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Antimicrobial Resistance, Biofilm Formation, and Associated Genetic Determinants in Clinical Staphylococcus aureus Isolates: An Integrated Phenotypic and Genotypic Analysis

Asmaa Samir Mohsin[1]*, Mays Jassim Abdalkareem[1], Maryam Jasim Hassan[1], Hiba Mohssin Ali[1]

[1]Department of Biology, College of Science, Mustansiriyah University, Baghdad, Iraq
*Corresponding author: Asmaa Samir Mohsin, Email: asmaa_s24@uomustansiriyah.edu.iq

ABSTRACT:

Background: Staphylococcus aureus remains a major clinical pathogen due to its ability to acquire antimicrobial resistance and develop biofilms that enhance survival during infection. The coexistence of resistance determinants and biofilm-associated mechanisms represents a significant challenge for effective treatment and infection control. Objectives: The objective of this research was to determine the patterns of antimicrobial resistance, multi-drug resistance (MDR), biofilm formation ability, and presence of resistance and biofilm-related genes in clinical isolates of S. aureus.

Methods: A total of 100 confirmed S. aureus isolates recovered from different clinical specimens were investigated. Antimicrobial susceptibility testing was performed against selected antibiotics, and multidrug resistance (MDR) classification followed the standardized category-based definition. Biofilm production was evaluated using the microtiter plate method. Polymerase chain reaction (PCR) targeting the nuc gene was included as a species-confirmatory molecular assay, while mecA, ermC, icaA, and icaD were detected to characterize resistance- and biofilm-associated determinants. Statistical analysis evaluated associations between phenotypic and genotypic characteristics.

Results: Wound specimens represented the most frequent source of isolates (25%), followed by urine (17%). Cefoxitin showed the highest resistance rate (47%), while linezolid showed the lowest resistance rate, with 98% of isolates remaining susceptible. Half of the isolates were classified as MDR. Biofilm production was detected in 89% of isolates, with moderate biofilm production being the predominant phenotype (48%). The most frequently detected gene was icaD (72%), followed by icaA (62%), mecA (48%), and ermC (34%). Associations were observed between mecA and cefoxitin resistance (OR = 103.20, 95% CI: 26.02–409.27) and between ermC and erythromycin resistance (OR = 15.56, 95% CI: 5.63–42.99), both with $p < 0.001$. The icaD gene was also significantly associated with biofilm formation (OR = 16.58, 95% CI: 3.30–83.27; $p < 0.001$).

Conclusion: Clinical S. aureus isolates demonstrated a high prevalence of biofilm formation and considerable antimicrobial resistance. The coexistence of resistance and persistence-associated determinants highlights the need for integrated phenotypic and molecular surveillance strategies.

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1. INTRODUCTION

Staphylococcus aureus is a major opportunistic bacterial pathogen associated with a broad spectrum of community- and healthcare-associated infections, including skin and soft-tissue infections, bloodstream infections, osteoarticular infections, pneumonia, and device-related infections. Its clinical importance is linked to the combination of virulence, immune evasion, ecological adaptability, and an exceptional capacity to acquire antimicrobial resistance, which enables persistence across both community and hospital settings (Tong et al., 2015; Lee et al., 2018).

The emergence and dissemination of methicillin-resistant *S. aureus* (MRSA) have complicated the treatment of staphylococcal infections because methicillin resistance compromises the activity of most β-lactam agents. The principal mechanism is acquisition of *mecA* within the staphylococcal cassette chromosome mec (SCCmec); *mecA* encodes the altered penicillin-binding protein 2a (PBP2a), which has reduced affinity for β-lactams and permits cell-wall synthesis to continue despite antibiotic exposure (Lee et al., 2018). Cefoxitin disk diffusion is widely used as a phenotypic surrogate for *mecA*-mediated resistance and has shown high diagnostic performance when compared with molecular detection of *mecA* by polymerase chain reaction (Broekema et al., 2009).

Formation of biofilm is another important survival strategy adopted by *S. aureus*. In a biofilm, the bacteria form an extracellular matrix that makes it difficult for antibiotics to gain access, allows for physiological diversity, and decreases the likelihood of elimination by the host immune system. The *icaADBC* operon facilitates the production of polysaccharide intercellular adhesion (PIA), also called poly-N-acetylglucosamine (PNAG). Here, *icaA* codes for an N-acetylglucosaminyltransferase, while *icaD* increases polysaccharide production efficiency. However, *S. aureus* can also produce a biofilm without the involvement of *ica* (Arciola *et al.*, 2015).

The association between biofilm formation and antibiotic resistance in *S. aureus* has been inconsistent in the clinical literature, with some studies indicating enhanced biofilm formation in antibiotic-resistant or MRSA strains while others showing weaker associations, suggesting that the phenotype is highly dependent on factors such as strain type, sample origin, culture conditions, presence of antibiotics, and biofilm assessment methods (Leshem *et al.*, 2022; Ali & Riaz, 2024). This is supported by current findings from Baghdad, where Mohsin *et al.* (2026) reported low levels of biofilm formation in a clinical *S. aureus* collection despite high levels of various biofilm-related genes and *mecA*.

Despite the considerable knowledge concerning both the methicillin resistance and the biofilm-forming ability of *S. aureus*, the exact nature of the relationships between the phenotypic resistance to antimicrobials, multidrug-resistance phenotype, ability to form biofilms and the presence of selected resistance- and biofilm-encoding genes in the same clinical isolate collection is not fully elucidated. It is especially important in the context of the Middle East countries since significant diversity has been described in the frequency of MRSA strains, the level of biofilm formation and the presence of *ica* genes (Hamad, 2023; Jomehzadeh & Seif Emrani, 2025; Mohsin *et al.,* 2026).

In order to fill the knowledge gap, the current study employed a combination of phenotypic and genotypic techniques for profiling 100 *S. aureus* strains by means of antimicrobial susceptibility testing, multidrug resistance (MDR) profiling, quantification of biofilm formation, and PCR identification of the presence of *nuc, mecA, ermC, icaA*, and *icaD* genes in the same set of samples. The study aimed to identify the correlation between resistance phenotypes and resistance genes, between *icaA* and *icaD* presence and biofilm production capacity, and genetic profiles associated with both resistance and biofilm formation.

2. MATERIALS AND METHODS

2.1 Study Design and Sample Collection

In this experiment, a total of 100 isolates of the bacterium *S. aureus* have been used. The isolates were obtained from wound, urine, pus, burn, blood, respiratory and catheter-associated specimens taken from patients at Medical City Hospital, Baghdad, Iraq from January to June 2026. All of the non-duplicate *S. aureus* isolates that were recovered during the specific period were used in the experiment in order to avoid over-representation. Only one isolate per patient has been considered for microbiological and statistical analysis. Samples have been processed following proper microbiological and biosafety procedures. The samples were transported and analyzed properly to maintain the survival and characteristics of bacteria.

2.2 Ethical Approval and Informed Consent

Ethics approval for this study protocol was sought and obtained from the Institutional Ethics Committee of Mustansiriyah University, Baghdad, Iraq. Informed consent was obtained in writing from all patients before inclusion of their clinical specimens and data into this study; in case of minors, consent was obtained from parents or authorized representatives. Participation in this study was on a voluntary basis, and the decision of not participating in this study would not impact their clinical management. To ensure privacy and confidentiality, all patient specimens and records were coded prior to analysis, and personally identifiable information was removed from the analytic data set and the manuscript.

2.3 Bacterial Identification

The clinical isolates were first identified as *S. aureus* through routine microbiological identification methods. At first, bacterial cultures were grown on standard culture media such as blood agar and mannitol salt agar, and they were characterized through colony characteristics. In addition, identification was done by routine biochemical tests such as Gram stain, catalase test, and coagulase test. The final phenotypic identification of *S. aureus* was carried out using the automated system of VITEK® 2 (France). For molecular identification, *S. aureus* was identified through PCR amplification of the *nuc* gene, which is a specific genetic marker for *S. aureus* identification (Brakstad *et al.*, 1992).

2.4 Antimicrobial Susceptibility Testing

Disk diffusion test for antimicrobial susceptibility was done on Mueller Hinton Agar (MHA) as per the guidelines mentioned in CLSI M02 and interpretation of results was done as per CLSI M100 guidelines (Clinical and Laboratory Standards Institute [CLSI], 2025, 35th ed.). Fresh cultures of bacteria were suspended in sterile saline solution and the density of suspension was adjusted to 0.5 McFarland standards (1.5 x 108 cfu/mL).The standardized suspension was inoculated uniformly onto MHA plates. The antimicrobial disks used were cefoxitin (FOX, 30 µg), ciprofloxacin (CIP, 5 µg), erythromycin (E, 15 µg), levofloxacin (LEV, 5 µg), tetracycline (TE, 30 µg), trimethoprim–sulfamethoxazole (SXT, 1.25/23.75 µg), clindamycin (DA, 2 µg), gentamicin (CN, 10 µg), and linezolid (LZD, 30 µg). The plates were incubated in aerobic condition at 35 ± 2°C for 16 to 18 hours. The quality control organism used was *Staphylococcus aureus* ATCC 25923. The diameter of inhibition zones was measured in mm using CLSI interpretation. MDR refers to non-susceptibility to one or more antimicrobial agents in three or more antimicrobial categories; this was done according to Magiorakos *et al.* (2012). Therefore, MDR was based on antimicrobial categories and not the total number of resistant agents.

2.5 Biofilm Formation Assay

Biofilm production was assessed using the crystal violet staining technique for microtiter plates, modified after Stepanović *et al.* (2000), which is a commonly used technique for quantification of attached staphylococcal biofilms. Overnight growth of bacteria was standardized to a turbidity level of 0.5 McFarland (1×10^8 CFU/mL), followed by dilution of the culture in 1:100 TSB containing 1% (w/v) glucose. An aliquot of 200 µL of the diluted bacterial suspension was inoculated in each well of sterile 96-well flat-bottom microtiter plates. Bacterial cultures were grown statically aerobically at 37°C for 24 h. This protocol was identical for all tested isolates.

After the incubation, the planktonic phase was aspirated and each well was washed three times with 200 µL of sterile PBS (pH 7.2). The biofilm that adhered to the surface was fixed by adding 200 µL of methanol for 15 minutes and air dried. The plate was stained using 200 µL of 0.1% (w/v) crystal violet solution for 15 minutes at room temperature. Excess stain was removed by washing each well three times with sterile distilled water and plates were allowed to air dry. Crystal violet stain was eluted with 200 µL of 33% (v/v) glacial acetic acid for 15 minutes, and the optical density (OD) of the samples was read at 570 nm using the microplate reader.

Negative control, which consisted of an uninoculated culture medium, was included in each test, while American Type Culture Collection (ATCC) 25923 strain of *S. aureus* was used as the quality control strain. Triplicate tests were performed for each isolate and the OD was calculated for analysis. The capacity of isolates to produce biofilm was determined based on the cut-off optical density (ODc), calculated by addition of three standard deviations to the average OD of the negative controls. According to the criteria by Stepanović *et al.* (2000, 2007), isolates were classified into four categories of biofilm producers: non-producers ($OD \leq ODc$), weak producers ($ODc < OD \leq 2 \times ODc$), moderate producers ($2 \times ODc < OD \leq 4 \times ODc$), and strong producers ($OD > 4 \times ODc$).

2.6 Molecular Confirmation and Detection of Resistance and Biofilm-Associated Genes

Genomic DNA was extracted from overnight cultures of confirmed *S.aureus* isolates using a commercial bacterial genomic DNA extraction kit (Promega, USA) according to the manufacturer's instructions. DNA concentration and purity were assessed spectrophotometrically, and DNA samples with an A260/A280 ratio of approximately 1.8–2.0 and a concentration of approximately 20–50 ng/µL were used for PCR amplification.

The extracted DNA was used as a template for amplification of the *nuc* gene for molecular confirmation of *S. aureus*, the resistance-associated genes *mecA* and *ermC*, and the biofilm-associated genes *icaA* and *icaD*. The *nuc* target was selected because of its high specificity for *S. aureus*, whereas *mecA* is the principal determinant of classical methicillin resistance, *ermC* encodes a methylase associated with macrolide-lincosamide-streptogramin B resistance, and *icaA* and *icaD* are involved in polysaccharide intercellular adhesin-dependent biofilm formation (Brakstad et al., 1992; Arciola et al., 2015; Lee et al., 2018).

PCR reactions were prepared in a final volume of 25 µL containing 12.5 µL of 2× PCR Master Mix (Promega, USA), 1 µL of each forward and reverse primer at a working concentration of 10 µM, corresponding to a final concentration of 0.4 µM for each primer, 2 µL of genomic DNA template, and nuclease-free water to complete the final reaction volume.

Primer sequences, target genes, melting temperature, and predicted size of amplicons for *nuc*, *mecA, ermC, icaA*, and *icaD* are provided in Table 1.

PCR amplification was done using a programmable thermal cycler with the initial denaturation step at 95°C for 5 min followed by 35 cycles consisting of denaturation at 95°C for 30 seconds, annealing at 55°C for 30 seconds for *nuc*, 55°C for *mecA*, 52°C for *ermC*, 55.5°C for *icaA*, and 55.5°C for *icaD*, and extension at 72°C for 45 seconds. A final extension at 72°C for 5 minutes was performed.

The PCR products were then separated using electrophoresis with 1.5% agarose gels dissolved in electrophoresis buffer and stained with a DNA safe fluorescent stain. DNA ladder 100 bp was used as the molecular marker. Electrophoresis was run at 100 volts for about 45 minutes, after which the amplified products were detected using ultraviolet illumination.

For each PCR run, the positive control included the DNA template of a previously identified *S. aureus* strain with the respective target gene and the negative control included nuclease-free water as the template rather than the DNA template. This was done to confirm amplification and avoid contamination in the PCR preparation.

Table 1. Primers used for molecular confirmation and detection of resistance- and biofilm-associated genes in *S. aureus*

Gene	Primer sequence (5'–3')	Amplicon size	Annealing temperature (°C)	Reference
mecA	**Forward:** AAA ATC GAT GGT AAA GGT TGG C **Reverse:** AGT TCT GCA GTA CCG GAT TTG C	533 bp	55	Louie *et al.* (2000)
ermC	**Forward:** GCT AAT ATT GTT TAA ATC GTC AAT TCC **Reverse:** GGA TCA GGA AAA GGA CAT TTT AC	572 bp	52	Lina *et al.* (1999)
icaA	**Forward:** TCT CTT GCA GGA GCA ATC AA **Reverse:** TCA GGC ACT AAC ATC CAG CA	188 bp	55.5	Arciola *et al.* (2001)
icaD	**Forward:** ATG GTC AAG CCC AGA CAG AG	198 bp	55.5	Arciola *et al.* (2001)

	Reverse: CGT GTT TTC AAC ATT TAA TGC AA			
nuc	**Forward:** GCG ATT GAT GGT GAT ACG GTT **Reverse:** AGC CAA GCC TTG ACG AAC TAA AGC	279 bp	55	Brakstad *et al.* (1992)

2.7 Statistical Analysis

Data analysis was performed using IBM SPSS Statistics software (version 26). Categorical variables were presented as frequencies and percentages. Associations between antimicrobial resistance patterns, biofilm production categories, and gene distribution were assessed using the Chi-square test or Fisher's exact test when appropriate. The strength of associations between gene presence and phenotypic characteristics was evaluated using odds ratios (ORs) with 95% confidence intervals (CIs) where estimable. Fisher's exact test was used when expected counts were small or a zero cell was present. A p-value < 0.05 was considered statistically significant.

RESULTS

3.1 Distribution of *S. aureus* isolates according to clinical sources

A total of 100 confirmed *S. aureus* isolates obtained from different clinical specimens were included in this study. The distribution of isolates varied according to the source of clinical samples. Wound specimens represented the most frequent source, accounting for 25% (25/100) of all isolates, followed by urine samples with 17% (17/100). Pus and burn specimens each contributed 15% (15/100) of the isolates, whereas blood samples represented 12% (12/100). Respiratory and catheter-associated samples showed lower frequencies, accounting for 9% and 7%, respectively (Table 2).

Table 2. Distribution of *S. aureus* isolates according to clinical sources (n = 100)

Clinical source	No. of isolates	Percentage (%)
Wound	25	25.0
Urine	17	17.0
Pus	15	15.0
Burn	15	15.0
Blood	12	12.0
Respiratory samples	9	9.0
Catheter	7	7.0
Total	**100**	**100**

3.2 Antimicrobial resistance profile of *S. aureus* isolates

The antimicrobial susceptibility profile of the 100 *S. aureus* isolates demonstrated variable resistance patterns against the tested antimicrobial agents (Table 3). The highest resistance rate was observed against cefoxitin (FOX), with 47 isolates (47.0%) classified as resistant, indicating a high prevalence of methicillin-resistant phenotypes among the studied isolates. Resistance to fluoroquinolones was also considerable, with ciprofloxacin and levofloxacin resistance rates of 36.0% and 33.0%, respectively.

Resistance to erythromycin and tetracycline was detected in 35.0% and 31.0% of isolates, respectively. Lower resistance rates were observed for trimethoprim-sulfamethoxazole (24.0%), clindamycin (23.0%), and gentamicin (22.0%). Linezolid exhibited the highest activity among the tested antibiotics, with only two isolates (2.0%) showing resistance.

Table 3. Antimicrobial resistance profile of *S. aureus* isolates (n = 100)

Antibiotic	**Resistant n (%)**	Susceptible n (%)
Cefoxitin (FOX)	47 (47.0)	53 (53.0)
Ciprofloxacin (CIP)	36 (36.0)	64 (64.0)
Erythromycin (E)	35 (35.0)	65 (65.0)
Levofloxacin (LEV)	33 (33.0)	67 (67.0)
Tetracycline (TE)	31 (31.0)	69 (69.0)
Trimethoprim-sulfamethoxazole (SXT)	24 (24.0)	76 (76.0)
Clindamycin (DA)	23 (23.0)	77 (77.0)
Gentamicin (CN)	22 (22.0)	78 (78.0)
Linezolid (LZD)	2 (2.0)	98 (98.0)

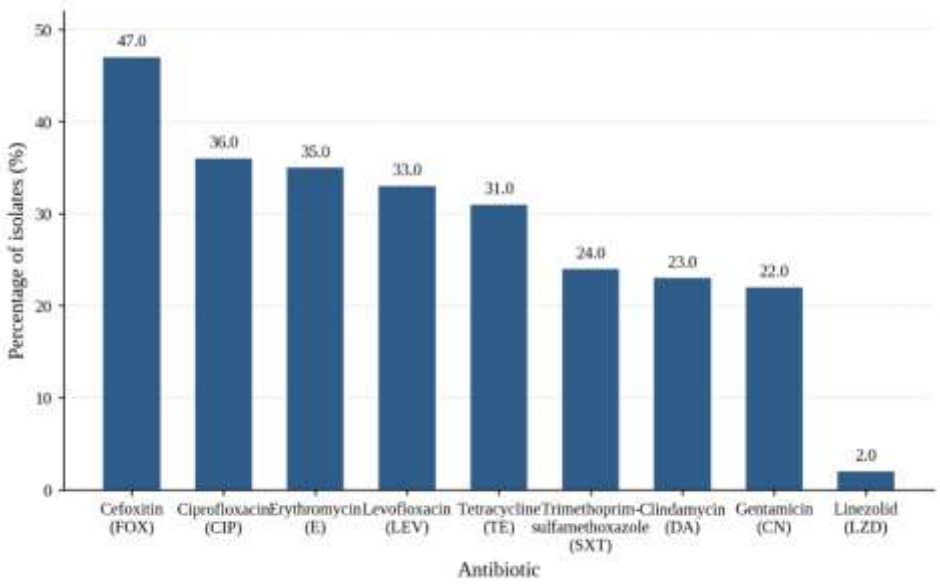

Figure 1. Distribution of antimicrobial resistance rates among clinical *S. aureus* isolates

3.3 Multidrug resistance pattern among *S. aureus* isolates

Among the 100 *S. aureus* isolates, 50 isolates (50.0%) were classified as multidrug-resistant (MDR) according to acquired non-susceptibility to at least one agent in three or more antimicrobial categories, while the remaining 50 isolates (50.0%) were categorized as non-MDR (Table 4).

Table 4. Multidrug resistance classification among *S. aureus* isolates

Category	**No. of isolates**	**Percentage (%)**
MDR	50	50.0
Non-MDR	50	50.0
Total	100	100

3.4 Phenotypic biofilm formation among *S. aureus* isolates

The capacity of the 100 S. aureus isolates to form a biofilm was assessed by the microtiter plate method. A majority of the isolates produced biofilm, of which 89 isolates (89.0%) were found to be biofilm producers. The category of moderate biofilm formation prevailed in 48 isolates (48.0%) followed by weak biofilm formation in 31 isolates (31.0%). Strong biofilm formation was found in 10 isolates (10.0%) while 11 isolates (11.0%) were found to be non-biofilm producers (Table5).

Table 5. Biofilm formation ability among *S. aureus* isolates (n = 100)

Biofilm category	No. of isolates	Percentage (%)
Strong producer	10	10.0
Moderate producer	48	48.0
Weak producer	31	31.0
Non-producer	11	11.0
Total producers	**89**	**89.0**

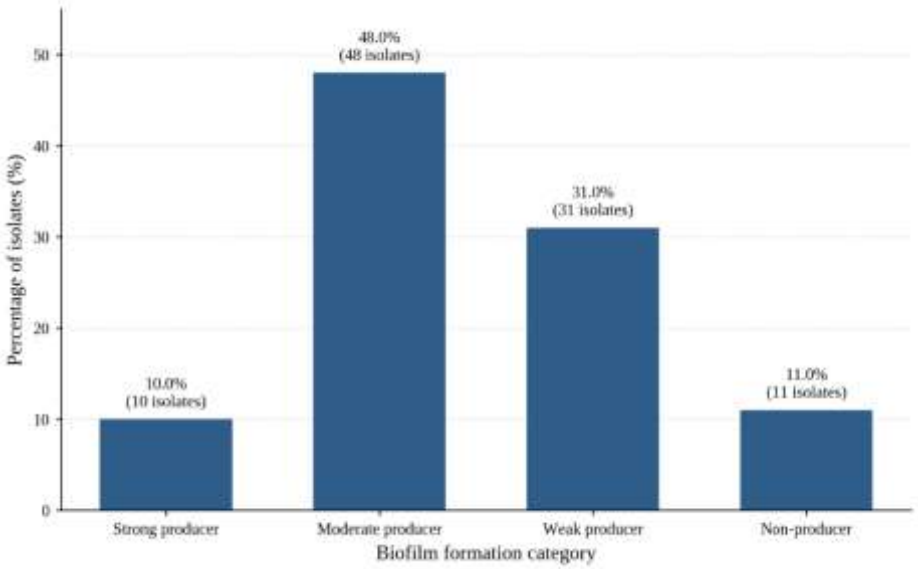

Figure 2. Distribution of biofilm production categories among *S. aureus* isolates

3.5 Association between resistance characteristics and biofilm formation

The relationship between MDR status and biofilm production ability was evaluated among *S. aureus* isolates. MDR isolates showed a higher proportion of moderate biofilm production compared with non-MDR isolates; however, the overall distribution of biofilm categories did not show a statistically significant association with MDR status (p = 0.168) (Table 6).

Table 6. Association between MDR status and biofilm formation among *S. aureus* isolates

MDR status	Strong biofilm n (%)	Moderate biofilm n (%)	Weak biofilm n (%)	Non-producer n (%)	Total
MDR	2 (4.0)	28 (56.0)	15 (30.0)	5 (10.0)	50
Non-MDR	8 (16.0)	20 (40.0)	16 (32.0)	6 (12.0)	50
Total	**10**	**48**	**31**	**11**	**100**

Chi-square test: p = 0.168

3.6 Molecular confirmation of *S. aureus* isolates

Molecular amplification of the *nuc* gene confirmed the identity of the investigated isolates as *S. aureus*. The expected 279-bp amplicon was detected in all 100 isolates (100%).

3.7 Detection of antimicrobial resistance and biofilm-associated genes

The distribution of antimicrobial resistance-associated and biofilm-associated genes among the 100 *S. aureus* isolates was investigated by PCR analysis. The biofilm-associated gene ***icaD*** showed the highest prevalence, being detected in 72 isolates (72.0%), followed by ***icaA*** in 62 isolates (62.0%). The methicillin resistance-associated gene ***mecA*** was detected in 48 isolates (48.0%), whereas the macrolide resistance gene ***ermC*** was identified in 34 isolates (34.0%) (Table 7).

Table 7. Distribution of resistance and biofilm-associated genes among *S. aureus* isolates (n = 100)

Gene	Positive isolates n (%)	Negative isolates n (%)
icaD	72 (72.0)	28 (28.0)
icaA	62 (62.0)	38 (38.0)
mecA	48 (48.0)	52 (52.0)
ermC	34 (34.0)	66 (66.0)

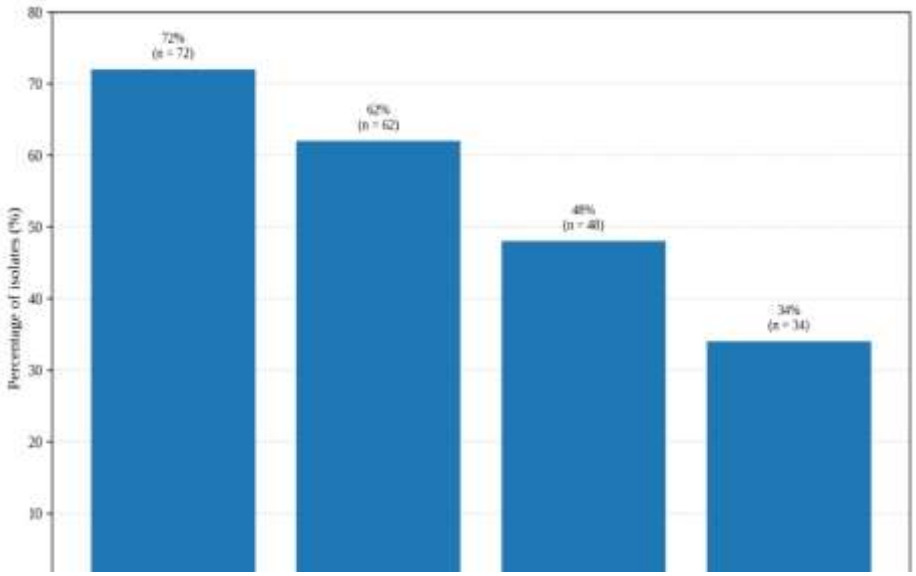

Figure 3. Frequency of antimicrobial resistance-associated (*mecA*, *ermC*) and biofilm-associated (*icaA*, *icaD*) genes among clinical *S. aureus* isolates (n = 100).

3.8 Association between genotypic and phenotypic antimicrobial resistance

3.8.1 Association between *mecA* gene detection and cefoxitin resistance

The association between the presence of the *mecA* gene and cefoxitin resistance phenotype was evaluated among the studied *S. aureus* isolates. A strong association was observed between *mecA* detection and cefoxitin resistance: 43 of 48 *mecA*-positive isolates (89.6%) were cefoxitin resistant, whereas only 4 of 52 *mecA*-negative isolates (7.7%) were resistant. The association was statistically significant ($p < 0.001$) (Table 8).

Table 8. Association between *mecA* gene detection and cefoxitin resistance among *S. aureus* isolates

***mecA* status**	**FOX resistant n (%)**	FOX susceptible n (%)	**Total**
Positive	43 (89.6)	5 (10.4)	48
Negative	4 (7.7)	48 (92.3)	52
Total	**47**	**53**	**100**

Statistical analysis: OR = 103.20, 95% CI = 26.02-409.27; Fisher's exact $p < 0.001$.

3.8.2 Association between *ermC* gene detection and erythromycin resistance

The relationship between the presence of the *ermC* gene and erythromycin resistance was investigated among the studied *S. aureus* isolates. Among the 34 isolates carrying the *ermC* gene, 25 isolates (73.5%) exhibited erythromycin resistance, whereas only 10 of the 66 *ermC*-negative isolates (15.2%) were resistant to erythromycin. A statistically significant association was observed between *ermC* detection and erythromycin resistance ($p < 0.001$), indicating that *ermC* carriage was strongly associated with the macrolide resistance phenotype (Table 9).

Table 9. Association between *ermC* gene detection and erythromycin resistance among *S. aureus* isolates

***ermC* status**	**Erythromycin resistant n (%)**	Erythromycin susceptible n (%)	**Total**
Positive	25 (73.5)	9 (26.5)	34
Negative	10 (15.2)	56 (84.8)	66
Total	**35**	**65**	**100**

Statistical analysis: OR = 15.56, 95% CI = 5.63-42.99; Fisher's exact $p < 0.001$.

3.9 Association between biofilm-associated genes and biofilm phenotype

The association between biofilm-associated genes (*icaA* and *icaD*) and phenotypic biofilm production was evaluated among *S. aureus* isolates. The presence of both genes was significantly associated with biofilm-forming ability.

All *icaA*-positive isolates (62/62, 100%) were classified as biofilm producers, whereas 27 of the *icaA*-negative isolates (71.1%) showed biofilm production. Similarly, most *icaD*-positive isolates (70/72, 97.2%) exhibited biofilm production compared with 19 of the *icaD*-negative isolates (67.9%). Both associations were statistically significant (Fisher's exact $p < 0.001$). For *icaD*, the odds ratio was 16.58 (95% CI = 3.30-83.27). For *icaA*, the crude odds ratio was not finite because no *icaA*-positive isolate was a non-producer; therefore, the exact test result is reported rather than an unstable crude OR (Table 10).

Table 10. Association between biofilm-associated genes and biofilm production phenotype among *S. aureus* isolates

Gene status	**Biofilm producer n (%)**	**Non-producer n (%)**	**p-value**
icaA positive	62 (100.0)	0 (0.0)	<0.001
icaA negative	27 (71.1)	11 (28.9)	
icaD positive	70 (97.2)	2 (2.8)	<0.001
icaD negative	19 (67.9)	9 (32.1)	

3.10 Integrated resistance- and biofilm-associated genetic profiles among *S. aureus* isolates

The distribution of combined resistance and biofilm-associated genetic profiles among the 100 *S. aureus* isolates is presented in Table 11. The most common profile was *icaA* + *icaD*, detected in 18 isolates (18.0%), followed by *mecA* + *icaA* + *icaD* (16.0%) and *icaA* + *icaD* + *ermC* (14.0%). The complete profile (*mecA* + *icaA* + *icaD* + *ermC*) was detected in 7 isolates (7.0%). Single-gene profiles and isolates lacking the investigated genes were also identified. The proportion of MDR isolates and biofilm production varied among different genetic profiles.

Table 11. Distribution of combined resistance- and biofilm-associated genetic profiles and corresponding phenotypic characteristics of *S. aureus* isolates

Genetic profile	No. (%)	MDR isolates, n (%)	Biofilm producers, n (%)	Strong/Moderate biofilm, n (%)
mecA + *icaA* + *icaD* + *ermC*	7 (7.0)	7 (100)	7 (100)	6 (85.7)
mecA + *icaA* + *icaD*	16 (16.0)	10 (62.5)	16 (100)	14 (87.5)
icaA + *icaD* + *ermC*	14 (14.0)	6 (42.9)	14 (100)	10 (71.4)
mecA + *icaD*	8 (8.0)	4 (50.0)	8 (100)	3 (37.5)
icaA + *icaD*	18 (18.0)	2 (11.1)	18 (100)	17 (94.4)
mecA + *icaA*	4 (4.0)	3 (75.0)	4 (100)	3 (75.0)
mecA + *ermC*	5 (5.0)	4 (80.0)	5 (100)	1 (20.0)
mecA + *icaD* + *ermC*	1 (1.0)	1 (100)	1 (100)	1 (100)
mecA only	7 (7.0)	6 (85.7)	5 (71.4)	1 (14.3)
icaD + *ermC*	4 (4.0)	2 (50.0)	3 (75.0)	1 (25.0)
icaD only	4 (4.0)	1 (25.0)	3 (75.0)	1 (25.0)
ermC only	2 (2.0)	2 (100)	1 (50.0)	0 (0)
icaA only	2 (2.0)	1 (50.0)	2 (100)	0 (0)
icaA + *ermC*	1 (1.0)	0 (0)	1 (100)	0 (0)
No detected genes	7 (7.0)	1 (14.3)	1 (14.3)	0 (0)
Total	**100 (100)**	**50 (50.0)**	**89 (89.0)**	**58 (58.0)**

3.11 Hierarchical clustering of antimicrobial resistance and genetic profiles among *S. aureus* isolates

Hierarchical clustering of the combined phenotypic and genotypic binary matrix revealed marked heterogeneity among the 100 *S. aureus* isolates (Figure 4). Rather than forming a single uniform pattern, the isolates were partitioned into multiple branches characterized by different combinations of antimicrobial resistance and gene carriage. The *nuc* gene was present in all isolates, whereas the biofilm-associated genes *icaD* and *icaA* occurred in 72% and 62%, respectively; 55 isolates carried both *icaA* and *icaD*. This recurrent co-carriage produced similar gene-presence patterns in a substantial subset of isolates, while other isolates showed partial or absent ica profiles.

Clustering also reflected the correspondence between selected resistance genes and their phenotypic counterparts. *mecA* was detected in 48 isolates, cefoxitin resistance in 47, and 43 isolates were positive for both; overall *mecA*–FOX concordance was 91%. The distribution of *ermC* similarly paralleled erythromycin resistance at the population level (34% vs. 35%), although discordant isolates remained evident. Resistance to the other antimicrobial agents was dispersed across several isolate clusters rather than concentrated in a single branch, consistent with the heterogeneous resistance pattern reported in Table 3. Linezolid resistance remained uncommon and was confined to two isolates. Figure 4 therefore provides an isolate-level overview of co-occurring resistance phenotypes and genetic determinants, while the statistical associations between specific genotype–phenotype pairs are reported separately in Tables 8–10.

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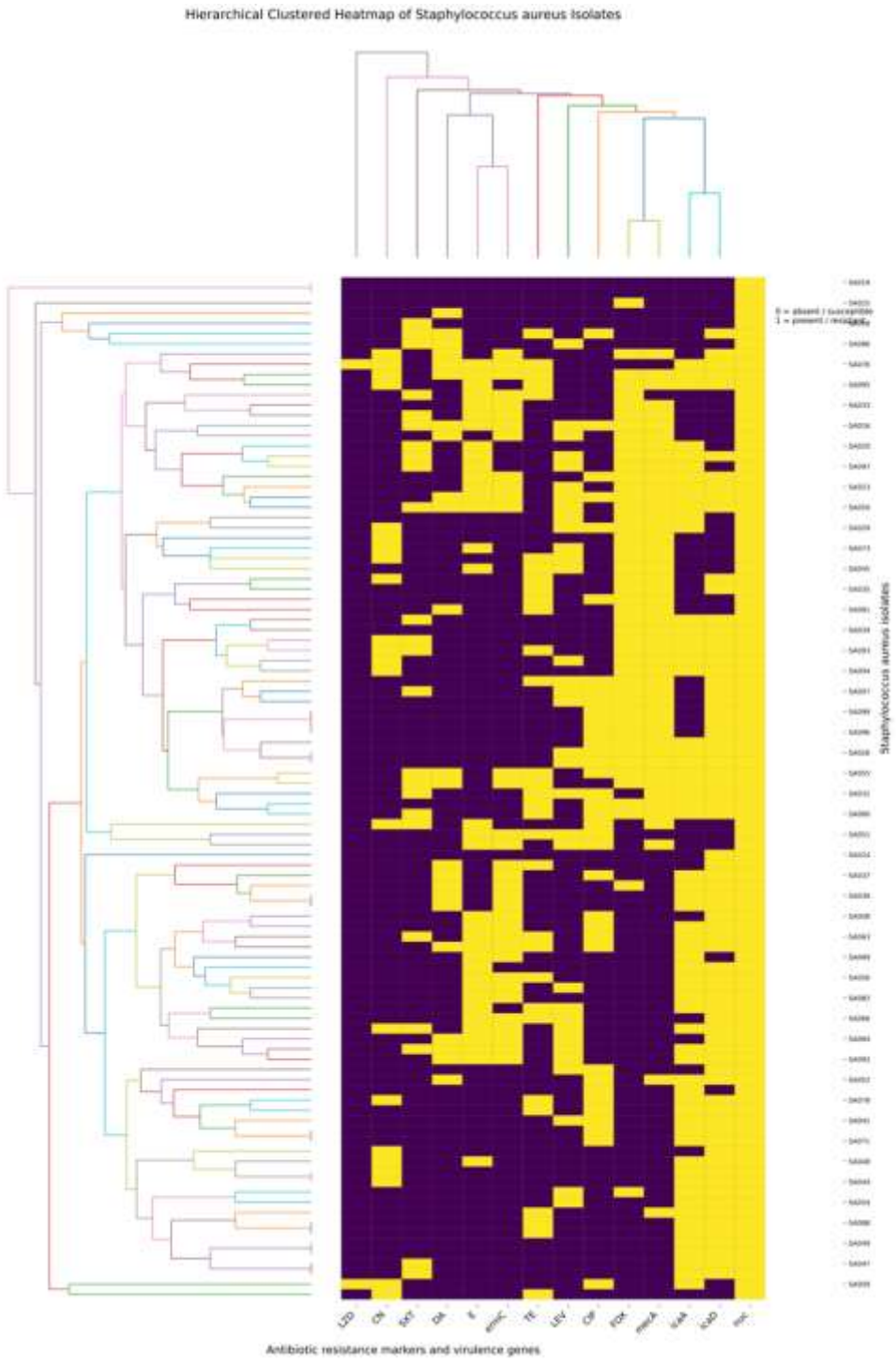

Figure 4. Hierarchical clustered heatmap of antimicrobial resistance phenotypes and selected gene profiles among 100 *S. aureus* isolates. Yellow indicates resistance/gene presence (1) and purple indicates susceptibility/gene absence (0). Rows represent isolates; columns represent clustered resistance phenotypes and gene markers (*ermC*, *mecA*, *icaA*, *icaD*, and *nuc*). Jaccard distance and average linkage were used.

DISCUSSION

The present study integrated antimicrobial susceptibility testing, standardized multidrug-resistance (MDR) classification, quantitative biofilm assessment, and PCR detection of selected resistance- and biofilm-associated genes in 100 clinical *S. aureus*

isolates. Assessing these phenotypic and genotypic features within the same isolate collection allowed the relationships among antimicrobial resistance, biofilm formation, and gene carriage to be examined directly. This is particularly relevant because antimicrobial resistance and biofilm-mediated persistence can coexist, yet they are controlled by partly independent genetic and regulatory mechanisms.

Samples of wounds were the most common source of isolation of *S. aureus* (25%), followed by urine samples (17%), pus and burn samples (15% each), and blood (12%). This is scientifically explainable due to the fact that disruption of the epithelial barrier provides the environment for the adherence and colonization of *S. aureus*, and surface adhesins help adhere to the host's extracellular matrix. Additionally, biofilm formation may enhance adherence and colonization (Tong *et al.,* 2015). However, considering the fact that the study was carried out in one medical center using samples collected within one particular timeframe, the specimen prevalence should be considered specific to the current isolate population.

The highest level of resistance was recorded with cefoxitin (47%), ciprofloxacin (36%), erythromycin (35%), levofloxacin (33%), and tetracycline (31%). On the other hand, linezolid had the lowest resistance rate, since 98% isolates were susceptible to it. The resistance rate with cefoxitin is close to that of MRSA (48.3%) in the case of clinical isolates of S. aureus from Iran, which is reported by Jomehzadeh and Seif Emrani (2025), although a direct comparison is impossible due to differences in sample composition and study design. Low resistance rates of linezolid show its in vitro susceptibility to the isolates in question but does not imply any treatment effectiveness of the antibiotic. Overall, the diversity of resistance rates in different antimicrobial classes indicates that local susceptibility data must be used to choose empiric antibiotics.

The presence of the *mecA* gene was found in 48% of isolates, and it significantly correlated with resistance to cefoxitin. Resistance to cefoxitin was observed in 89.6% of *mecA*-positive isolates compared to 7.7% of *mecA*-negative isolates, producing an OR of 103.20 (95% CI = 26.02-409.27; $p < 0.001$). This high genotype-phenotype correlation makes biological sense because of the known role of the *mecA* gene in producing a penicillin-binding protein (PBP2a), which exhibits low binding activity toward beta-lactam drugs (Lee *et al.*, 2018). It also agrees with previous literature that supports the use of cefoxitin disk diffusion as a phenotype predictor of *mecA*-induced resistance to methicillin (Broekema *et al.*, 2009). Nevertheless, the very large 95% CI suggests some ambiguity in the true effect size despite the significant relationship due to the sample size and the distribution of discordant isolates. The small number of genotype-phenotype differences should therefore be carefully considered since heterogenous expression, borderline phenotypes, or resistance mechanisms other than those targeted by the PCR could lead to discrepancies.

Fifty percent (50%) of isolates were MDR by the standardized definition. Such an MDR percentage is clinically significant in terms of non-susceptibility to many antimicrobial classes which can limit the treatment options available to clinicians and make the use of one class of antibiotics likely to result in the survival of resistant subpopulations. It should be borne in mind, however, that the MDR prevalence rate is very context-dependent and depends on the usage of antibiotics, patient population characteristics, hospital environment, type of sample, presence of circulating clones, and antimicrobial classes tested. Studies of *S. aureus* strains in Iraq have found considerable resistance to antibiotics, but the rates of antimicrobial resistance differ depending on the criteria used to select isolates and methodology of the study (Hamad, 2023; Mohsin *et al.,* 2026). For this reason, the current proportion of MDR strains is best assessed against the background of the category-based definition of Magiorakos *et al.* (2012).

Biofilm production was found in 89% of the isolates, and most had moderate biofilm production (48%), followed by weak (31%) and strong (10%). Such a high rate of biofilm production is much greater than 32% phenotypic biofilm production frequency mentioned by Jomehzadeh and Seif Emrani (2025) and 20% in a more recent study done on 40 clinical isolates of *S. aureus* in Baghdad (Mohsin *et al.*, 2026). On the other hand, in the study done on *S. aureus* isolates in Erbil, Hamad (2023) found that biofilm production was seen in all the *S. aureus* isolates, and there were variations in biofilm intensity depending on whether the isolates were MRSA or MSSA isolates. These results, therefore, imply that biofilm frequency is highly affected by isolates composition, clinical source, sampling time, culture conditions, technique of assay, and cutoff point of optical density, among others.

Although MDR isolates showed a greater proportion of moderate biofilm production, the overall association between MDR status and biofilm category was not statistically significant ($p = 0.168$). This finding indicates that antimicrobial resistance and biofilm formation should not be treated as interchangeable phenotypes. Previous *S. aureus* studies have likewise reported variable

relationships among methicillin resistance, antimicrobial-resistance patterns, ica gene carriage, and quantitative biofilm production (Hamad, 2023; Jomehzadeh & Seif Emrani, 2025; Mohsin et al., 2026). Mechanistically, the absence of a statistically significant association is plausible because resistance can arise through target modification, drug inactivation, altered permeability, or efflux, whereas biofilm formation depends on adhesion, matrix production, regulatory networks, and physiological adaptation. These processes may coexist under antimicrobial selection without being obligatorily linked. The present result therefore supports a context-dependent relationship between MDR and biofilm formation, while the sample size should also be considered when interpreting the absence of statistical significance.

The biofilm-associated genes were detected more frequently than the selected resistance genes: *icaD* was present in 72% of isolates and *icaA* in 62%, compared with 48% for *mecA* and 34% for *ermC*. The observed *icaA* and *icaD* frequencies fall within the broad range reported in regional *S. aureus* studies. Jomehzadeh and Seif Emrani (2025) reported high carriage of adhesion-associated genes in clinical isolates, while Hamad (2023) detected *icaA* and *icaD* in 84% and 82% of isolates, respectively. Differences among studies are expected because carriage of the ica locus varies with clonal background and isolate source, and the presence of a gene does not establish its transcriptional activity or the amount of biofilm biomass produced.

Both *icaA* and *icaD* were significantly associated with the biofilm phenotype (Fisher's exact $p < 0.001$). All *icaA*-positive isolates were biofilm producers, while 97.2% of *icaD*-positive isolates produced biofilm. The association for *icaD* was substantial (OR = 16.58, 95% CI = 3.30-83.27), although the broad confidence interval again indicates limited precision in the estimated effect size. A finite crude OR for *icaA* could not be calculated because no *icaA*-positive isolates occurred in the non-producer category. Biologically, these results are consistent with the role of the *icaADBC* locus in polysaccharide intercellular adhesin/poly-N-acetylglucosamine-dependent biofilm accumulation (Arciola et al., 2015). Importantly, biofilm production was also observed among isolates lacking one or both tested ica targets. Thus, ica carriage is informative but not sufficient as a stand-alone predictor of biofilm phenotype, because protein-mediated adhesion, extracellular DNA, autolytic processes, and additional regulatory pathways can support ica-independent biofilm development.

The *ermC* gene was detected in 34% of isolates and was strongly associated with erythromycin resistance. Resistance was observed in 73.5% of *ermC*-positive isolates compared with 15.2% of *ermC*-negative isolates (OR = 15.56, 95% CI = 5.63-42.99; $p < 0.001$). This association is consistent with the established role of *ermC*-mediated ribosomal methylation in reducing macrolide binding. Nevertheless, erythromycin resistance observed in those strains which lack the *ermC* gene indicates that this gene is not responsible for the whole phenotype. There could be other methylase genes, efflux pumps, mutations in the target sites, or unknown resistance genes that could affect the residual resistance.

Further analysis of combined profiles revealed that the resistance and biofilm-related genes occurred together in isolates. The predominant profile was *icaA* + *icaD* (18%), followed by *mecA* + *icaA* + *icaD* (16%) and *icaA* + *icaD* + *ermC* (14%). The significance of the co-occurrence of these genes lies in the fact that resistance genes may contribute to bacterial survival in the presence of antimicrobials, while biofilm formation may facilitate persistence due to limited penetration of antimicrobials, heterogeneity in metabolism, and protection from host factors. However, such co-occurrences cannot be considered as an indication of any kind of interactions between the identified genes.

Regarding the isolate level, the hierarchical heatmap is an extension of the pairwise association analyses in that there were several combinations of resistance/biofilm determinants that frequently occur rather than just one single common pattern. The close proximity of *mecA* and cefoxitin resistance corresponds to the strong association shown in Table 8, and the known role of *mecA*-encoded PBP2a in methicillin resistance (Lee *et al.*, 2018; Broekema *et al.*, 2009). The co-presence of *icaA* and *icaD* in 55 isolates suggests frequent possession of *ica*-dependent biofilm pathway components, although gene possession does not mean transcriptional activity and biofilm formation (Arciola *et al.,* 2015). It is important to note that the dendrogram can be used only as a similarity map in exploratory analysis, since the proximity of variables/isolates only means a common pattern of binary associations, but not causality. Since clonal typing was not done, isolates in the same clusters may have similar phenotypic patterns either due to common ancestry, independent possession of determinants, or convergent evolution.

In summary, the results indicate the frequent occurrence of both antibiotic resistance and biofilm characteristics in clinical strains of *S. aureus* while proving that antibiotic resistance or biofilm phenotype cannot be reliably predicted by any of the tested markers. The principal advantage of this study was the simultaneous phenotypic and genotypic examination within the same strain series, allowing for direct determination of the degree of agreement and disagreement between measured phenotypes and

specific genotypes. The results should, nevertheless, be taken into account in terms of the single center study design, small sample size, restricted panel of resistance genes, lack of clonal typing and gene expression analysis. In future multicenter studies, wider panels of resistance and biofilm genes, transcriptional profiling, and clonal typing should be done in order to establish whether the observed gene profiles were related to local clonal spread or independent acquisition of resistance determinants, or to different regulation of gene expression. Evaluation of *icaA, icaD, mecA*, and *ermC* expression under relevant environmental and antibiotic exposure could additionally help differentiate the mere carriage from functional involvement in a particular phenotype.

CONCLUSION

Antimicrobial resistance and biofilm formation were found to be prevalent traits among clinical *S. aureus* isolates. Of the 100 clinical *S. aureus* isolates used for this study, 47% exhibited cefoxitin resistance, 50% of which were MDR strains while 89% were biofilm producers. The presence of *mecA, ermC*, *icaA*, and *icaD* genes and their associations with resistance and biofilm formation underscore their roles in bacterial survival. This study provides a complete description of the resistance and biofilm-forming characteristics of the clinical isolates studied, supporting further surveillance of antimicrobial resistance.

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