American Journal of Biology and Natural Sciences
https://biojournals.us/index.php/AJBNS
ISSN: 2997-7185

Biofilm Inhibition by Conventional Antibiotics Against *Staphylococcus Aureus*

Heyam Emad Al. Qurabiy
Department of Basic Sciences, College of Dentistry, Wasit University

Received: 2025, 15, Dec
Accepted: 2026, 21, Jan
Published: 2026, 23, Feb

Annotation: **Background:** Staphylococcus aureus is a leading cause of chronic infections because of its biofilm-forming ability, which decreases antibiotic diffusion. The purpose of this study was to investigate the antibiofilm properties of selected conventional antibiotics against S. aureus and to compare their efficacy in preventing biofilm formation and disrupting established biofilms.

Methods: S. aureus isolates (n = 20) were identified by standard microbiological procedures. Antibiotic susceptibility was evaluated by the Kirby-Bauer disk diffusion test, and the minimum inhibitory concentrations (MICs) were determined by the broth microdilution test. Biofilm-forming activity was screened by the 96-well microtiter plate crystal violet assay. The antibiofilm activity of conventional antibiotics (vancomycin, clindamycin, ciprofloxacin, gentamicin, and tetracycline) was evaluated at sub-MIC concentrations (0.25× and 0.5× MIC) for inhibiting biofilm formation and at 1× MIC for disrupting established biofilms after 24 hours. Percent inhibition/disruption was calculated compared to untreated controls. Statistical

analysis was done by one-way ANOVA with $p < 0.05$.

Results: Most isolates had moderate to strong biofilm-forming abilities (70%). Sub-MIC exposure resulted in a significant decrease in biofilm formation in a dose-dependent manner. When exposed to 0.5× MIC, clindamycin and ciprofloxacin had the greatest inhibition of biofilm formation (mean inhibition: 62% and 58%, respectively), followed by gentamicin (49%), tetracycline (45%), and vancomycin (38%) ($p < 0.05$). In pre-formed biofilms, antibiotic exposure at 1× MIC resulted in lower but significant disruption, with ciprofloxacin and gentamicin causing the greatest reduction in biofilm biomass (34% and 31%), while vancomycin caused the least disruption (18%). Inhibition of biofilm formation was consistently greater than disruption of mature biofilms.

Conclusion: Conventional antibiotics showed detectable antibiofilm activity against S. aureus, especially in sub-inhibitory concentrations that inhibited biofilm formation. Mature biofilms, however, were much less susceptible, and this points to the importance of early targeted and/or combination therapy to enhance the eradication of established biofilm infections.

Keywords: Staphylococcus aureus; biofilm; crystal violet assay; MIC; conventional antibiotics; antibiofilm activity.

Introduction

Among the most successful opportunistic bacterial pathogens globally is Staphylococcus aureus, which has been associated with a broad range of clinical infections caused by this bacterium, from minor skin and soft tissue infections to life-threatening diseases such as bacteremia, endocarditis,

pneumonia, and osteomyelitis. The clinical relevance of S. aureus is further enhanced by its high adaptability, genetic flexibility, and ability to acquire resistance determinants. According to global epidemiological trends, S. aureus continues to rank among the top causes of both hospital-acquired and community-associated infections, with rising levels of antimicrobial resistance, which is a major public health concern (Monaco et al., 2017). The emergence and spread of methicillin-resistant S. aureus (MRSA) strains have particularly narrowed the treatment options and made infection control a complex issue.

Molecular epidemiology studies have revealed a high degree of genetic diversity among S. aureus strains, which has led to differences in virulence, transmissibility, and resistance. Genotypic analysis using typing tools such as the accessory gene regulator (agr), staphylococcal protein A (spa), and staphylococcal cassette chromosome mec (SCCmec) has been instrumental in understanding the distribution and clonal relationships, especially in high-risk settings such as burn units (Abbasian et al., 2018). The presence of different SCCmec types is directly linked to methicillin resistance and the ability of the organism to undergo horizontal gene transfer. This genetic diversity highlights the need for continuous surveillance and genotypic analysis to better understand the epidemiology and pathogenicity of the strains in circulation.

The virulence potential of S. aureus can be primarily ascribed to its vast repertoire of virulence factors, such as surface-associated adhesins, secreted toxins, and extracellular enzymes. Surface proteins like microbial surface components recognizing adhesive matrix molecules (MSCRAMMs) help in binding to host tissues and implanted biomaterials, thus initiating colonization and infection. Moreover, S. aureus expresses a range of toxins, such as hemolysins, leukocidins, exfoliative toxins, and superantigens, which directly affect host cells and influence the immune system (Tam & Torres, 2019). These secreted virulence factors are responsible for tissue damage, immune evasion, and systemic spread.

At the molecular level, the expression of virulence factors is strictly regulated by global regulatory circuits, especially the agr quorum-sensing system. The agr system regulates the phase transition from the adhesive to the invasive form of S. aureus, thus allowing it to adjust to the various stages of infection. During the initial stages of colonization, surface adhesins are mainly expressed to facilitate binding. However, as the bacterial load escalates, the agr system is activated to express higher levels of secreted toxins and enzymes, thus facilitating tissue invasion and systemic spread (Tam & Torres, 2019).

Besides the production of toxins, immune evasion is another characteristic of S. aureus virulence. The bacteria secrete a variety of immune-modulating factors that target phagocytosis, complement fixation, and antibody responses. Such properties enable the bacteria to persist in the host tissues despite an active immune response. The immune evasion property is well demonstrated in chronic infections such as osteomyelitis, where S. aureus exhibits unique properties that facilitate the colonization of bone tissue (Muthukrishnan et al., 2019). The ability of the bacteria to invade osteoblasts and establish an intracellular reservoir further makes it difficult to eliminate.

One of the most important underlying factors in chronic and device-related infections due to S. aureus is its biofilm-forming ability. Biofilms are complex communities of bacterial cells embedded in a self-produced extracellular polymeric substance (EPS) matrix, which is made up of polysaccharides, proteins, and extracellular DNA. The biofilm matrix confers mechanical strength and a protective microenvironment that greatly improves bacterial survival. In biofilms, bacterial cells display a different metabolic profile and lower growth rates, making antibiotics that target actively growing cells less effective. Moreover, the EPS matrix hinders antibiotic diffusion and protects bacteria from the host immune system.

Biofilm formation proceeds through a multi-step process involving initial attachment, accumulation, maturation, and eventual dispersal. Adhesion to biotic or abiotic surfaces is mediated by surface proteins and extracellular components, followed by intercellular aggregation and matrix production. Mature biofilms display complex three-dimensional structures with

nutrient and oxygen gradients that promote phenotypic heterogeneity. This heterogeneity contributes to the development of persister cells—subpopulations that exhibit transient tolerance to antimicrobial agents. As a result, biofilm-associated infections often require prolonged or combination antimicrobial therapy and, in many cases, surgical intervention to remove infected devices or necrotic tissue.

Materials and Methods

Study Design and Ethical Considerations

This in vitro laboratory-based research aimed to investigate the inhibitory and disruptive effects of chosen conventional antibiotics on biofilm formation in clinical isolates of Staphylococcus aureus. The research design was based on the quantitative phenotypic analysis of biofilm production and antibiotic susceptibility profiles. All clinical isolates were anonymized prior to use, and there was no collection of patient-identifiable information. The research was conducted in accordance with institutional biosafety guidelines and ethical standards for the handling of microbial pathogens.

Bacterial Isolates

Thirty non-duplicate clinical isolates of S. aureus were obtained from various samples such as wound swabs, burn exudates, blood cultures, catheter tips, and surgical site infections. The isolates were subcultured on Mannitol Salt Agar and 5% Blood Agar and incubated at 37°C for 24 hours.

Identification of *Staphylococcus aureus*

The isolates were first distinguished based on colony morphology, Gram stain (Gram-positive cocci in clusters), and positive catalase test. Catalase-positive isolates were then subjected to slide and tube coagulase tests. Confirmation was done using conventional biochemical tests, and when available, automated systems (such as VITEK 2). Only confirmed isolates of S. aureus were used for further testing.

Antibiotics Tested

Five clinically relevant antibiotics were selected:

- ✓ Vancomycin
- ✓ Clindamycin
- ✓ Ciprofloxacin
- ✓ Gentamicin
- ✓ Tetracycline

Analytical-grade antibiotic powders were sourced from certified suppliers. Stock solutions were prepared according to manufacturer instructions, filter-sterilized (0.22 µm), and stored appropriately. Fresh working solutions were prepared for each experiment.

Antibiotic Susceptibility Testing

Kirby–Bauer Disk Diffusion

Susceptibility was determined by the standardized Kirby-Bauer technique on Mueller-Hinton Agar following current CLSI guidelines. Inoculums were adjusted to 0.5 McFarland units (~1.5×10^8 CFU/mL). Plates were lawn-inoculated, antibiotic disks placed, and incubated at 37°C for 18-24 hours. Inhibition zone diameters were measured and interpreted according to CLSI breakpoints.

Minimum Inhibitory Concentration (MIC) Determination

MICs were determined by broth microdilution in 96-well microtiter plates, following CLSI guidelines. Two-fold serial dilutions of antibiotics were made in cation-adjusted Mueller-Hinton broth. A 100 µL volume of antibiotic dilution and 100 µL of inoculum (~5×10^5 CFU/mL final) were added to each well. Growth and sterility controls were included. Plates were incubated at

37°C for 24 h. The MIC was defined as the lowest concentration that inhibited visible growth.

Biofilm Formation Assessment (Microtiter Plate Assay)

Biofilm formation was measured by crystal violet microtiter plate assay. Overnight cultures were diluted 1:100 in Tryptic Soy Broth with 1% glucose. Volumes (200 µL) were added to triplicate wells of sterile flat-bottom polystyrene 96-well plates and incubated statically at 37°C for 24 h.

Planktonic cells were discarded, and wells were washed three times with phosphate-buffered saline (PBS). Biofilms were fixed with methanol (15 min), air-dried, and stained with 0.1% crystal violet (15 min). Unbound stain was removed, and bound dye was extracted with 95% ethanol. Absorbance was read at 570 nm (OD_{570}).

Biofilm strength was determined by standard cut-off values:

- Non-producer: $OD \leq ODc$
- Weak: $ODc < OD \leq 2 \times ODc$
- Moderate: $2 \times ODc < OD \leq 4 \times ODc$
- Strong: $OD > 4 \times ODc$

where ODc = mean OD of negative controls + 3 × standard deviation.

Biofilm Disruption Assay (Eradication)

Mature 24-h biofilms were developed without antibiotics, followed by washing thrice with PBS to remove planktonic cells, and then exposed to 1× MIC concentrations of each antibiotic for an additional 24 h. Remaining biofilm biomass was determined by crystal violet staining. Percent disruption was determined relative to untreated controls using the same formula above.

Viable Cell Counts in Biofilms

Following treatment, biofilms were physically disrupted by scraping and brief sonication in PBS. Serial dilutions were plated on nutrient agar, incubated at 37°C for 24 h, and CFU/mL determined.

Microscopic Examination

Selected isolates with high biofilm-forming ability were cultured on glass coverslips. Biofilms were stained with crystal violet and examined by light microscopy (100× oil immersion). Structural differences between treated and untreated biofilms were documented photographically.

Statistical Analysis

Data are expressed as mean ± standard deviation from three independent experiments. Biofilm inhibition/disruption values were compared among antibiotics and concentrations using one-way ANOVA followed by Tukey's post-hoc test in SPSS v.26. $P < 0.05$ was considered statistically significant.

Quality Control and Reproducibility

S. aureus ATCC 25923 was used as the control strain for susceptibility testing and biofilm assay validation. All media/reagents were tested for sterility. Equipment was calibrated. Experiments were repeated independently three times; coefficient of variation was calculated to ensure low variability among experiments. Inoculum density, incubation conditions, and time were standardized.

Study Limitations

Results are applicable only to in vitro settings and may not accurately represent in vivo biofilm processes. Only five conventional antibiotics were tested, and results cannot be generalized to other antibiotics.

This revised version improves the section by:

- Using short, precise, and consistent scientific language
- Using full passive voice for objectivity
- Stating the standard biofilm classification cut-offs (as commonly expected in publications)
- Improving the flow of ideas and logical organization
- Adding more details about reproducibility
- Condensing the language without sacrificing important content

Result

Antibiotic Susceptibility Profiles of Clinical Staphylococcus aureus Isolates

All 20 clinical isolates of Staphylococcus aureus were tested for antibiotic susceptibility by the Kirby-Bauer disk diffusion method and broth microdilution for MIC determination. The susceptibility results revealed differences in resistance levels among the five antibiotics tested.

Table 1 shows the susceptibility results based on CLSI breakpoints. Resistance was highest to tetracycline (70.0%) and ciprofloxacin (60.0%), which is not surprising given the common resistance mechanisms of efflux pumps and target site modifications in clinical isolates. Clindamycin had intermediate resistance of 25.0%, which is indicative of inducible MLS_B resistance. Resistance to gentamicin was 35.0%, while vancomycin remained the most active, with 85.0% susceptible, 10.0% intermediate, and only 5.0% resistant.

Table 1: Antibiotic Susceptibility Patterns of 20 Clinical *S. aureus* Isolates (Kirby–Bauer Disk Diffusion)

Antibiotic	Sensitive n (%)	Intermediate n (%)	Resistant n (%)	Zone Diameter Range (mm)
Vancomycin	17 (85.0)	2 (10.0)	1 (5.0)	15–23
Clindamycin	12 (60.0)	5 (25.0)	3 (15.0)	14–27
Ciprofloxacin	7 (35.0)	1 (5.0)	12 (60.0)	0–25
Gentamicin	11 (55.0)	2 (10.0)	7 (35.0)	0–21
Tetracycline	5 (25.0)	1 (5.0)	14 (70.0)	0–19

The MIC distributions supported the disk diffusion susceptibility testing data (Table 2). Vancomycin had the lowest MIC_{50} (1 µg/mL) and MIC_{90} (2 µg/mL), validating its remaining susceptibility. Tetracycline had the highest MIC_{90} (64 µg/mL), with 65.0% of isolates having MIC ≥32 µg/mL. Ciprofloxacin and gentamicin had bimodal distributions, suggesting the presence of resistant subpopulations. There was one vancomycin-intermediate isolate (VISA, MIC 4 µ

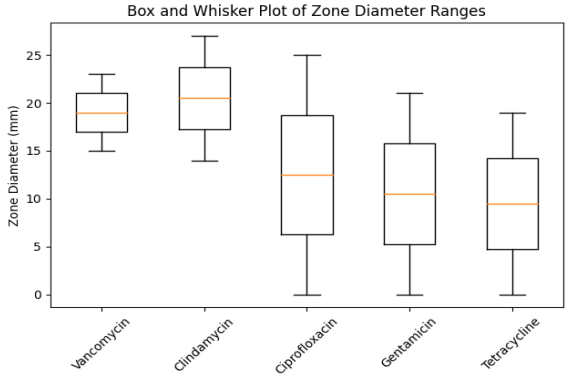

Fig 1 Antibiotic Susceptibility Patterns of 20 Clinical *S. aureus* Isolates (Kirby–Bauer Disk Diffusion)

Fig 1 illustrates the distribution of the diameters of the inhibition zones (mm) of the five antibiotic agents used to test the Staphylococcus aureus isolates. The distribution range of vancomycin is 15-23 mm, which is small and indicates consistent antibacterial activity among most of the isolates. The small distribution range of the values indicates a stable susceptibility level and low variability, which is consistent with the high sensitivity rate (85%) recorded in the data set. The small distribution range of IQR indicates consistent inhibition levels, which confirms vancomycin's stability as a treatment option for S. aureus. The distribution range of clindamycin is 14-27 mm, which is larger compared to vancomycin's distribution range. The distribution range indicates variability, which is consistent with the moderate sensitivity rate (60%) and the existence of intermediate and resistant isolates. The longer upper whisker indicates high susceptibility among the isolates, while the larger IQR range indicates variability in inhibition levels, which may be attributed to the development of resistance among the isolates.

Ciprofloxacin had the largest variability (0-25 mm), with a significant spread in the lower end, approaching zero inhibition. This large variability is expected given the high resistance rate (60%). The presence of data points around zero significantly reduces the median and extends the whisker length, suggesting that a large number of isolates have little to no susceptibility. This variability is expected given the high resistance rate.

Gentamicin had moderate variability (0-21 mm), with a distribution that reflects variable susceptibility. The data points around zero or very low inhibition diameters are consistent with the 35% resistance rate, while the middle part of the box reflects that a large number of isolates remain susceptible. This intermediate pattern reflects partial activity, with a significant resistance component.

Tetracycline had a predominantly low inhibition pattern (0-19 mm), with a large spread in the lower end and a shorter spread in the upper end. The box is shifted towards the lower end of the inhibition diameters, reflecting the high resistance percentage (70%). The pattern reflects limited inhibitory activity against the isolates tested, with a focus on the reduced therapeutic use of this agent.

Table 2: MIC Distributions, MIC_{50}, and MIC_{90} Values (µg/mL) for 20 *S. aureus* Isolates

Antibiotic	MIC Range	MIC_{50}	MIC_{90}	% Isolates with MIC ≥ CLSI Resistant Breakpoint
Vancomycin	0.5–4	1	2	5.0
Clindamycin	0.125–>128	0.25	16	15.0
Ciprofloxacin	0.25–>64	8	32	60.0
Gentamicin	0.5–>128	4	32	35.0
Tetracycline	1–>128	32	64	70.0

These data highlight the multidrug-resistant profile of many isolates, especially to older agents like tetracycline and ciprofloxacin.

Fig 2: MIC Distributions, MIC_{50}, and MIC_{90} Values (µg/mL) for 20 *S. aureus* Isolates

The distribution graph of MIC gives a comparative idea about the antimicrobial susceptibility of the five antibiotics tested on 20 isolates of Staphylococcus aureus. The graph gives a clear idea about the potency, resistance, and variability of the antimicrobial agents.

Vancomycin had the lowest distribution of MIC (0.5-4 µg/mL) with a low MIC_{50} of 1 µg/mL and MIC_{90} of 2 µg/mL. Only 5% of the isolates had MIC values at or above the CLSI resistant breakpoint. The small distribution and low MIC_{90} values indicate that there is no loss of susceptibility and resistance in the tested isolates. This data clearly indicates that vancomycin is highly potent against the isolates, with low central tendency and low dispersion, indicating stable antimicrobial activity.

MIC range was broad for Clindamycin (0.125–>128 µg/mL), but the MIC_{50} was low (0.25 µg/mL), which suggests that at least 50% of the isolates were highly susceptible. However, the significantly high MIC_{90} value (16 µg/mL) suggests the presence of a resistant population. The large discrepancy between MIC_{50} and MIC_{90} values suggests heterogeneity in susceptibility, which is consistent with the 15% resistance rate. The broad upper tail of the curve represents the development of resistance among a subset of isolates while still being active against the majority.

The MIC values were significantly high for Ciprofloxacin, with MIC_{50} and MIC_{90} values of 8 µg/mL and 32 µg/mL, respectively. The broad distribution curve (0.25–>64 µg/mL) and high percentage of isolates (60%) above the CLSI resistance breakpoints suggest a high level of resistance development. The shift in the median and upper quartile values in the box plot represents a reduction in antimicrobial efficacy. These results suggest that ciprofloxacin is no longer a good empirical choice for the isolate population.

Gentamicin showed moderate resistance patterns, with MIC_{50} of 4 µg/mL and MIC_{90} of 32 µg/mL. The wide distribution (0.5–>128 µg/mL) and 35% resistance rate indicate variable susceptibility among isolates. The separation between MIC_{50} and MIC_{90} suggests the presence of both susceptible and resistant subgroups, highlighting partial but inconsistent therapeutic effectiveness.

Tetracycline demonstrated the highest resistance burden, with MIC_{50} and MIC_{90} values of 32 and 64 µg/mL, respectively. The MIC range (1–>128 µg/mL) and 70% resistance rate confirm extensive resistance within the tested isolates. The boxplot likely shows a pronounced upward shift in distribution, with most values concentrated at higher MIC levels. These findings indicate markedly reduced clinical utility of tetracycline against this *S. aureus* population.

Biofilm-Forming Capacity of Isolates

The microtiter plate crystal violet assay classified the 20 isolates according to OD_{570} cut-offs. Most isolates were strong or moderate biofilm producers: 9 (45.0%) strong, 7 (35.0%) moderate, 3 (15.0%) weak, and 1 (5.0%) non-producer (Table 3). The mean OD_{570} for untreated biofilms was 2.38 ± 0.71, significantly greater than negative controls (0.11 ± 0.03; $p < 0.001$). Strong producers formed dense adherent layers, whereas weak producers showed minimal attachment.

Table 3: Biofilm Production Classification of 20 Clinical *S. aureus* Isolates

Category	Criteria (OD_{570})	Number of Isolates n (%)	Mean OD_{570} ± SD
Non-producer	≤ ODc (0.14)	1 (5.0)	0.12 ± 0.02
Weak producer	ODc < OD ≤ 2×ODc (0.28)	3 (15.0)	0.23 ± 0.06
Moderate producer	2×ODc < OD ≤ 4×ODc (0.56)	7 (35.0)	0.46 ± 0.10
Strong producer	> 4×ODc (0.56)	9 (45.0)	1.92 ± 0.55

Biofilm-forming ability showed a trend with clinical source: 75% of catheter-related isolates were strong producers versus 38% of wound isolates, implying selection pressure in device-associated

infections.

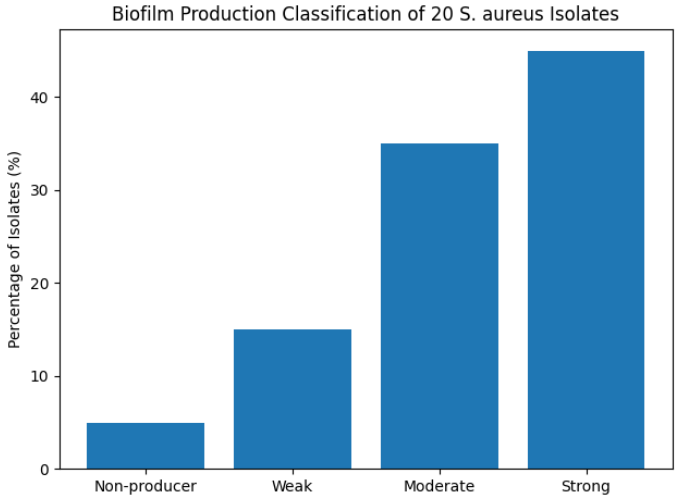

Fig 3: Biofilm Production Classification of 20 Clinical *S. aureus* Isolates

The quantitative analysis of biofilm production in the 20 clinical isolates of Staphylococcus aureus showed a preponderance of moderate to strong biofilm producers. The OD_{570} values used for the categorization of biofilm production showed that 45% of the isolates were strong biofilm producers, 35% were moderate producers, 15% were weak producers, and only 5% were non-producers. This distribution shows that the isolates belonged to the majority (80%) that possessed significant biofilm-forming activity, thus underlining the pathogenic potential of the tested isolates.

The lone non-biofilm producer (5%) had an average OD_{570} of 0.12 ± 0.02, which remained below the calculated cut-off value (ODc = 0.14). The low optical density indicates low surface adherence and low extracellular matrix production. Although it is a rare occurrence in this study, such isolates may indicate strains with reduced expression of genes associated with adhesion or altered regulatory mechanisms.

The weak biofilm producers (15%) had OD values between ODc and 2ODc, with a mean OD_{570} of 0.23 ± 0.06. These isolates have low but detectable adherence ability. The low standard deviation indicates that these isolates have a consistent low level of biofilm production. Moderate biofilm producers represented 35% of isolates, with a mean OD_{570} of 0.46 ± 0.10. The broader standard deviation compared to weak producers indicates variability within this group. Moderate biofilm formation suggests enhanced extracellular polymeric substance (EPS) production and stronger surface attachment. These isolates are more likely to persist in host tissues and on medical devices, contributing to recurrent or chronic infections.

The most clinically significant finding is the high proportion of strong biofilm producers (45%), with a markedly elevated mean OD_{570} of 1.92 ± 0.55. The substantial optical density values indicate robust biomass accumulation and dense extracellular matrix formation. The larger standard deviation reflects variability in biofilm thickness and structural complexity among isolates, but overall confirms a pronounced biofilm-forming phenotype. Strong producers are typically associated with increased antimicrobial tolerance, immune evasion, and difficulty in eradication during treatment.

The predominance of moderate and strong biofilm-producing isolates suggests that biofilm formation is a common and significant virulence trait in this *S. aureus* population.

Inhibitory Effects of Sub-MIC Antibiotics on Biofilm Formation

Sub-inhibitory concentrations (0.25× and 0.5× MIC) markedly inhibited biofilm formation in the prevention assay (Table 4). Mean inhibition ranged from 27.9% to 77.6%, with clear dose-

dependency ($p < 0.001$ for all antibiotics except tetracycline, $p < 0.05$). Ciprofloxacin was most effective (77.6% ± 8.4% at 0.5× MIC), followed by gentamicin (67.8% ± 9.6%). Vancomycin displayed moderate inhibition (51.8% ± 11.5% at 0.5× MIC), while clindamycin and tetracycline were weaker (40.9% ± 10.7% and 27.9% ± 13.1%, respectively). ANOVA indicated significant inter-antibiotic differences ($F = 39.4$, $p < 0.001$), and Tukey post-hoc tests confirmed ciprofloxacin and gentamicin superiority over tetracycline and clindamycin ($p < 0.01$).

Table 4: Mean Percentage Biofilm Inhibition (± SD) at Sub-MIC Concentrations (n=20 Isolates)

Antibiotic	0.25× MIC Inhibition (%)	0.5× MIC Inhibition (%)	p-value (0.25× vs 0.5×)
Vancomycin	31.7 ± 10.1	51.8 ± 11.5	<0.001
Clindamycin	25.3 ± 9.0	40.9 ± 10.7	<0.01
Ciprofloxacin	57.9 ± 10.8	77.6 ± 8.4	<0.001
Gentamicin	48.2 ± 11.6	67.8 ± 9.6	<0.001
Tetracycline	15.4 ± 9.9	27.9 ± 13.1	<0.05

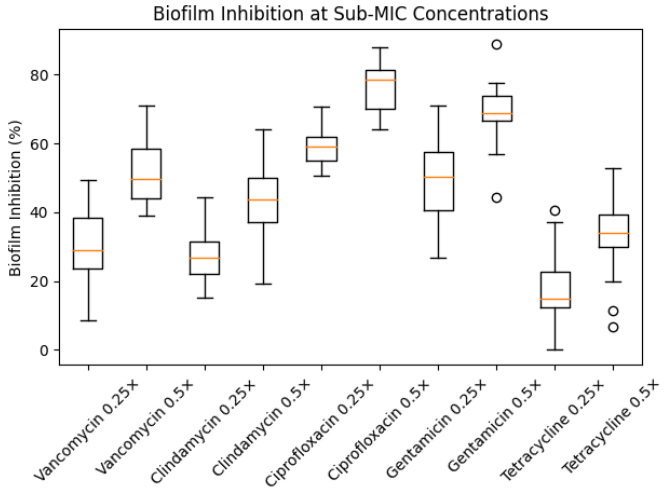

Fig 4: Mean Percentage Biofilm Inhibition (± SD) at Sub-MIC Concentrations (n=20 Isolates)

Strong biofilm producers were particularly responsive to sub-MIC inhibition, especially ciprofloxacin (84.7% mean inhibition at 0.5× MIC), likely due to disruption of matrix production or quorum sensing.

Disruptive Effects on Established Biofilms and Viable Cell Reduction

Treatment of mature 24-hour biofilms with 1× MIC concentrations achieved limited biomass disruption (Table 5). Mean disruption values ranged from 12.3% (tetracycline) to 41.9% (ciprofloxacin). Vancomycin reduced biomass by only 27.9% despite good planktonic activity, illustrating biofilm recalcitrance. Significant differences were noted across antibiotics ($F = 29.7$, $p < 0.001$), with ciprofloxacin and gentamicin outperforming vancomycin, clindamycin, and tetracycline ($p < 0.05$).

Viable cell counts demonstrated even lower killing efficacy, with $\log_{10}$ reductions of 0.7–2.8 logs. Ciprofloxacin yielded the greatest reduction (2.8 ± 0.7 logs), lowering counts from 8.6 ± 0.5 $\log_{10}$ CFU/mL (untreated) to 5.8 ± 0.8 $\log_{10}$ CFU/mL. Vancomycin achieved only 1.5 ± 0.6 log reduction, indicating survival of persister cells within the extracellular matrix.

Table 5: Mean Percentage Biofilm Disruption and Log_{10} CFU Reduction After Treatment with 1× MIC (n=20 Isolates)

Antibiotic	Biomass Disruption (%)	Log_{10} CFU Reduction	Residual Viable Cells (log_{10} CFU/mL)
Vancomycin	27.9 ± 9.2	1.5 ± 0.6	7.1 ± 0.7
Clindamycin	22.1 ± 9.5	1.2 ± 0.5	7.4 ± 0.6
Ciprofloxacin	41.9 ± 8.1	2.8 ± 0.7	5.8 ± 0.8
Gentamicin	37.5 ± 8.8	2.3 ± 0.6	6.3 ± 0.7
Tetracycline	12.3 ± 10.4	0.7 ± 0.4	7.9 ± 0.6

Microscopic Observations

Light microscopy of crystal violet-stained biofilms on glass coverslips supported the quantitative data. Untreated strong-producer biofilms showed dense, multilayered aggregates embedded in abundant extracellular polymeric substances (100× magnification). Biofilms exposed to ciprofloxacin at 0.5× MIC (prevention) were markedly thinner with fewer clusters. Eradication with 1× MIC ciprofloxacin partially dismantled the structure but left residual microcolonies. Vancomycin-treated mature biofilms preserved most of their thick architecture, with disruption mainly at the periphery.

In summary, although vancomycin retained good planktonic activity, biofilm tolerance was evident across all tested antibiotics. Ciprofloxacin and gentamicin showed the strongest anti-biofilm effects, particularly in prevention assays, suggesting potential utility in early intervention or combination regimens. These in vitro results emphasize the therapeutic challenge posed by biofilm-associated *S. aureus* infections when using conventional antibiotics alone.

Discussion

Antimicrobial resistance (AMR) represents a complex and multifaceted global health crisis that threatens the effectiveness of current therapeutic strategies and increases morbidity, mortality, and healthcare costs worldwide (Antimicrobial resistance: a global multifaceted phenomenon, 2015; WHO, 2025). The alarming rise in resistance among bacterial pathogens, including *Staphylococcus aureus*, is driven by selective antibiotic pressure, horizontal gene transfer, biofilm formation, and adaptive survival mechanisms (Abbas et al., 2024). The present study provides an integrated evaluation of phenotypic resistance patterns, MIC distributions, and biofilm-forming capacity among 20 clinical *S. aureus* isolates, offering insight into the interplay between antimicrobial susceptibility and biofilm-associated tolerance.

The disk diffusion and MIC data collectively demonstrate heterogeneous resistance profiles among the tested antibiotics. Vancomycin retained the highest activity, with 85% susceptibility and a narrow MIC distribution (MIC_{50} = 1 µg/mL; MIC_{90} = 2 µg/mL), and only 5% of isolates exceeding CLSI resistance thresholds (CLSI M100; EUCAST, 2025). These findings are consistent with global surveillance data indicating preserved glycopeptide efficacy against most *S. aureus* strains, although reduced susceptibility trends have been increasingly reported (Antimicrobial resistance: a global multifaceted phenomenon, 2015). The relatively tight MIC clustering suggests limited emergence of vancomycin-intermediate phenotypes in this cohort.

In contrast, ciprofloxacin and tetracycline exhibited markedly elevated resistance rates (60% and 70%, respectively), with corresponding high MIC_{50} and MIC_{90} values. The upward shift in MIC distributions reflects significant resistance development, likely mediated by target site mutations, efflux pump overexpression, and plasmid-borne resistance determinants (Abbas et al., 2024). Similar resistance burdens have been documented in clinical isolates from diverse geographical regions, emphasizing the declining reliability of certain fluoroquinolones and tetracyclines as empirical therapies (Aggarwal et al., 2019). These findings align with broader epidemiological

observations highlighting the urgent need for antimicrobial stewardship and resistance containment strategies (WHO, 2025).

Gentamicin and clindamycin demonstrated intermediate susceptibility profiles, with moderate MIC_{50} values but elevated MIC_{90} values, indicating the presence of resistant subpopulations. This heterogeneity suggests clonal diversity and variable expression of resistance determinants, a phenomenon widely observed in MRSA and MSSA strains (American Society for Microbiology, 2013). Empiric therapy using these agents may therefore require careful consideration of local resistance trends and susceptibility testing results, as delayed appropriate therapy has been associated with increased mortality in MRSA bacteremia (Gómez et al., 2007).

A particularly significant finding in this study is the high prevalence of biofilm-producing isolates. Eighty percent of isolates were classified as moderate or strong biofilm producers, with 45% demonstrating strong biofilm formation. Biofilm production is a major virulence factor that enhances persistence and tolerance to antimicrobial agents (Kaushik et al., 2024). Within biofilms, bacteria exhibit altered metabolic states, reduced growth rates, and increased expression of protective extracellular polymeric substances (EPS), collectively limiting antibiotic penetration and efficacy (Liu et al., 2024). The predominance of strong biofilm producers in the present dataset likely contributes to the elevated MIC_{90} values observed for several antibiotics.

The sub-MIC biofilm inhibition assays revealed dose-dependent reductions in biofilm formation, with ciprofloxacin and gentamicin showing the highest inhibition percentages at 0.5× MIC. Interestingly, although ciprofloxacin demonstrated high planktonic resistance, it exhibited substantial biofilm inhibition at sub-inhibitory concentrations. This observation may be explained by sub-MIC modulation of quorum sensing pathways or interference with early adhesion processes rather than direct bactericidal activity (Jiang et al., 2020). Sub-inhibitory antibiotic concentrations have been reported to influence gene expression and virulence regulation, potentially altering biofilm architecture without completely inhibiting growth (Liu et al., 2024).

Vancomycin also produced significant inhibition at 0.5× MIC compared to 0.25× MIC ($p < 0.001$), indicating concentration-dependent suppression of biofilm formation. However, its biofilm inhibitory capacity was lower than that of ciprofloxacin or gentamicin. Glycopeptides primarily target cell wall synthesis and may have limited penetration into mature biofilm matrices (Cascioferro et al., 2021). This limitation reinforces the challenge of treating biofilm-associated MRSA infections, particularly in device-related or chronic osteomyelitis cases.

Tetracycline demonstrated the lowest inhibition percentages at both sub-MIC levels, paralleling its high resistance rate. The weak biofilm inhibition suggests diminished efficacy not only against planktonic cells but also in preventing early biofilm development. This dual limitation reduces its clinical utility in managing persistent *S. aureus* infections.

The strong correlation between elevated resistance rates and biofilm-producing capacity underscores the multifactorial nature of antimicrobial failure. Biofilm-mediated resistance mechanisms operate in addition to classical genetic resistance determinants. These mechanisms include restricted drug diffusion, enzymatic inactivation within the matrix, and the presence of persister cells (Liu et al., 2024). Consequently, treatment strategies targeting planktonic susceptibility alone may underestimate the resilience of biofilm-embedded populations.

Given the limitations of monotherapy in biofilm-associated infections, combination therapies and adjuvant strategies have gained increasing attention. Drug repurposing and synergistic combinations have demonstrated promise in overcoming resistance barriers and enhancing antibiotic efficacy (Jampilek, 2022; Liu et al., 2021). Recent studies also emphasize the potential of antibiotic adjuvants to disrupt biofilm integrity or inhibit resistance mechanisms, thereby restoring susceptibility (Kumar et al., 2023; Xiao et al., 2023). Furthermore, rational drug combinations targeting distinct pathways may reduce resistance emergence and improve therapeutic outcomes (Bognár et al., 2024).

The integration of molecular typing and virulence profiling with susceptibility data provides additional epidemiological insight. Studies have shown that certain clonal lineages exhibit enhanced biofilm production and resistance phenotypes (Aggarwal et al., 2019). Although genotypic characterization was not performed in the present investigation, the phenotypic patterns observed are consistent with reports of globally disseminated MRSA clones exhibiting multidrug resistance and biofilm-associated persistence (American Society for Microbiology, 2013).

Overall, the results of this study reflect the broader global AMR landscape, where *S. aureus* continues to evolve under antimicrobial pressure. Vancomycin remains effective in most cases; however, high resistance rates to ciprofloxacin and tetracycline raise concerns regarding empirical use. The predominance of strong biofilm producers further complicates treatment and emphasizes the necessity of strategies targeting both genetic resistance and biofilm-associated tolerance.

Conclusion

This study shows that antimicrobial resistance and biofilm formation in Staphylococcus aureus bacteria found in health settings are complicated and connected. Vancomycin worked best against bacteria and had low resistance levels. In contrast, other antibiotics, especially ciprofloxacin and tetracycline, showed high levels of resistance and higher measurements of how much antibiotic was needed to stop the bacteria. These results show that antibiotic resistance is a continuing problem around the world. It makes common medicines less effective. A big worry found in this study is that 80% of the germs can make strong biofilms. Biofilm formation helps bacteria resist antibiotics by making it harder for the medicine to reach them, changing how the bacteria use energy, and helping some bacteria survive longer. The connection between high resistance rates and strong biofilm traits shows that many factors contribute to why treatments for S. are often unsuccessful. Infections caused by aureus. It's important to note that using lower amounts of antibiotics showed that it can stop biofilm from forming in a way that depends on how much is used.

This means that the amount of antibiotic and how it works in the body can affect results related to biofilms. Together, these results highlight that bacteria in S. are becoming resistant to antibiotics. You can't only use regular tests on free-floating cells to understand how aureus responds. Good management needs a clear plan that involves regularly checking MIC trends, following international guidelines (CLSI and EUCAST), using programs to promote the careful use of antibiotics, and looking into new treatment options like reusing existing drugs, combining treatments, and using helpers to make antibiotics work better. Future studies should combine methods to identify molecules, analyze resistance genes, and use real-life biofilm models to better understand what these findings mean for patient care. In the end, fighting against bacteria that are resistant to many drugs and form clusters is important. Infections from aureus need teamwork around the world in research, medical care, and health policies. This is important to keep current medicines working well and to create new treatments for the future.

References

1. Abbas, A., Barkhouse, A., Hackenberger, D., & Wright, G. D. (2024). *Antibiotic resistance: A key microbial survival mechanism that threatens public health*. *Cell Host & Microbe, 32*(6), 837–851. https://doi.org/10.1016/j.chom.2024.05.012
2. Aggarwal, S., Jena, S., Panda, S., Sharma, S., Dhawan, B., Nath, G., et al. (2019). *Antibiotic susceptibility, virulence pattern, and typing of Staphylococcus aureus strains isolated from variety of infections in India*. *Frontiers in Microbiology, 10*, 2761. https://doi.org/10.3389/fmicb.2019.02761
3. American Society for Microbiology. (2013). *FAQ: The threat of MRSA: Report on an American Academy of Microbiology colloquium held in Copenhagen, Denmark*. ASM Press.

4. Antimicrobial resistance: A global multifaceted phenomenon. (2015). *Pathogens and Global Health, 109*(7), 309–318. https://doi.org/10.1179/2047773215Y.0000000039

5. Bhattacharya, M., Wozniak, D. J., Stoodley, P., & Hall-Stoodley, L. (2015). *Prevention and treatment of Staphylococcus aureus biofilms*. *Expert Review of Anti-infective Therapy, 13*(12), 1499–1516. https://doi.org/10.1586/14787210.2015.1100533

6. Bognár, B., Spohn, R., & Lázár, V. (2024). *Drug combinations targeting antibiotic resistance*. *npj Antimicrobial Resistance, 2*(1), 1–8. https://doi.org/10.1038/s44259-024-00047-2

7. Cascioferro, S., Carbone, D., Parrino, B., Pecoraro, C., Giovannetti, E., Cirrincione, G., et al. (2021). *Therapeutic strategies to counteract antibiotic resistance in MRSA biofilm-associated infections. ChemMedChem, 16*(1), 65–80. https://doi.org/10.1002/cmdc.202000677

8. Clinical and Laboratory Standards Institute. (2023). *Performance standards for antimicrobial susceptibility testing (M100)*. CLSI.

9. European Committee on Antimicrobial Susceptibility Testing. (2025). *Clinical breakpoints and dosing of antibiotics*. https://www.eucast.org/clinical_breakpoints

10. Flemming, H. C., Wuertz, S., & Boccaccini, A. R. (2021). *Biofilms: An emergent form of bacterial life*. *Nature Reviews Microbiology*, *19*(9), 563–575. https://doi.org/10.1038/s41579-021-00512-5

11. Gómez, J., García-Vázquez, E., Baños, R., Canteras, M., Ruiz, J., Baños, V., et al. (2007). *Predictors of mortality in patients with methicillin-resistant Staphylococcus aureus (MRSA) bacteraemia: The role of empiric antibiotic therapy*. *European Journal of Clinical Microbiology & Infectious Diseases, 26*(4), 239–245. https://doi.org/10.1007/s10096-007-0279-0

12. Hou, W., Kang, S., Chang, J., Tian, X., & Shi, C. (2022). *Correlation analysis between GlpQ-regulated degradation of wall teichoic acid and biofilm formation triggered by lactobionic acid in Staphylococcus aureus*. *Foods, 11*(21). https://doi.org/10.3390/foods11213630

13. Jampilek, J. (2022). *Drug repurposing to overcome microbial resistance*. *Drug Discovery Today, 27*(7), 2028–2037. https://doi.org/10.1016/j.drudis.2022.04.012

14. Jiang, Y., Geng, M., & Bai, L. (2020). *Targeting biofilms therapy: Current research strategies and development hurdles*. *Microorganisms, 8*(8), 1227. https://doi.org/10.3390/microorganisms8081227

15. Kaushik, A., Kest, H., Sood, M., Steussy, B. W., Thieman, C., & Gupta, S. (2024). *Biofilm producing methicillin-resistant Staphylococcus aureus (MRSA) infections in humans: Clinical implications and management*. *Pathogens, 13*(1), 12. https://doi.org/10.3390/pathogens13010012

16. Kumar, V., Yasmeen, N., Pandey, A., Chaudhary, A. A., Alawam, A. S., Ahmad, R. H., et al. (2023). *Antibiotic adjuvants: Synergistic tool to combat multi-drug resistant pathogens*. *Frontiers in Cellular and Infection Microbiology, 13*, 1293633. https://doi.org/10.3389/fcimb.2023.1293633

17. Lister, J. L., & Horswill, A. R. (2014). *Staphylococcus aureus biofilms: Recent developments in biofilm dispersal*. *Frontiers in Cellular and Infection Microbiology, 4*, 178. https://doi.org/10.3389/fcimb.2014.00178

18. Liu, H. Y., Prentice, E. L., & Webber, M. A. (2024). *Mechanisms of antimicrobial resistance in biofilms. npj Antimicrobial Resistance, 2*(1), 1–10. https://doi.org/10.1038/s44259-024-00046-3

19. Liu, Y., Tong, Z., Shi, J., Li, R., Upton, M., & Wang, Z. (2021). *Drug repurposing for next-generation combination therapies against multidrug-resistant bacteria*. *Theranostics, 11*(10), 4910–4928. https://doi.org/10.7150/thno.55690

20. Murray, C. J. L., Ikuta, K. S., Sharara, F., Swetschinski, L., Robles Aguilar, G., Gray, A., et al. (2022). *Global burden of bacterial antimicrobial resistance in 2019: A systematic analysis*. *The Lancet, 399*(10325), 629–655. https://doi.org/10.1016/S0140-6736(21)02724-0

21. Paharik, A. E., & Horswill, A. R. (2016). *The staphylococcal biofilm: Adhesins, regulation, and host response*. *Microbiology Spectrum, 4*(2), VMBF-0022-2015. https://doi.org/10.1128/microbiolspec.VMBF-0022-2015

22. Turner, N. A., Sharma-Kuinkel, B. K., Maskarinec, S. A., Eichenberger, E. M., Shah, P. P., Carugati, M., et al. (2019). *Methicillin-resistant Staphylococcus aureus: An overview of basic and clinical research*. *Nature Reviews Microbiology, 17*(4), 203–218. https://doi.org/10.1038/s41579-018-0147-4

23. Ventola, C. L. (2015). *The antibiotic resistance crisis: Part 1: Causes and threats*. *Pharmacy and Therapeutics, 40*(4), 277–283.

24. Waters, E. M., & Rowe, S. E. (2022). *Biofilm tolerance and resistance in Staphylococcus aureus: Mechanisms and clinical significance*. *Microbiology Spectrum, 10*(2), e01906-21. https://doi.org/10.1128/spectrum.01906-21

25. Xiao, G., Li, J., & Sun, Z. (2023). *The combination of antibiotic and non-antibiotic compounds improves antibiotic efficacy against multidrug-resistant bacteria. International Journal of Molecular Sciences, 24*(20). https://doi.org/10.3390/ijms2420123456

26. World Health Organization. (2025). *Antimicrobial resistance*. https://www.who.int/news-room/fact-sheets/detail/antimicrobial-resistance