Molecular and Phenotypic Characterization of Biofilm Formation, Antibiotic Resistance, and Virulence Determinants in Clinical Staphylococcus aureus Isolates

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ABSTRACT

Staphylococcus aureus is a very important pathogen in medicine. This is due to the various mechanisms involved in the initiation and maintenance of infection including the formation of biofilms, antibiotic resistance and the presence of genetic determinants of virulence. The aim of this virtual study was to evaluate the association between biofilm formation, antibiotic resistance patterns and the presence of specific virulence and resistance genetic markers in clinical isolates of this bacterium. The model was consisted of 100 clinical isolates. Biofilm formation ability, antibiotic susceptibility profiles and prevalence of icaA, fnbA, hla and mecA genes were determined. The results indicated that 80% of the isolates were able to form biofilms to different degrees:20% was strong biofilm-forming ability, 35% moderate, and 25% weak, while 20% was no biofilm-forming ability and according to the these criteriait was classified. Methicillin resistance was observed in 42% of the isolates, and multidrug resistance in 48%, with the highest resistance rate recorded against penicillin (82%). At the molecular level, the detection rates for the icaA, fnbA, hla, and mecA genes were approximately 68%, 72%, 81%, and 42%, respectively. The data revealed an increase in the proportion of multidrug-resistant isolates from 20% among non-biofilm-forming isolates to 80% among strong biofilm-forming isolates, suggesting a potential link between the intensity of biofilm formation and multidrug resistance. These results highlight the importance of comprehensively studying biofilms, virulence determinants, and genetic resistance to understand the pathogenic characteristics of S. aureus.

Keywords: Staphylococcus aureus, biofilms, antibiotic resistance, virulence determinants, multidrug resistance.

INTRODUCTION

Staphylococcus aureus is considered one of the most important human pathogens, with a wide spectrum of conditions from skin and soft-tissue infections and wound-related infections to more severe cases, such as bacteremia, endocarditis and pneumonia. Medical relevance is associated with a wide range of virulence factors which are responsible for adhesion to host cells, evasion of immune defenses and tissue damage. Moreover, the ability of the bacterium to acquire mechanisms of resistance to antibiotics poses a continuing challenge to the control of the infections it causes in healthcare settings (Tong et al., 2015). The spread of antibiotic-resistant strains, in particular methicillin-resistant Staphylococcus aureus (MRSA) exacerbates S. aureus infections. Such resistance is mainly linked to the acquisition of the mecA gene , encoding for a low-affinity penicillin-binding protein, called PBP2a. This allows the bacteria to keep on synthesizing their cell walls in the presence of various beta-lactam antibiotics. The emergence of MRSA has complicated the treatment of healthcare-associated and community-acquired infections, and has highlighted the need to study the association between antibiotic resistance and other biological features of this bacterium (Turner et al., 2019). Another key feature of *S. aureus* is biofilm formation, the ability of this bacterium to form microbial communities attached to surfaces and embedded in a multi-component extracellular matrix composed of polysaccharides, proteins, and extracellular nucleic acids. This structural organization allows the bacteria to

colonize a wide variety of environments and contributes to the persistence of infections,making them difficult to eradicate. Biofilms are particularly significant in the context of chronic and medical device-associated infections, as they provide an environment that supports bacterial survival and enhances tolerance to environmental and therapeutic stressors (Flemming et al., 2016).

Genetic resistance to antibiotics is not the only reason for increased bacterial tolerance within a biofilm; reduced response to treatment may be caused by factors such as poor diffusion of antibiotics into some parts of the biofilm, slow metabolic growth of some cells, and the presence of tolerant cells. Thus, it is important to distinguish between genetic resistance, which can be evaluated by conventional susceptibility tests, and tolerance related to the physiological state of cells in the biofilm. This distinction is important in the interpretation of the relationship between the extent of biofilm formation and phenotypic resistance patterns (Hall & Mah, 2017). The formation of biofilms is influenced by a variety of genetic determinants that impact cell adhesion and biofilm structure and stability. Prominent among these are the icaA gene, involved in the synthesis of intercellular polysaccharide in strains using this pathway, and the fnbA gene, which encodes a surface protein that facilitates bacterial binding to fibronectin, a component of the host tissue's extracellular matrix. The hla gene is also a major virulence-associated gene because it encodes alpha-toxin, which can damage host cells. However, the presence of these genes does not necessarily mean that they are expressed at the same level in all the isolates. Moreover, biofilm formation in S. aureus may depend on alternative pathways and mechanisms that are strain- and environment-dependent (Flemming et al., 2016; Hall & Mah, 2017).

This underscores the necessity of a united analytical framework to investigate the relationship between biofilm formation, antibiotic resistance, and virulence factors. While these traits may overlap for some isolates, an observed association does not necessarily indicate a direct causal relationship between the traits. The combination of phenotypic assays of biofilm formation, antibiotic susceptibility testing and molecular analysis of selected genes may help to elucidate the patterns of association between these traits and to identify factors that deserve further investigation. Thus, the present study was designed to evaluate the association of level of biofilm formation, antibiotic resistance profiles, virulence factors and genetic resistance in clinical isolates of S. aureus.

This involves determining biofilm formation levels, characterizing antibiotic susceptibility patterns, estimating the prevalence of the icaA, fnbA, hla, and mecA genes, and analyzing the relationships between these variables using appropriate statistical methods. This design is intended to provide an educational framework for understanding the interplay between the phenotypic and molecular characteristics of the bacterium

MATERIALS AND METHODS

Study Design and Collection of Bacterial Isolates

The current study was carried out with descriptive-analytical design to investigate the relationship between biofilm formation, antibiotic resistance and virulence determinants in clinical isolates of Staphylococcus aureus. One hundred bacterial isolates were obtained from different clinical samples and studied. The study was conducted to evaluate the phenotypic and molecular characteristics of the isolates and to study the correlations between the studied variables. The clinical samples were collected from diverse sites of infection such as wound swabs, urine, blood and nasal swabs as per the source of sample and clinical diagnosis. The isolates were preserved by suitable microbiological storage techniques until laboratory examination.

Identification of Bacterial Isolates

The isolates were identified based on phenotypic characteristics and conventional microbiological tests. These tests included colony morphology on nutrient agar and blood agar, Gram staining, the catalase test, and the coagulase test. Additional identification tests were employed as needed to confirm the identity of the isolates.

Evaluation of Biofilm-Forming Ability

The ability of the isolates to form biofilms was determined by microtiter plate assay method with crystal violet staining, according to the protocol described by Stepanović et al. (2000), with appropriate modifications to suit laboratory conditions. The isolates were grown in a suitable growth medium and bacterial cultures were transferred into wells of a multi-well microtiter plate, including negative control wells with the medium without the bacteria. After the specified incubation period, the non-adherent cultures were removed and the wells were gently washed to remove cells not attached to the surface. Adherent cells were fixed and stained with crystal violet, the excess stain was removed and absorbance was measured by a microplate reader at a wavelength appropriate for the adopted protocol. Negative control readings were used to determine the cutoff point between biofilm-forming and non-biofilm-forming isolates, and the isolates were classified into four categories—non-forming, weak, moderate, and strong formers—based on pre-established classification criteria.

Antibiotic Susceptibility Testing

Antibiotic susceptibility patterns were determined by the disk diffusion method according to the guidelines of Clinical and Laboratory Standards Institute (CLSI). The tests were carried out with some antibiotics of therapeutic importance for infections by Staphylococcus aureus, using Mueller-Hinton agar as a culture medium and preparing the bacterial suspensions according to standardized criteria of turbidity. Plates were incubated under the recommended conditions and the diameters of the zones of growth inhibition were measured in mm. Results were interpreted as susceptible, intermediate or resistant, using interpretive breakpoints for each antibiotic. Methicillin resistance in S. aureus was phenotypically detected by cefoxitin disk test as a validated surrogate method following the relevant CLSI guidelines.

Determination of Multidrug Resistance

Isolates were classified as multidrug-resistant (MDR) were classified according if they exhibited resistance to at least one agent in three or more different antibiotic categories, (Magiorakos et al. 2012). Susceptibility results were used to determine the proportion of MDR isolates, and this proportion was then compared across different biofilm-forming categories.

Molecular Detection of Virulence- And Resistance-Associated Genes

Polymerase chain reaction (PCR) was performed to identify a panel of genes involved in biofilm formation, adhesion, virulence and methicillin resistance. The targeted genes were:

icaA: Involved in intercellular polysaccharide formation in some isolates.

fnbA: Encodes a surface protein that facilitates binding to fibronectin.

hla: produces alpha-toxin, a factor associated with damage of host cells.

mecA: encodes penicillin-binding protein PBP2a and is associated with methicillin resistance.

DNA extraction was carried out for bacterial isolates by appropriate extraction method and PCR reactions were performed using gene specific primers under suitable amplification conditions. Amplification products were run on agarose gels with appropriate positive and negative controls to determine the presence of genes based on expected sizes of the products.

Table 1. Primer Sequences for Detection of Biofilm-Associated, Virulence, and Methicillin-Resistance Genes in Staphylococcus aureus

Target gene	Primer	Sequence (5′–3′)	Amplicon size (bp)	Reference
icaA	Forward	GATTATGTAATGTGCTTGGA	770	Rasmi et al. (2022)
icaA	Reverse	ACTACTGCTGCGTTAATAAT	770	Rasmi et al. (2022)

fnbA	Forward	GCGGAGATCAAAGACAA	1279	Rasmi et al. (2022)
fnbA	Reverse	CCATCTATAGCTGTGTGG	1279	Rasmi et al. (2022)
hla	Forward	CTGATTACTATCCAAGAAATTCGATTG	209	Rasmi et al. (2022)
hla	Reverse	CTTTCCAGCCTACTTTTTTATCAGT	209	Rasmi et al. (2022)
mecA	Forward	GTAGAAATGACTGAACGTCCGATGA	310	Eslamnezhad et al. (2025)
mecA	Reverse	CCAATTCCACATTGTTTCGGTCTAA	310	Eslamnezhad et al. (2025)

Statistical Analysis

Data were analyzed using appropriate statistical software. Categorical variables were presented as frequencies and percentages, and the relationships between biofilm formation levels, antibiotic resistance patterns, and the presence of genes were assessed using the Chi-square test.

RESULTS

Distribution of Isolates Based on Biofilm-Forming Ability

The results of the study showed that 80 (80%) of 100 clinical isolates of Staphylococcus aureus could form biofilms to varying degrees. These isolates classified into weak, moderate and strong biofilm forming categories. 20 isolates (20%) had no biofilm forming ability according to the classification criteria used in this study. Moderate biofilm-forming isolates were the largest group (35 isolates; 35%), followed by weak biofilm-forming isolates (25 isolates; 25%) and strong biofilm-forming isolates (20 isolates; 20%).

Table 2 . Distribution of clinical Staphylococcus aureus isolates according to their biofilm formation capacity

	Number of isolates	Percentage (%)
Non-biofilm formers	20	20.0
Weak biofilm formers	25	25.0
Moderate biofilm formers	35	35.0
Strong biofilm formers	20	20.0
Total	100	100

Antibiotic Resistance Patterns

Analysis of the antibiotic susceptibility patterns showed different resistance rates for the tested antibiotics. The highest resistance rate was observed for penicillin (82%), followed by erythromycin (48%). Resistance to cefoxitin, the agent used in this model to identify phenotypic isolates classified as MRSA, was 42%. Moreover, multidrug-resistant isolates accounted for 48% of the total isolates.

Table 3. Antibiotic Resistance Patterns Among Clinical Staphylococcus aureus Isolates

Antibiotic	Resistant isolates (n)	Resistance (%)
Penicillin	82	82.0
Erythromycin	48	48.0
Cefoxitin	42	42.0
Tetracycline	35	35.0
Clindamycin	31	31.0
Trimethoprim-sulfamethoxazole	28	28.0

Gentamicin	24	24.0
Rifampicin	12	12.0

Detection of Genes Associated with Biofilm Formation, Virulence, and Methicillin Resistance

The molecular analysis showed differences in the detection rate of the target genes among the studied isolates. The hla gene was the most common with 81 isolates (81%), followed by fnbA gene with 72 isolates (72%) and icaA gene with 68 isolates (68%). mecAgene was detected in 42 isolates (42%) which is equal to the number of isolates phenotypically classified as methicillin-resistant.

Table 4. Prevalence of Target Genes Among Clinical Staphylococcus aureus Isolates

Target gene	Gene function	Positive isolates (n)	Positive isolates (%)
icaA	Polysaccharide intercellular adhesin-associated biofilm formation	68	68.0
fnbA	Fibronectin-binding protein and adhesion	72	72.0
hla	Alpha-hemolysin production	81	81.0
mecA	Methicillin resistance	42	42

Relationship between Biofilm Formation and Multidrug Resistance

When the categories of biofilm formation were compared, the proportion of multidrug resistant isolates was found to increase with the intensity of biofilm formation. The rate was 20% in non-biofilm formers, 32% in weak biofilm formers, 57.1% in moderate biofilm formers and 80% in strong biofilm formers. These results indicate an increase in the rate of multidrug resistance with an increase in the intensity of biofilm formation.

Table 5. Association Between Biofilm Formation Capacity and Multidrug Resistance

Biofilm formation category	Total isolates (n)	MDR-positive isolates (n)	MDR-positive isolates (%)
Non-biofilm formers	20	4	20.0
Weak biofilm formers	25	8	32.0
Moderate biofilm formers	35	20	57.1
Strong biofilm formers	20	16	80.0
Total	100	48	48

The chi-square test, based on the frequencies presented in the table, demonstrated a statistical association between biofilm formation categories and multidrug resistance ($\chi^2 = 18.22$, df = 3, $p < 0.001$).

Table 6. Summary of Major Study Findings

Study parameter	Main finding
Overall biofilm formation	80%
Strong biofilm formation	20%
Methicillin resistance	42%
Multidrug resistance	48%
Highest antibiotic resistance	Penicillin (82%)
Most prevalent target gene	hla (81%)

icaA prevalence	68%
fnbA prevalence	72%
mecA prevalence	42%
MDR among strong biofilm formers	80%
MDR among non-biofilm formers	20%

DISCUSSION

Staphylococcus aureus is a pathogen of clinical importance due to its wide array of virulence factors, its ability to form biofilms and its acquisition of resistance to several antibiotics. The aim of the present study was to analyze the relationship between biofilm formation, antibiotic resistance and the presence of specific virulence and resistance genes in clinical isolates of this bacterium. The results showed that Based on the classification used, 80% of the isolates were able to form biofilms to varying degrees, classified as weak, moderate and strong formation at rates of 25%, 35% and 20% respectively, while 20% of the isolates did not show biofilm forming ability. These results indicate the ability of biofilm formation is a common characteristic among the studied isolates and may be involved in the bacteria's ability to colonize host environments and survive adverse conditions. This interpretation is in line with the observations of Flemming et al. (2016) on the importance of biofilms as structured microbial communities surrounded by an extracellular matrix. The role of biofilms in reducing the effectiveness of antibiotics via mechanisms such as restricted drug penetration is also discussed by Hall and Mah (2017).

Staphylococcus aureus is a human pathogen that colonizes host tissues, produces a wide range of virulence factors, and forms biofilms and is resistant to antimicrobial drugs. In this study, 100 clinical S. aureus isolates were characterized for antibiotic susceptibility patterns, multidrug resistance (MDR), biofilm formation, and the presence of icaA, fnbA, hla, and mecA genes. The results represented a combination of phenotypic and molecular findings among the investigated isolates and suggested a possible relationship between biofilm formation and antimicrobial resistance (Radhi et al. 2026).

Among the isolates, 15 (80%) were biofilm formers (weak, 25%; moderate, 35%, and strong, 20%), but no biosfilm was detected with 5 (20%) isolates under the conditions test. The high prevalence of biofilm producing isolates indicates that surface associated growth was a common trait among the isolates studied. Biofilms are communities of microorganisms encased within an extracellular matrix of polysaccharides, proteins and extracellular DNA. This organization facilitates bacterial attachment and persistence and may increase survival upon exposure to antimicrobial agents and other environmental stresses (Flemming et al., 2016).

Furthermore, Hall and Mah (2017) described how the decreased antimicrobial susceptibility in biofilms can be attributed to a variety of interacting mechanisms, including interactions between antimicrobial agents and matrix components, lower cellular growth rates, and the presence of antibiotic-tolerant subpopulations. The high incidence of biofilm formation in the present study is biologically important for the persistence of S. aureus infections. However, biofilm formation per se is not enough to establish clinical treatment failure and its effect size depends on the bacterial strain, the antimicrobial agent and the experimental conditions (Khdhair et al.2024).

Antimicrobial susceptibility testing showed a lot of variation in resistance to the tested agents. The highest resistance rate was found for penicillin (82%), followed by erythromycin (48%) and cefoxitin (42%). The high prevalence of penicillin resistance is consistent with the known capacity of S. aureus to resist β-lactam antibiotics, often via production of β-lactamases. Resistance to erythromycin further demonstrates the presence of resistance to a different antimicrobial class. The results emphasize the significance of susceptibility to multiple antibiotics in the characterization of clinical isolates. The clinical significance of S. aureus infections and its management challenges have been described previously (Tong et al., 2015). Methicillin-resistant S. aureus (MRSA) is still relevant in clinical practice (Turner et al., 2019). However, the resistance rates obtained

in this study should be compared with those of other studies with caution as antimicrobial panels, patient populations, geographic settings and laboratory procedures may differ.

Resistance to cefoxitin was detected in 42% isolates and mecA gene was detected in 42 %. The mecA gene encodes penicillin-binding protein 2a (PBP2a) with decreased affinity for most β-lactam antibiotics, and it is responsible for the methicillin-resistant phenotype.

Cefoxitin disk diffusion is commonly used as a phenotypic surrogate for detection of methicillin resistance in S. aureus, according to standardized susceptibility-testing guidance (Clinical and Laboratory Standards Institute [CLSI], 2024). In general, the matching percentages obtained in this study are consistent with the distribution of phenotypic and genotypic resistance in the dataset. However, identical percentages do not necessarily imply the same isolates were positive by both methods. Concordance can only be established by an isolate-level comparison. Moreover, mecA presence doesn't always imply multidrug resistance that is defined as resistance to different antimicrobial classes and not a single resistance gene (Jassim, et al.2025).

They also looked at the distribution of icaA, fnbA, and hla in connection with antimicrobial resistance, demonstrating the need of evaluating virulence-associated factors in addition to resistance phenotypes. Since the isolate sources, antimicrobial drugs tested, and demographic variables may affect the observed prevalence, the discrepancy between the MDR percentages reported in the two studies should not be taken as a direct difference in clinical severity.

The most commonly found target gene, according to molecular analysis, was hla (81%), followed by fnbA (72%), icaA (68%), and mecA (42%). The high frequency of hla suggests that the isolates under investigation shared the genetic determinant for alpha-hemolysin. alpha-hemolysin is an important virulence factor involved in host-cell injury and tissue damage, but PCR detection identifies gene presence rather than the amount of toxin produced. Gene expression may differ according to regulatory mechanisms, bacterial genetic background and environmental conditions. Thus, the high prevalence of hla supports the significance of investigating toxin-associated genetic determinants in clinical S. aureus, but does not independently prove hla-positive isolates caused more severe infections (Tong et al., 2015; Rasmi et al., 2022).

The fnbA gene was found in 72% of the isolates. It encodes fibronectin-binding protein A, a surface-associated adhesin that mediates interactions with fibronectin in the host extracellular matrix. Such interactions can promote bacterial attachment and colonization. Thus, the relatively high prevalence of fnbA is consistent with the importance of adhesion mechanisms in S. aureus pathogenicity. However, the presence of fnbA alone cannot be used to determine whether an isolate forms a stronger biofilm than an isolate without the gene. A direct comparison of fnbA-positive and fnbA-negative isolates across the biofilm-forming categories would be needed to demonstrate such a relationship.

The icaA gene was detected in 68% of the isolates. This gene is involved in the synthesis of polysaccharide intercellular adhesin via the ica-dependent pathway and may participate in intercellular accumulation and biofilm structure. Its prevalence is of interest to the observation that 80% of the isolates formed biofilms. However, the overall prevalence of icaA and the percentage of isolates producing biofilm cannot be used to establish whether the gene was present in each isolate forming biofilm. Other ica-independent mechanisms, such as protein-associated mechanisms, may also contribute to biofilm formation in S. aureus (Flemming et al., 2016; Hall & Mah, 2017). Therefore, a gene-by-biofilm-category analysis would be needed to determine if icaA carriage was associated with weak, moderate or strong biofilm formation in the present collection. Rasmi et al. (2022) also investigated the prevalence of icaA and fnbA in clinical isolates, providing relevant published context for evaluating these adhesion- and biofilm-associated determinants. Hassan, et al.2025.

The most striking relationship in the present study was the increase in the prevalence of MDR across biofilm-forming categories. MDR was found in 20% of non-biofilm forming isolates, 32% of weak biofilm formers, 57.1% of moderate biofilm formers and 80% of strong biofilm formers. The reported chi-square analysis showed a statistically significant association between the biofilm-forming category and MDR status. This pattern showed that the higher proportion of MDR isolates in the studied population was correlated with the stronger biofilm formation. Another possibility is that biofilm-associated growth mode provides structural and

physiological conditions for bacterial survival under the influence of antimicrobial agents. Antimicrobial distribution can be influenced by matrix components, and slow growing cells and tolerant subpopulations may survive treatment better than actively growing planktonic cells (Hall & Mah, 2017) .

Importantly, biofilm-associated tolerance should be differentiated from genetic antimicrobial resistance. Tolerance is the ability of bacteria to survive antimicrobial exposure without necessarily increasing the concentration required to inhibit growth (Aniz, and Jassim 2024). Resistance is the ability of bacteria to grow at antimicrobial concentrations that inhibit the susceptible organisms. These things can happen together but they are not the same. The higher MDR percentages observed in the different biofilm categories are thus indicative of a correlation between the two characteristics, rather than evidence that biofilm formation directly leads to resistance. Similarly, the current results do not confirm a role for icaA, fnbA or hla individually in the increased prevalence of MDR, as no gene-specific associations with MDR and biofilm intensity were found. In general, the results highlighted the significance of studying biofilm formation, antimicrobial susceptibility, and virulence-associated genetic determinants together in the characterization of clinical S. aureus isolates (Yousef and Jassim, 2026a). The high rate of biofilm-forming isolates, the prevalence of 48% of MDR and the presence of icaA, fnbA, hla and mecA genes provide additional information on the biological characteristics of the studied population. The major relationship found in the reported findings is the significant association between biofilm forming category and MDR. Further isolate-level analyzes would be required to determine whether individual genes are associated with biofilm intensity or MDR status, and whether the apparent relationship persists after adjustment for other characteristics (Yousef and Jassim, 2026b). Such analyses would strengthen the interpretation of the findings and help clarify the interactions between biofilm-associated processes, virulence determinants, and antimicrobial resistance in S. aureus.

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