibility

Table 1 shows resistance rates in counts and percentages for antibiotics tested via disc diffusion. The antibiotics include cefoxitin (30 µg), fusidic acid (10 µg), erythromycin (15 µg), ciprofloxacin (5 µg), linezolid (30 µg), trimethoprim–sulfamethoxazole (25 µg), clindamycin (2 µg), and levofloxacin (5 µg). Chi-square tests compared strong biofilm producers with non-producers, with p-values indicating this comparison.

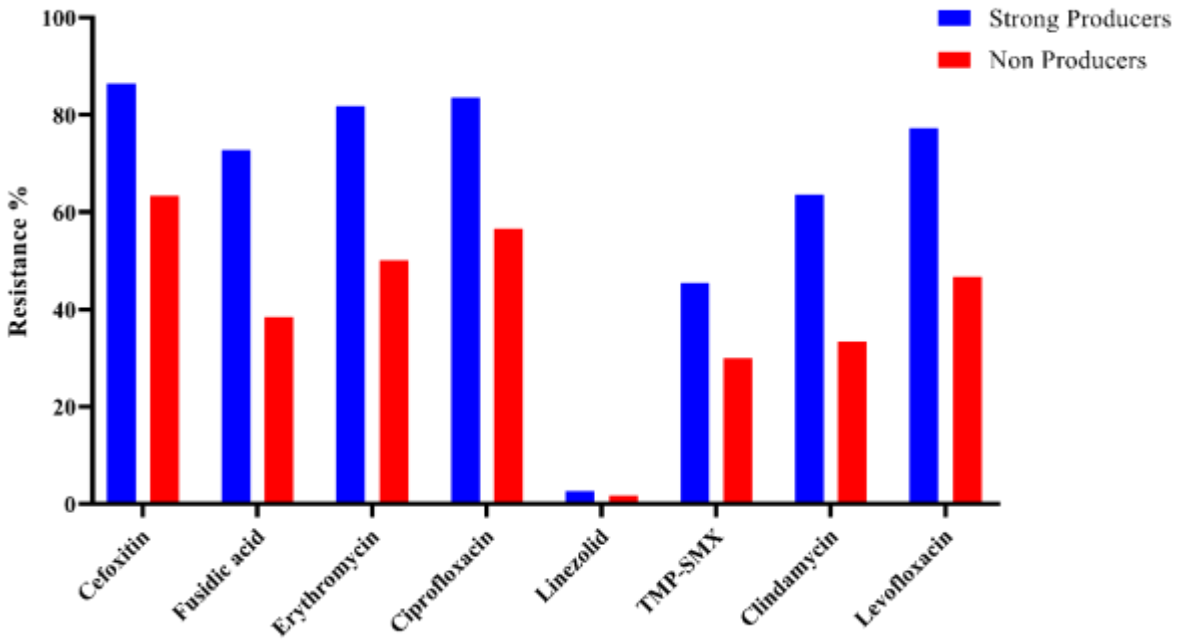

Figure 4. Resistance % of Biofilm producing and non-biofilm producing S. aures.

Table 2. Antibiotic resistance among strong biofilm producers versus non-producers (counts and %).

Antibiotic	Strong producers (n = 550) Resistant n (%)	Non-producers (n = 300) Resistant n (%)	χ^2 value	p-value
Cefoxitin	475 / 550 (86.36%)	190 / 300 (63.33%)	62.84	<0.001

Fusidic acid	400 / 550 (72.73%)	115 / 300 (38.33%)	95.21	<0.001
Erythromycin	450 / 550 (81.82%)	150 / 300 (50.00%)	97.64	<0.001
Ciprofloxacin	460 / 550 (83.64%)	170 / 300 (56.67%)	74.88	<0.001
Linezolid	15 / 550 (2.73%)	5 / 300 (1.67%)	0.19	0.663 (ns)
Trimethoprim–sulfamethoxazole	250 / 550 (45.45%)	90 / 300 (30.00%)	3.88	0.049
Clindamycin	350 / 550 (63.64%)	100 / 300 (33.33%)	86.32	<0.001
Levofloxacin	425 / 550 (77.27%)	140 / 300 (46.67%)	92.55	<0.001

Notes: chi-square test used for each 2×2 comparison (resistant vs non-resistant, producer vs non-producer). "ns" = not significant.

Interpretation: Biofilm-producing strains showed significantly higher resistance compared to non-producers when tested against cefoxitin, fusidic acid, erythromycin, ciprofloxacin, clindamycin, and levofloxacin, with p-values below 0.001. An exception was observed with TMP-SMX, which was marginally significant at p = 0.049. Resistance to linezolid was rare and showed no significant difference between groups.

3.2 Vancomycin MIC distribution (broth microdilution)

Vancomycin minimum inhibitory concentrations (MICs) were evaluated by broth micro dilution according to Clinical and Laboratory Standards Institute (CLSI) guidelines, as detailed in Methods. The prevalence of vancomycin non-susceptibility, including vancomycin-intermediate Staphylococcus aureus (VISA) and vancomycin-resistant Staphylococcus aureus (VRSA), was higher among strains with strong biofilm production.

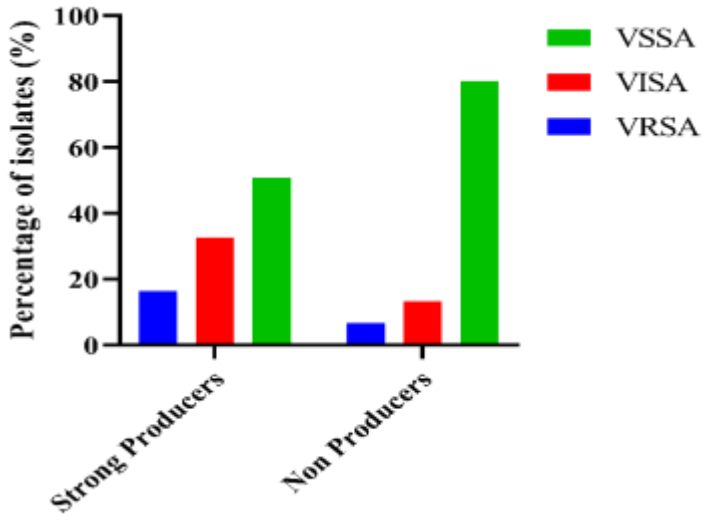

Figure 5. Isolate % of Biofilm producing and non-biofilm producing S. aures.

Table 3. Vancomycin MIC categories among biofilm phenotypes.

Category	Strong producers (n = 550)	Non-producers (n = 300)
VRSA	90 / 550 (16.36%)	20 / 300 (6.67%)
VISA	180 / 550 (32.73%)	40 / 300 (13.33%)
VSSA	280 / 550 (50.91%)	240 / 300 (80.00%)

The chi-square test of Vancomycin non-susceptibility (VRSA + VISA) was significantly higher in strong biofilm producers (270/550; 49.1%) compared to non-producers (60/300; 20.0%) (p < 0.001).

3.3 Multidrug resistance (MDR) and antibiotic resistance profile of MRSA

Among a representative subset of MRSA isolates (n=380), 340 (89.5%) were multidrug-resistant, showing resistance to three or more antimicrobial classes. The isolates showed high resistance to ciprofloxacin 335/380 (88.2%),

erythromycin 325/380 (85.5%), and fusidic acid 258/380 (67.8%). Resistance to linezolid was rare, with only 8 isolates (2.0%) showing resistance.

Table 4. Representative susceptibility profile of MRSA isolates (n = 380)

Antibiotic	Resistant n (%)
Cefoxitin	380 (100%)
Ciprofloxacin	335 (88.2%)
Erythromycin	325 (85.5%)
Fusidic acid	258 (67.8%)
Clindamycin	225 (59.2%)
TMP-SMX	170 (44.7%)
Levofloxacin	275 (72.4%)
Linezolid	8 (2.0%)
Vancomycin (VRSA)	65 (17.1%)

3.4 Antibiogram Studies of Biofilm-Forming Staphylococcus aureus

The study assessed antibiotic resistance profiles of S. aureus isolates relative to biofilm formation. Strong biofilm producers (n = 550) exhibited a high prevalence of multidrug resistance (480/550; 87.3%) compared to non-producers (140/300; 46.7%). Moderate biofilm producers (n = 125) showed intermediate resistance levels, with 70/125 (56.0%) displaying multidrug resistance, whereas weak or non-biofilm producers demonstrated the lowest resistance rates. A similar trend was observed for vancomycin non-susceptibility. Strong biofilm producers showed the highest rate of VRSA (90/550; 16.4%), followed by moderate producers (10/125; 8.0%), while weak/non-producers exhibited minimal resistance (5/225; 2.2%). Statistical analysis confirmed a significant association between biofilm formation and both multidrug resistance and vancomycin resistance ($\chi^2 = 28.72$, $p < 0.001$). These findings indicate S. aureus strains forming biofilms, especially those with strong production and icaA and icaD genes, present therapeutic challenges due to antibiotic resistance.

3.3. Amplification and Partial Sequencing of mecA and vanA

PCR assays were conducted to detect mecA and vanA genes using specific primers, as outlined in Table 6, to confirm genetic resistance. 150 isolates showing phenotypic resistance to methicillin and vancomycin were selected for molecular analysis. The partial sequence of the mecA gene was amplified in 50 (33.3%) of these 150 MRSA isolates. The methodology for detecting and sequencing the vanA gene is described in the subsequent section (Figure 1).

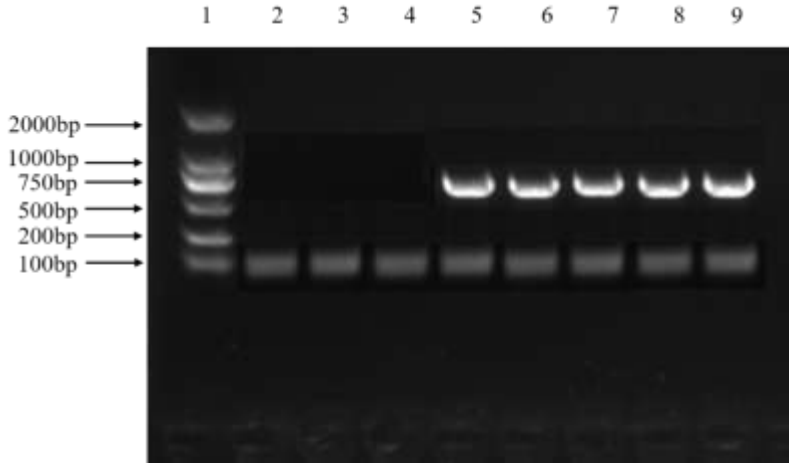

Figure 6. PCR amplification of the mecA gene in

Staphylococcus aureus isolates.

The amplification of the mecA gene produced a 583 bp PCR fragment**.** Lane 1: 2000 bp DNA ladder (Generalbio, RTU, China). Lanes 2–4: mecA-negative control isolates**.** Lanes 5–9: clinical isolates positive for the mecA gene**.**

The partial amplification of mecA yields a 583 base pair (bp) PCR product. Lane 1 contains the 2000 bp DNA ladder (Generalbio, RTU, China) as a molecular weight reference. Lanes 2-4 correspond to sensitive S. aureus strains. Lanes 5-9 show S. aureus isolates with amplified mecA gene fragment. To verify PCR product specificity and exclude nonspecific amplification, partial sequences of mecA and vanA amplicons underwent Sanger sequencing using the dideoxy chain termination method. The sequence analysis confirmed the identity of the resistance genes.

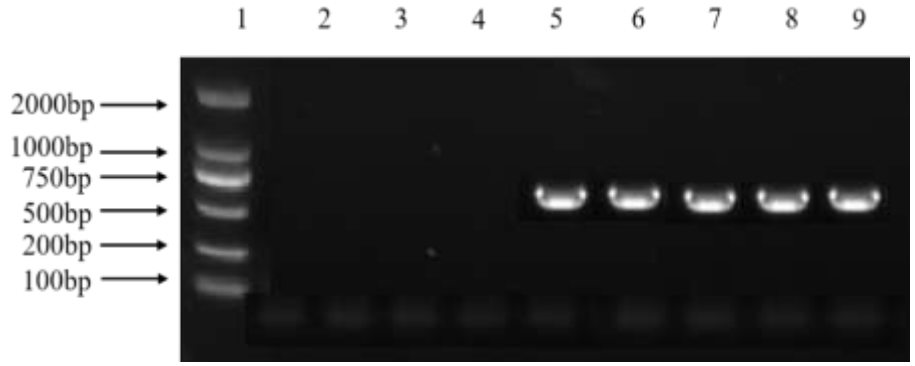

Figure 7. PCR amplification of the vanA gene in

Staphylococcus aureus isolates.

The vanA gene produced a 713 bp PCR product. Lane 1: 2000 bp DNA ladder. Lanes 2–4: negative isolates without amplification**.** Lanes 5–9: clinical isolates showing successful amplification of the vanA gene**.**

The vanA gene amplification yielded a 713 base pairs (bp) fragment. Lane 1 shows the 2000 bp DNA ladder (Generalbio, RTU, China). Lanes 2-4 contain unamplified isolates, while lanes 5-9 show successful vanA gene amplification.

Amplification of icaC and icaD

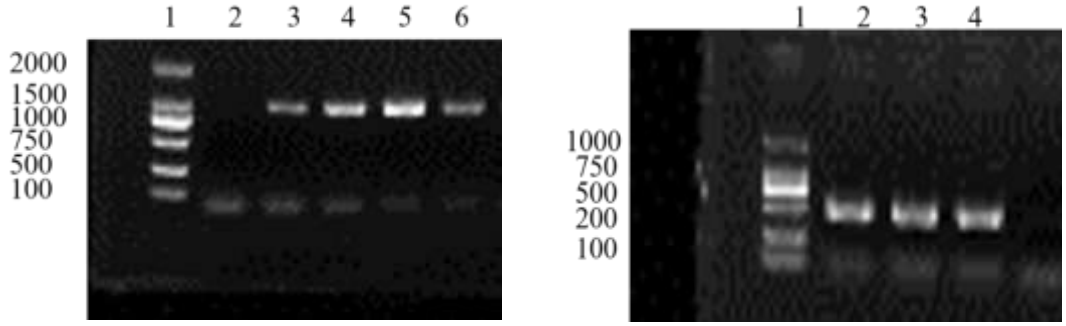

Figure 8. PCR detection of biofilm-associated genes in S. aureus. (a)

Amplification of the icaC gene produced a 1100 bp PCR fragment. Lane 1: 2000 bp DNA ladder. Lane 2: negative control isolate. Lanes 3–6: clinical isolates positive for icaC. **(b)** Amplification of the icaD gene produced a 198 bp PCR fragment. Lane 1: 2000 bp DNA ladder. Lanes 2–4: clinical isolates showing positive amplification of the icaD gene.

4. DISCUSSION

This study shows a high prevalence of methicillin resistance and reduced vancomycin susceptibility among clinical Staphylococcus aureus isolates from a tertiary-care setting. The phenotypic MRSA prevalence (760/1000; 76.0%) and vancomycin non-susceptibility rates (VRSA 14.0% and VISA 30.0%) have important implications for treatment and infection control. These findings indicate that many S. aureus isolates are resistant to primary β-lactams or show decreased susceptibility to glycopeptides, limiting empirical treatment options and increasing treatment failure risk if alternative medications are not properly used.

A key finding is the robust association between biofilm formation capability and multidrug resistance. Strong biofilm producers (n = 550) were more likely to exhibit multidrug resistance (480/550; 87.3%) compared to non-producers (140/300; 46.7%) Moderate biofilm producers (n = 125) showed intermediate resistance levels, with 70/125 (56.0%) displaying multidrug resistance, whereas weak or non-biofilm producers demonstrated the lowest resistance rates. A

similar trend was observed for vancomycin non-susceptibility, with strong biofilm producers showing the highest rate of VRSA (90/550; 16.4%), followed by moderate producers (10/125; 8.0%), while weak/non-producers exhibited minimal resistance (5/225; 2.2%). Statistical analysis confirmed a significant association between biofilm formation and both multidrug resistance and vancomycin resistance ($\chi^2 = 28.72$, $p < 0.001$). These findings are biologically plausible, as biofilms form a protective extracellular matrix that limits antibiotic penetration, promotes metabolic heterogeneity, and facilitates horizontal gene transfer, thereby enhancing bacterial survival and resistance development.

The high prevalence of icaA/icaD among strong producers provides molecular evidence for phenotypic observations, as the ica operon drives polysaccharide intercellular adhesin production and biofilm formation [12, 13]. The detection of fibronectin-binding genes (fnbA/fnbB) in many isolates reinforces the connection between adhesive capacity, biofilm development, and persistence on host and non-living surfaces.

Molecular screening further revealed that among selected resistant isolates, mecA was detected in 50 (33.3%) and vanA in 45 (30.0%)**.** The detection rate of mecA, lower than expected compared to the phenotypic MRSA rate, suggests variability in the genetic basis of β-lactam resistance in this cohort. This variability may be due to alternative determinants like mecC, borderline oxacillin resistance from β-lactamase overproduction or PBP alterations [14], or false negatives from assay sensitivity issues. This discrepancy highlights the need to integrate phenotypic testing with expanded genotypic panels for precise MRSA characterization. The detection of vanA in isolates is concerning, indicating an acquired, transferable mechanism that can cause high-level vancomycin resistance if not controlled. Conversely, VISA isolates typically relate to cell wall modifications and altered autolysis rather than van genes, a distinction critical for understanding transmission risks and therapeutic decisions [15].

The antibiogram results from the panel of cefoxitin, fusidic acid, erythromycin, ciprofloxacin, linezolid, trimethoprim–sulfamethoxazole, clindamycin, and levofloxacin show that several common antibiotics have limited efficacy. Ciprofloxacin, erythromycin, and levofloxacin showed high resistance rates, while clindamycin and fusidic acid were often ineffective. Conversely, linezolid maintained substantial activity. The sustained susceptibility to linezolid and tigecycline in other study parts offers viable treatment options for challenging infections [16, 17]. However, these medications have significant drawbacks, including cost, side effects, and pharmacokinetics, requiring judicious use. The prevalence of multidrug resistance (MDR) among biofilm producers complicates treatment decisions and highlights the need for early laboratory identification of biofilm-associated phenotypes in severe or device-related infections.

From the perspective of infection control and antibiotic stewardship, the findings advocate for several immediate measures. First, routine laboratory procedures should incorporate standardized vancomycin MIC assessments, alongside disc-diffusion tests, to ensure accurate detection of VISA/VRSA phenotypes. Second, integrating phenotypic with expanded genotypic screening, including mecC and broader van gene panels, would enhance surveillance precision and guide containment efforts. Third, infection-control strategies—such as contact precautions for colonized or infected patients, thorough cleaning of surfaces prone to biofilm formation, and antimicrobial stewardship to curb misuse of glycopeptides and broad-spectrum antibiotics—are essential to prevent selection of resistant strains. Lastly, public health initiatives addressing behavioral factors like incomplete antibiotic courses and low awareness will be crucial in reducing community reservoirs that contribute to hospital infections.

The study was conducted at a single tertiary-care center, its cross-sectional design provides a snapshot rather than longitudinal data, limiting result generalizability to other hospitals without additional surveillance. Molecular testing was restricted to few isolates and a limited gene panel; whole-genome sequencing would offer better understanding of resistance determinants, mobile genetic elements, and clonal relationships. The study lacks linked clinical outcomes, preventing assessment of biofilm phenotype or vancomycin MIC impact on patient morbidity, length of stay, or mortality. While in vitro susceptibility guides treatment, success is influenced by pharmacodynamic and clinical factors, so empirical conclusions about therapeutic efficacy, such as with tigecycline, require caution.

This study underscores the local impact of MRSA and alarming vancomycin resistance, noting that strong biofilm producers are more prone to multidrug resistance. These findings highlight the need to integrate biofilm evaluation into standard microbiological assessments for clinically relevant S. aureus, expand molecular surveillance, and implement targeted infection-control strategies. Future research should prioritize multicenter monitoring, genomic analysis of resistance mechanisms and clonal lineages, assessment of antibiofilm approaches (including device-related prevention), and investigations linking laboratory phenotypes to clinical outcomes to inform evidence-based management of biofilm-associated, drug-resistant S. aureus infections.

5. CONCLUSION

The research shows high prevalence of methicillin-resistant Staphylococcus aureus (760/1000; 76.0%) and reduced vancomycin susceptibility (VRSA 14.0%, VISA 30.0%) among clinical samples from a tertiary-care setting. Strong

biofilm producers with icaA/icaD showed high multidrug resistance (480/550; 87.3%) and were more susceptible to vancomycin non-susceptibility compared to non-producers. Molecular analysis revealed mecA in 50/150 (33.3%) and vanA in 45/150 (30.0%), confirming traditional and acquired resistance genes in local S. aureus strains.

The findings show that biofilm formation plays a significant role in S. aureus infection persistence and treatment challenges. The combination of biofilm-forming capacity, high multidrug resistance rates, and van gene presence increases treatment failure and nosocomial transmission risk. Consequently, an integrated strategy is needed, including vancomycin MIC assessment, molecular surveillance of resistance markers like mecC, and early detection of biofilm-associated infections.

To address clinical and public health implications, the study advocates enhancing infection control measures and antimicrobial stewardship. It recommends targeted diagnostic testing for biofilm and resistance phenotypes in severe or device-related infections, alongside community initiatives to promote appropriate antibiotic use. Future efforts should focus on multicenter surveillance, genomic analysis of resistance and clonality, and translational research into effective antibiofilm and therapeutic strategies against biofilm-associated, drug-resistant S. aureus.

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Authors' Contribution

Anam Tanveer: Designed and performed experimental work, data analysis and manuscript writing; Zeeshan Nawaz: Supervised the overall research and critically reviewed the manuscript; Abu Bakar Siddique and Saima Muzammil: Contributed to literature review, data interpretation and Manuscript editing.

All authors read and approved the final version of the manuscript.

Declarations

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Data availability statement

All data generated or analyzed during this study are included in this published article. Additional data can be made available from the corresponding author upon reasonable request.

Ethical approval

The study protocol was approved by the institutional ethical committee

Conflict of interest

The authors declare no conflict of interest.

REFERENCES

1. Nunes Nascimento, J.C., et al., Staphylococcus aureus in food safety: antimicrobial resistance, detection technologies, and future perspectives. Critical Reviews in Food Science and Nutrition, 2025: p. 1-24.
2. Tigabu, A. and A. Getaneh, Staphylococcus aureus, ESKAPE Bacteria Challenging Current Health Care and Community Settings: a Literature Review. Clinical Laboratory, 2021. **67**(7).
3. Ugwuoke, P.C., et al., Time-dependent Reduction in Bacterial Population by Antimicrobial Agents.
4. Yang, Y., et al., Recent development and fighting strategies for lincosamide antibiotic resistance. Clinical Microbiology Reviews, 2024. **37**(2): p. e00161-23.
5. Portman Ross, C., Mechanistic understanding of β-lactam resistance in Staphylococcus aureus. 2024, University of Sheffield.
6. Hajhamed, N.M., et al., Current status and recent trends in innovative tactics and the One Health approach to address the challenge of methicillin-resistant Staphylococcus aureus infections: a comprehensive review. Discover Medicine, 2025. **2**(1): p. 1-22.
7. Yassin, A.S., et al., Phenotypic and Genetic Detection of Healthcare Associated Multi-drug Resistant Staphylococcus aureus Infection: A Guideline Step of Infection Control Strategy in an Outbreak Prevention. Egyptian Journal of Medical Microbiology, 2025. **34**(2): p. 251-258.
8. Radford-Smith, D.E. and D.C. Anthony, Vancomycin-Resistant E. faecium: Addressing Global and Clinical Challenges. Antibiotics, 2025. **14**(5): p. 522.

9. Zhao, Y.-C., et al., Unmasking MRSA antibiotic resistance: the impact of environment, pollution, and healthcare quality—a nationwide exploration in China: a comprehensive analysis spanning 2014 to 2021 in mainland China. Environmental Sciences Europe, 2025. **37**(1): p. 22.
10. SheezmaáNaziráShah, M., MudasiráAhmadáBhat, andáArifáTasleemáJan. Nanotechnology Based Strategies foráCombating Antimicrobial Resistance: p. 1.
11. Mirzaee, M., S. Najar Peerayeh, and A.-M. Ghasemian, Detection of icaABCD genes and biofilm formation in clinical isolates of methicillin resistant Staphylococcus aureus. Iranian Journal of Pathology, 2014. **9**(4): p. 257-262.
12. Lerch, M.F., et al., A non-coding RNA from the intercellular adhesion (ica) locus of Staphylococcus epidermidis controls polysaccharide intercellular adhesion (PIA)-mediated biofilm formation. Molecular microbiology, 2019. **111**(6): p. 1571-1591.
13. Arciola, C.R., et al., Polysaccharide intercellular adhesin in biofilm: structural and regulatory aspects. Frontiers in cellular and infection microbiology, 2015. **5**: p. 7.
14. Hess, K.A., et al., Failure of mecA/mecC PCR testing to accurately predict oxacillin resistance in a patient with Staphylococcus aureus infective endocarditis. Antimicrobial Agents and Chemotherapy, 2023. **67**(10): p. e00437-23.
15. Cafiso, V., et al., Modulating activity of vancomycin and daptomycin on the expression of autolysis cell-wall turnover and membrane charge genes in hVISA and VISA strains. PLoS One, 2012. **7**(1): p. e29573.
16. Eckmann, C. and M. Dryden, Treatment of complicated skin and soft-tissue infections caused by resistant bacteria: value of linezolid, tigecycline, daptomycin and vancomycin. European journal of medical research, 2010. **15**(12): p. 554.
17. Chen, H., et al., Role of linezolid combination therapy for serious infections: review of the current evidence. European Journal of Clinical Microbiology & Infectious Diseases, 2020. **39**(6): p. 1043-1052.