Molecular Characterization of Biofilm Formation-Associated Genes, and Antibiotic Resistance Burden in MDR Staphylococcus Aureus

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Abstract: *Staphylococcus aureus* is an important opportunistic pathogen associated with antimicrobial resistance, biofilm formation, and persistent infections, representing a major challenge in clinical treatment. This study aimed to evaluate antimicrobial resistance profiles, biofilm formation ability, and the distribution of biofilm- and virulence-associated genes among multidrug-resistant (MDR) and extensively drug-resistant (XDR) *S. aureus* isolates. A total of 250 clinical bacterial isolates were examined, from which 50 *S. aureus* isolates obtained from different clinical sources were identified using conventional microbiological methods and confirmed by the automated VITEK® 2 system. Antimicrobial susceptibility testing was performed according to CLSI guidelines, and isolates were classified as MDR and XDR. Biofilm production was assessed using the microtiter plate method. All isolates demonstrated biofilm-forming ability, including strong (84%), moderate (10%), and weak (6%) producers. A significant difference in resistance burden was observed between MDR and XDR isolates, with mean resistance scores of 9.62 and 14.10, respectively ($p = 5.66 \times 10^{-7}$). Twenty highly resistant isolates were selected for molecular analysis by PCR targeting the *agrA*, *icaA*, *sarA*, and *fnbA* genes. The detected prevalence of these genes was *fnbA* (100%), *icaA* (90%), *sarA* (85%), and *agrA* (80%). The findings indicate that biofilm formation and the presence of associated virulence genes may contribute to enhanced antimicrobial resistance and bacterial persistence. Integration of phenotypic and molecular approaches is essential for improved surveillance, diagnosis, and management of resistant *S. aureus* infections.

1 INTRODUCTION

Staphylococcus aureus may cause skin infections, bacteremia, endocarditis, and necrotizing pneumonia, and antibiotic resistance and host adaptation make it clinically important [1]. The high MDR/XDR of S. aureus requires complex treatment and higher global healthcare costs, and Multidrug-resistant (MDR) isolates resist three or more antimicrobial classes, whereas Extensively drug-resistant (XDR) isolates resist practically all antibiotics, limiting treatment options [2], [3].

Bacterial adaptability and pathogenicity are regulated by agrA, sarA, icaA, and fnbA, with these genes regulating adhesion [4], [5], stress response, and antibiotic resistance, which may help bacteria resist antimicrobial strains, and impact of gene distribution on antibiotic resistance is unclear [6]. This work molecularly identified select virulence-associated genes with biofilms formation (agrA, sarA, icaA, and fnbA) in clinical isolates of S. aureus and evaluated their relationship with antibiotic resistance burden in MDR and XDR groups using statistical analysis. Resistance escalation may be detected, and clinical management improved.

2 MATERIALS AND METHODS

2.1 Bacterial Isolates

A total of 250 bacterial isolates from various clinical sources, including blood, wounds, burns, and urine, were collected from samples obtained from Baquba General Hospital (Diyala, IRAQ), this study was conducted during the period from 11/2024 to 3/2025, According to the ethics book numbered (C E P 4 0

3)which included colony morphology, Gram staining, and biochemical testing, and current study included only confirmed isolates of S. aureus bacteria, while duplicate isolates, contaminated cultures and uncertain isolates were excluded and 50 isolates were identified as S. aureus. Next, antibiotic resistance was assessed on certified S. aureus isolates. The twenty most antibiotic-resistant isolates were molecularly analyzed, and these highly resistant isolates were tested for biofilms formation genes using PCR [7].

2.2 Bacterial Identification

Initially, S. aureus isolates were characterized using colony morphology, Gram staining, and biochemical assays. Colonies resembling S. aureus were chosen for confirmation. The isolates were examined for identification confirmation using the automated VITEK® 2 system (bioMérieux, France) according to the manufacturer's instructions. Gram-positive bacteria were identified using cards after the adjustment of bacterial solutions to the appropriate turbidity. This approach was swiftly and accurately identified using molecular profiling. Only S. aureus isolates verified using conventional and VITEK® 2 methodologies were assessed for antibiotic susceptibility and molecular analysis.

2.3 Antibiotic Susceptibility Testing

The automated VITEK® 2 system (bioMérieux, France) assessed antimicrobial susceptibility per manufacturer instructions. Bacterial suspensions were adjusted to 0.5 McFarland before analysis. The approach examined isolates for multi-class antibiotic susceptibility. results were interpreted as MIC values with a final interpretation for each antibiotic were interpreted following CLSI criteria. Resistance profiles identified MDR/XDR isolates. MDR isolates were resistant to at least one agent in three or more antimicrobial categories, whereas XDR isolates were resistant to practically all agents with limited susceptibility [8].

2.4 Biofilm Formation Assay

The formation of biofilm was evaluated using microtiter plates, and biofilm presence was investigated as reference [9] and isolates were categorized as non-adherent, weak, moderate, or robust biofilm producers based on their values.

2.5 DNA Template Preparation by the Boiling Method

The DNA template was produced using the boiling process outlined by Ali et al. (2025) [10], and 5 separate colonies of overnight-cultivated bacteria were completely suspended in 2 ml of distilled water and subjected to boiling in a water bath for 10 minutes. Following centrifugation, the supernatant served as the template DNA for the PCR.

2.6 Polymerase Chain Reaction Amplification

The PCR amplification process for identifying local isolates of S. aureus at the genetic level [11], as shown in Table 1, follows these steps: the final volume of the PCR mixture was 25 µl, comprising 12.5 µl of 2x Master Mix, 5 µl of template DNA, 1 µl of each forward and reverse primer, and 5.5 µl of nuclease-free water, prepared in uniplex PCR Eppendorf tubes. The quantities were modified for multiplex PCR, followed by brief vortex mixing before placement in the thermocycler for polymerase chain reaction. The protocol for each multiplex PCR combination is shown in Tables 1 and 2.

2.7 Statistical Analysis

The IBM SPSS Statistics (version 28.0) (IBM Corp., Armonk, NY, USA) was used for statistical analysis, and data normality was established before analysis [12]. Due to the non-normal distribution of antibiotic resistance scores, the non-parametric Wilcoxon rank-sum test (Mann-Whitney U test) was employed to compare MDR and XDR groups. Finally, statistical significance was specified as p-value<0.05. Graphics showing group resistance score distribution and variability.

3 RESULTS

3.1 Distribution of Bacterial Isolates

Initial clinical samples produced 250 bacterial isolates; the S. aureus was confirmed in 50 isolates from differentiated clinical sources, by standard microbiological and biochemical methods. Antibiotic susceptibility testing was conducted on these confirmed isolates to identify resistance.

Table 1: PCR amplification conditions of primers.

Amplified gene	Initial DENATURATION	NO. OF CYCLE	DENATURATION	ANNEALING	ELONGATION	Final extension
agrA	95°C/ 5min	35	95°C/ 30 SEC	55°C/1MIN	72°C/30 SEC	72°C/2MIN
icaA	94°C/ 3min	35	95°C/ 40 SEC	61°C/40 SEC	72°C/40 SEC	72°C/2MIN
sarA	94°C/ 5min	35	95°C/ 1MIN	60°C/1MIN	72°C/1 MIN	72°C/2MIN
fnbA	94°C/ 5min	35	95°C/ 1MIN	60°C/1MIN	72°C/1 MIN	72°C/2MIN

Table 2: Primer sequences used for PCR amplification.

Gene	Nucleotide sequence (5′–3′)	Product size (bp)	Reference
agrA	F: TCGTAAGCATGACCCAGTTG R: AAATCCATCGCTGCAACTTT	96	[13]
icaA	F: CTTGCTGGCGCAGTCAATAC R: GTAGCCAACGTCGACAACTG	109	[14]
sarA	F: CCCAGAAATACAATCACTGTGR: AGTGCCATTAGTGCAAAACC	720	[15]
fnbA	F: CATAAATTGGGAGCAGCATCAR: ATCAGCAGCTGAATTCCCATT	127	[16]

Table 3: Antibiotic resistance pattern of s. aureus isolates.

Antibiotic Class	Example Antibiotic	Resistance Trend
Sulfonamides	Trimethoprim/Sulfamethoxazole	High
Rifamycins	Rifampicin	High
Fusidic acid	Fusidic acid	High
Tetracyclines	Tetracycline	Moderate
Glycopeptides	Teicoplanin	Moderate
Macrolides	Erythromycin	High
Fluoroquinolones	Ciprofloxacin	High
Aminoglycosides	Gentamicin	Moderate-High
β-lactams	Oxacillin, Benzylpenicillin	Very high

3.2 Profiles of Antibiotic Resistance

Various S. aureus isolates have shown resistance to many kinds of antibiotics, including sulfonamides, rifamycins, tetracyclines, glycopeptides, macrolides, fluoroquinolones, aminoglycosides, and β-lactams. Elevated resistance rates were seen for rifampicin, fusidic acid, and β-lactam antibiotics, particularly oxacillin and benzylpenicillin; However, several isolates exhibited greater susceptibility to tigecycline and levofloxacin. Resistance patterns categorized isolates as multidrug-resistant (MDR) or extensively drug-resistant XDR, and these XRD isolates exhibited a more pronounced resistance profile, being resistant to almost all antibiotics. The antibiotic-resistant isolates underscored the clinical difficulties in managing S. aureus infections, as shown in Table 3.

3.3 Antibiotic Resistance Burden

A resistance score was assigned to each isolate to objectively evaluate resistance, based on the quantity of antibiotics to which it exhibited resistance. A significant difference in resistance load was seen between the MDR and XDR groups. The mean resistance score for MDR isolates was 9.62, whereas XDR isolates had a significantly higher average score of 14.10. The Wilcoxon rank-sum test indicated a statistically significant difference between the two groups ($p = 5.66 \times 10^{-7}$), showing that XDR isolates had a much greater resistance burden than MDR isolates, as shown in Figure 1.

3.4 Biofilm Formation

All Isolates of S. aureus exhibited powerful (84%), moderate (10%), and mild (6%) responses. Absence of non-adherent isolates. Substantial biofilm proliferation across isolates ($p < 0.0001$) indicates a pronounced propensity for biofilm formation, and this study indicates that biofilm formation may enhance bacterial resistance to antimicrobials.

3.5 Selecting Highly Resistant Isolates for Molecular Analysis

The molecular analysis of 20/50from every source and selected the strongest isolate, verified from S. aureus isolates with the highest resistance scores, and this selection focused on therapeutically relevant isolates with higher resistance profiles to study genetic variables.

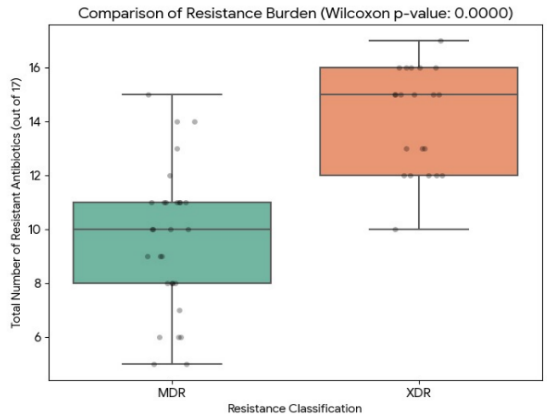

Figure 1: Comparison of resistance burden between MDR and XDR.

3.6 Virulence-Linked Gene Molecular Identification

The PCR was used to screen the isolates for virulence genes, such as agrA, icaA, sarA, and fnbA, and the distribution of these genes was found in the isolates. From these results, the FnbA was found more commonly than icaA, agrA, and sarA. Isolate genetic diversity shows varied gene contributions to bacterial adaptability, as shown in Table 4.

Table 4: Genes among selected S. aureus isolates.

Gene	Positive isolates (n=20)	Percentage (%)
fnbA	20	100%
icaA	18	90%
sarA	17	85%
agrA	16	80%

3.7 Agarose Gel Electrophoresis

The PCR results showed, using agarose gel electrophoresis, discrete DNA bands that matched the sizes of the amplified genes, and successful amplification was confirmed by bands at 96, 109, 720, and 127 bp for agrA, icaA, sarA, and fnbA. The gel images, as shown in Figure 2, show distinct target gene bands.

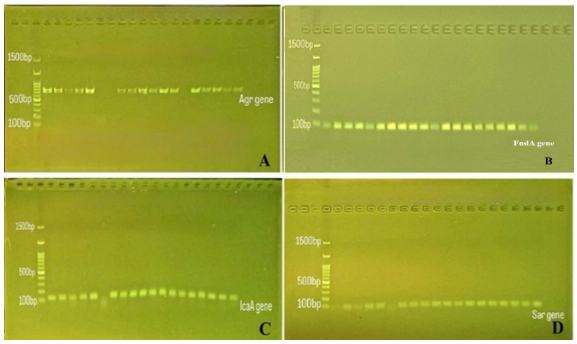

Figure 2: Agarose gel electrophoresis of different amplifications of target genes: (A) agrA gene; (B) fnbA gene; (C) icaA gene; (D) sarA gene.

4 DISCUSSION

S. aureus showed full mannitol sugar fermentation as colony the yellow verify the diagnosis of bacterial isolates extracted from clinical samples, the System Compact Vitek-2 was confirmed [17].This research found that XDR S. aureus isolates were far more drug-resistant than MDR isolates. Research shows that resistance factors cause MDR-XDR phenotypes [18], [19], with the study's substantial difference ($p = 5.66 \times 10^{-7}$) demonstrating how selection pressure and genetic adaptation increase resistance. While the efflux pumps, target alteration, and antibiotic enzymatic degradation may make XDR isolates resistant [20], S. aureus resists excessive antibiotic exposure in drug-overusing hospitals via several mechanisms. This supports recent researchers' results that XDR strains extend hospital stays and increase death [3], and high amounts of icaA, agrA, and sarA were found, but fnbA was low. The presence of icaA shows that bacteria may withstand antibiotics, and influence polysaccharide synthesis and bacterial defense, which may indirectly alter resistance [21].

This research found that 84% of S. aureus isolates generated robust biofilms, suggesting that biofilms enhance antibiotic resistance. By obstructing antibiotics, biofilms enable bacteria to endure extreme circumstances, with metabolic alterations in biofilm cells diminish antibiotic sensitivity and extend longevity [22].

Prior studies have shown that robust biofilm makers exhibit multidrug resistance and virulence. Antibiotics and host immune responses are ineffective in eliminating biofilm-associated illnesses [23]. The significant biofilm formation rate observed in this investigation may explain the increased resistance burden, particularly in XDR isolates.

The research also identified elevated rates of antibiotic resistance for β-lactams, rifamycins, and macrolides. Enzymatic degradation, target alteration, and efflux mechanisms may precipitate treatment failure because to this extensive resistance spectrum [24]. Prolonged antimicrobial exposure leads to the accumulation of resistance mechanisms in XDR isolates.

The genes agrA and sarA are found in many isolates, indicating they affect gene expression and stress response, in addition these regulatory mechanisms may alter metabolic activity and antibiotic tolerance to help bacteria adapt [25]. While the low frequency of fnbA shows that adhesion-related variables may play a smaller role in resistance than regulatory and structural genes, supporting Jomehzadeh, Nabi, and Sogol Seif Emrani [26].

S. aureus isolate genetic variation and population heterogeneity determine pathogenicity and resistance, and variability makes S. aureus infection treatment difficult [27]. Genetic studies using highly resistant samples provide concentrated insight into clinically relevant disorders, but this may restrict generalizability [28]-[30]. So, the inference is that virulence-associated gene distribution may promote XDR isolate resistance. Integrating genetic and phenotypic investigations helps understand antibiotic resistance. Resistance and gene prevalence are linked.

In this study suggests ongoing monitoring strategies to track MDR and XDR S. aureus in clinical settings, and early discovery resistance associated and biofilm-related genes requires molecular and phenotypic methodologies. The antimicrobial overuse reduced by strict infection control and reasonable antibiotic management. In future study will examine different size, such as bigger sample sizes, gene expression analysis, and innovative biofilm-targeted therapies.

5 CONCLUSIONS

This study demonstrated that clinical *Staphylococcus aureus* isolates possess high levels of antimicrobial resistance and biofilm-forming capacity, particularly among XDR isolates, which showed a significantly greater resistance burden compared with MDR isolates. The high prevalence of biofilm production among isolates indicates that biofilm formation may represent an important mechanism supporting bacterial persistence and reduced antimicrobial susceptibility.

Molecular analysis revealed a high distribution of biofilm- and virulence-associated genes, with *fnbA* detected in all examined isolates (100%), followed by *icaA* (90%), *sarA* (85%), and *agrA* (80%). These findings suggest that adhesion, biofilm regulation, and virulence-associated mechanisms may contribute to the survival and adaptation of resistant *S. aureus* strains under antimicrobial pressure.

The combination of antimicrobial susceptibility testing, biofilm evaluation, and molecular characterization provides a more comprehensive understanding of resistance mechanisms in *S. aureus*. Continuous monitoring of MDR and XDR strains, together with rational antibiotic use and effective infection-control measures, is essential to limit the spread of resistant pathogens.

This study was limited by the molecular investigation of selected highly resistant isolates rather than the entire collection of isolates. Further studies involving larger populations, genomic approaches, and gene expression analysis are required to clarify the functional role of resistance and virulence determinants and to support the development of improved therapeutic strategies.

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