



Characterization of Clonal Groups of Antibiotic-Resistant Biofilm-Forming *Staphylococcus aureus* Strains Isolated from Patients with Urinary Tract Infections in Isfahan, Iran

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Abstract

Background: Over the past decades, the role of biofilm-forming *Staphylococcus aureus* strains in urinary tract infections (UTIs) has garnered significant attention.

Objectives: This study aimed to determine the epidemiological characteristics and diversity of *S. aureus* strains isolated from patients with UTIs in Isfahan, Iran, in 2017, with regard to their antimicrobial resistance, biofilm formation, and phylogenetic profiles. Additionally, the study investigated potential relationships among these factors statistically to develop efficient control and treatment approaches.

Methods: All patients with symptomatic UTIs who had positive urine cultures for *S. aureus* during the study period at the laboratory of a referral hospital in Isfahan were included. All isolates were identified using specific primers for the *nucA* gene. Their biofilm formation capacity was evaluated using a combination of the microtiter plate and Congo-red agar methods. Antibiotic susceptibility testing was performed using the disk diffusion method. The presence of genes involved in biofilm formation and resistance to ceftazidime, aminoglycosides, and fluoroquinolones was detected using polymerase chain reaction (PCR). *Staphylococcal cassette chromosome mec* (SCC*mec*) typing, *agr* typing, and phage typing (PhP) were employed to investigate the diversity of collected strains.

Results: Results showed that 19%, 57%, and 24% of confirmed *S. aureus* strains were strong, intermediate, and non-biofilm formers, respectively. The highest rate of resistance was against nalidixic acid (77%), followed by streptomycin (73%). The *icaD* and *icaA* genes had the highest frequency among biofilm-producing strains. *gyrA* (44%) and *griA* (35%) were the most frequent genes among fluoroquinolone-resistant strains, while *aph(3')-IIIa* was the most prevalent aminoglycoside-modifying enzyme gene. The majority of bacterial strains harbored SCC*mec* type III and *agr* type I. PhP typing of strains revealed the presence of 8 common types (CTs) and 14 single types (STs), with CT2 being the dominant type.

Conclusions: The present investigation revealed various biofilm production capacities, antimicrobial resistance profiles, and clonal lineages in *S. aureus* isolated from patients with UTIs. These findings provide further insights into the epidemiology and pathogenicity of *S. aureus* strains in Iran, thereby improving the quality of surveillance and therapeutic protocols.

Keywords: Biofilms, Drug Resistance, Urinary Tract Infections, Fluoroquinolones, Aminoglycosides, Bacterial Typing

1. Background

Urinary tract infections (UTIs) are the second most common infections in humans after the common cold (1, 2). Urinary tract infections are a serious public health concern and can lead to severe, life-threatening conditions, primarily due to their recurring nature (3).

Recurrent UTIs are directly related to biofilms formed within the urinary tract or on indwelling devices (4). *Staphylococcus aureus* is considered the second most prevalent etiologic agent in UTIs, causing persistent biofilm-related infections (2). In this bacterium, the main component of the biofilm matrix is polysaccharide intercellular adhesin (PIA), encoded by

the intercellular adhesion operon (5). In addition to the *ica* operon, proteins belonging to the microbial surface components recognizing adhesive matrix molecules (MSCRAMMs), such as fibronectin-binding proteins A and B (*fnbA* and *fnbB*) and clumping factors A and B (*clfA* and *clfB*), mediate interactions between the bacterial cells and host extracellular matrix proteins. Additionally, the accessory gene regulator (*agr*) locus in *S. aureus* encodes the quorum sensing system, which plays a crucial role in regulating biofilm development (6, 7). *Staphylococcus aureus* isolates can be classified into four major *agr* groups (types I to IV) based on the sequences of *agrC* [encoding the receptor of the auto-inducing peptide (AIP)] and *agrD* (encoding a cyclic AIP) genes. Previous studies have emphasized that different *agr* types have varying effects on disease severity due to the relationship between the *agr* group and the genetic background of each strain (8).

Biofilm formation also facilitates the exchange and dissemination of genes involved in antibiotic resistance, resulting in limited treatment options, especially in infections caused by methicillin-resistant *S. aureus* strains (MRSA) (9, 10). Methicillin resistance is due to the presence of *mecA* and *mecC* genes, which are part of a large and mobile genetic element known as staphylococcal cassette chromosome *mec* (SCC*mec*). To date, 13 SCC*mec* types (according to the combination of *ccr* gene complexes and *mec* gene complexes) have been identified among MRSA strains (11). While vancomycin remains the primary treatment for MRSA infections, its high cost, intravenous administration, and several side effects limit its routine use. Although quinolone antibiotics have been proposed as an oral alternative to parenteral vancomycin therapy, the widespread use of these drugs in recent years has led to the emergence of fluoroquinolone-resistant *S. aureus*, especially among MRSA strains (9).

Aminoglycosides, one of the important drugs that enhance the activity of anti-staphylococcal antibiotics, are closely associated with methicillin resistance in *S. aureus* (10, 12). Therefore, understanding the frequency of genes involved in antibiotic resistance and analyzing epidemic *S. aureus* clones is essential to developing effective approaches for controlling the spread, improving antimicrobial therapies, and decreasing treatment-related costs (13). Among various phenotyping and genotyping methods, the phene plate (PhP) system and SCC*mec* typing are simple, fast, and

inexpensive methods that provide beneficial information for epidemiologic studies of bacteria (14).

2. Objectives

This study aimed to determine the prevalence of clonal groups of *S. aureus* strains isolated from patients with UTIs in Isfahan, Iran. Additionally, we evaluated the possible genetic relationships between *agr* types, biofilm formation, and resistance to aminoglycosides and quinolones.

3. Methods

3.1. Sample Collection and Characterization of *Staphylococcus aureus* Isolates

In this study, a convenience sampling method was used for the collection of bacterial isolates. The sample size was calculated using the following formula based on the prevalence of biofilm formation among *S. aureus* strains (95.45%) reported in previous studies (15):

$$n = \frac{z^2(p \times q)}{e^2}$$

Where n = required sample size, p = prevalence of biofilm formation (95.45%), q = 1 - p, e = margin of error (5%), z = 2.58 at 99% CI.

A total of 119 suspected *S. aureus* isolates were obtained from patients with symptomatic UTIs at the laboratory of a referral hospital in Isfahan, Iran, in 2017. All patients exhibited symptoms of UTIs, such as frequent or urgent urination, dysuria, hematuria, fever, cloudy urine, tenderness, and suprapubic and flank pain. All bacterial isolates were cultured on nutrient agar (Biolife, Italy) medium (16). Pure colonies were collected and characterized as *S. aureus* strains using molecular tests.

3.2. Biofilm Formation Assay

3.2.1. Qualitative Method

Qualitative detection of biofilm production was performed using the Congo-red agar (CRA) method. The confirmed *S. aureus* strains were streaked on CRA plates (Merck, Germany). After 48 hours of incubation at 37°C and an additional overnight incubation at room temperature, the characteristics of each colony were examined to evaluate the potential for biofilm

formation. Strains showing rough black colonies were interpreted as slime-positive (strong biofilm formation), those showing dark red colonies were considered suspected colonies (intermediate biofilm formation), and those showing light red colonies were interpreted as slime-negative (biofilm negative) (5).

3.2.2. Quantitative Method

The microtiter plate (MTP) method was employed to detect bacterial adherence to an abiotic surface. *S. aureus* strains were grown in tryptic soy broth (TSB) (Merck, Darmstadt, Germany) with 0.25% glucose for 24 hours at 37°C. Then, 200 µL of the bacterial suspension (final concentration ~ 10⁶ CFU/mL) was added to each well of polystyrene MTPs (Greiner, Louis, MO, USA). After 24 hours of incubation at 37°C, the supernatant was discarded, and the wells were washed three times with phosphate-buffered saline (PBS). The adhered cells were stained using 0.3% crystal violet (Merck, Darmstadt, Germany) for 5 - 10 minutes, and then sterile water was used to rinse off the unbound dye. Finally, the stained biofilms were solubilized with an ethanol:acetone (80:20) solution, and absorbance was measured using an ELISA reader (Stat Fax-2100) at 570 nm.

The cut-off OD (OD_c) was calculated as three standard deviations (SD) above the mean OD of the negative control: OD_c = average OD of negative control + (3 * SD of negative control). The OD value of each strain was defined as the average OD value of the strain reduced by the OD_c value (OD = average OD of a strain - OD_c). The biofilm formation ability of each strain was categorized as follows: OD₅₇₀ ≥ 1 = strong biofilm producer, 0.2 ≤ OD₅₇₀ < 1.0 = moderate biofilm producer, OD₅₇₀ < 0.2 = weak biofilm producer, OD₅₇₀ ≤ OD_c = biofilm negative.

All experiments were done in triplicate, and the wells without bacterial cells were considered negative controls (5).

3.3. Antimicrobial Susceptibility Testing

The susceptibility of all biofilm-producing strains was tested against 15 antibiotics on Mueller-Hinton agar (MHA) plates (Merck, Germany) using the Kirby-Bauer disk diffusion method according to Clinical Laboratory Standard Institute (CLSI) guidelines. The antibiotic disks used in the study were as follows: Kanamycin (30 µg), nalidixic acid (30 µg), ciprofloxacin (5 µg), levofloxacin

(5 µg), ofloxacin (5 µg), norfloxacin (10 µg), streptomycin (10 µg), gentamicin (10 µg), amikacin (30 µg), tobramycin (10 µg), erythromycin (15 µg), azithromycin (15 µg), tetracycline (30 µg), doxycycline (30 µg), and ceftiofloxacin (30 µg). Additionally, the resistance of strains to vancomycin was determined using the broth microdilution assay as recommended by CLSI (17).

3.4. DNA Extraction

The DNA template of all biofilm-positive strains was extracted using a polymerase chain reaction (PCR) template preparation kit (SinaClon, Tehran, Iran) according to the manufacturer's instructions.

3.5. Identification of *Staphylococcus aureus* Strains

All 119 suspected isolates were identified and confirmed as *S. aureus* using a PCR assay with species-specific primers for the *nucA* gene (Appendix 1 in Supplementary File) (18).

3.6. Detection of Biofilm-Related Genes

Biofilm-positive *S. aureus* strains were screened for the presence of *icaA*, *icaD*, *cna*, *fnbPA*, and *clfA* genes using specific primers and PCR conditions mentioned in Appendix 1 in Supplementary File (19).

3.7. Detection of Antibiotic Resistance Genes

Separate PCR reactions were employed to detect *aac(6')-Ie/aph(2'')*, *ant(4')-Ia*, and *aph(3')-IIIa* (encoding aminoglycoside-modifying enzymes) (20), as well as *gyrA*, *gyrB*, *grlA*, and *grlB* (fluoroquinolone resistance) genes, according to the conditions described in Appendix 1 in Supplementary File (12).

3.8. Typing of Bacterial Isolates

The amplification of the *mecA* gene was carried out using PCR with specific primers for the detection of MRSA strains (18). Moreover, a multiplex-PCR assay was employed to identify various types and subtypes of SCC_{mec} (I, II, III, IVa, IVb, IVc, IVd, and V) among MRSA strains (21). Additionally, the frequency distribution of different *agr* types (groups I-IV) was determined according to a previously reported protocol for *agr* typing (19). The sequence of primers and PCR conditions are summarized in Appendix 1 in Supplementary File.

3.9. Biochemical Fingerprinting

Biochemical phenotyping of bacterial strains was carried out using the PhP-CS plates (PhPlate AB, Stockholm, Sweden), which contain four sets of 24 dehydrated reagents. The microplates were incubated at 37°C, and the image of each plate was scanned at 16, 40, and 64-hour intervals with an HP Scanjet 4890 scanner (Hewlett-Packard, Palo Alto, CA, USA). The diversity of the bacterial populations was then calculated using PhPWin software (PhPWin ver. 4.2; PhP Microplate Techniques AB) (22).

3.10. Statistical Analysis

Statistical analyses were performed using GraphPad Prism software (version 8.0). Data for categorical variables were expressed as percentages. Statistical significance was assessed using Fisher's exact test for categorical variables. Differences were considered statistically significant at $P \leq 0.05$.

4. Results

4.1. Identification of *Staphylococcus aureus* Isolates

All 119 isolates collected from patients with symptomatic UTIs were identified and confirmed as *S. aureus* strains using specific *nucA* primers.

4.2. Biofilm Formation

Biofilm formation was evaluated in this study using two different methods: The qualitative CRA and the quantitative MTP methods. On the CRA plates, 12% ($N = 14$) of *S. aureus* strains produced slime and black colonies, while 27% ($N = 32$) were suspected biofilm producers, and 61% ($N = 73$) were non-biofilm producers. The results of the MTP assay showed that 10 (19%), 68 (57%), and 41 (24%) of *S. aureus* strains were classified as strong, moderate, and non-biofilm-forming strains, respectively.

4.3. Antibiotic Susceptibility Testing

Results of antibiotic susceptibility testing revealed that the highest antibiotic resistance rate was to nalidixic acid (77%), followed by streptomycin (73%) (Table 1). Additionally, 41%, 36%, 35%, and 32% of strains

showed resistance to tobramycin, kanamycin, gentamicin, and amikacin (as aminoglycoside antibiotics), respectively. Furthermore, 36% of strains were resistant to different fluoroquinolone antibiotics tested (ciprofloxacin, ofloxacin, levofloxacin, and norfloxacin). None of the strong biofilm-producing strains were resistant to doxycycline, and 50% of strong and moderate biofilm-forming strains showed resistance to tetracycline. Compared to strong biofilm-forming strains, the rate of resistance to all antibiotics (except for streptomycin, nalidixic acid, and tetracycline) was higher among moderate biofilm-producing isolates. The MICs of different strains to vancomycin were less than 1 µg/mL, indicating susceptibility to vancomycin.

Strong and moderate biofilm-producing strains were categorized into 8 and 32 antibiotypes, respectively (Table 2). The most frequent resistance type was antibiotype 35 (resistance to 14 different antibiotics), occurring in 24% ($N = 19$) of isolates. Additionally, 6% ($N = 4$) of the moderate biofilm producers were susceptible to all studied antibiotics (Table 2). In total, 40% of strong biofilm-producer strains and 36% of moderate biofilm-producer strains were resistant to at least one antibiotic in three or more classes of antibiotics, categorizing them as multidrug-resistant (MDR) strains. Moreover, 34 strains (44%) showed resistance to ceftiofur and were positive for the *mecA* gene, identifying them as MRSA.

4.4. Biofilm Related Genes

Results of PCR using specific primers showed that the *icaA* and *icaD* genes were present in 78% and 82% of strains, respectively. Additionally, the prevalence of the *clfA*, *fnbA*, and *cna* genes was 68%, 59%, and 42% of strains, respectively (Figure 1). Among the 10 strong biofilm-producing strains, 80% harbored both *icaA* and *icaD* genes. These frequencies among moderate biofilm formers were 78% and 82%, respectively. Moreover, the frequency of *clfA* and *cna* among moderate biofilm-producing strains was significantly higher ($P < 0.0001$) than among strong biofilm-forming strains.

4.5. Antibiotic Resistance Genes

Among aminoglycoside-modifying enzymes, the *aph(3')-IIIa* gene was the dominant gene, detected in 45% of strains, followed by the *ant(4)-Ia* (32%) and *aac(6')-Ie-aph(2'')-Ia* (17%) genes (Figure 2). The *aph(3')-IIIa* gene was

Table 1. Frequency of Antibiotic Resistance Among Strong and Intermediate Biofilm Forming *Staphylococcus aureus* Strains^a

Antibiotics	Strong Biofilm-Forming Strains	Moderate Biofilm-Forming Strains	Total	P-Value
FOX	3 (30)	31 (46)	34 (44)	0.0286
E	3 (30)	31 (46)	34 (44)	0.0286
AZM	4 (40)	32 (47)	36 (46)	0.3922
D	0	4 (6)	4 (5)	0.0289
TE	5 (50)	34 (50)	39 (50)	1.0000
NA	8 (80)	52 (76)	60 (77)	0.6089
CIP	2 (20)	26 (38)	28 (36)	0.0078
LEV	2 (20)	26 (38)	28 (36)	0.0078
OFX	2 (20)	26 (38)	28 (36)	0.0078
NOR	2 (20)	26 (38)	28 (36)	0.0078
S	8 (80)	49 (72)	57 (73)	0.2463
GM	2 (20)	25 (37)	27 (35)	0.0118
TOB	3 (30)	29 (43)	32 (41)	0.0776
AN	2 (20)	23 (34)	25 (32)	0.0378
K	2 (20)	26 (38)	28 (36)	0.0078
VAN	0	0	0	-

Abbreviations: AN, amikacin; AZM, azithromycin; CIP, ciprofloxacin; D, doxycycline; E, erythromycin; FOX, cefoxitin; GM, gentamicin; K, kanamycin; LEV, levofloxacin; NA, nalidixic acid; NOR, norfloxacin; OFX, ofloxacin; S, streptomycin; TE, tetracycline; TOB, tobramycin; VAN, vancomycin.

^a Values are expressed as No. (%) unless otherwise indicated.

the most frequent among strong (30%) and moderate (47%) biofilm-producing strains, while the *aac(6')-Ie-aph(2'')-Ia* variant was the least detected AME-encoding gene type.

Among quinolone resistance genes, *gyrA* and *grlA* were the most prevalent among all biofilm-producing strains, detected in 44% and 35% of strains, respectively (Figure 2). The frequency of *grlA* (40%) and *gyrB* (20%) among strong biofilm-producing strains was higher than among moderate biofilm-forming ones. Conversely, *gyrA* and *grlB* were the most frequent genes among moderate biofilm formers.

4.6. SCCmec and agr Typing

Among all 34 MRSA strains, five different SCCmec types were detected: Types I, III, IVa, IVc, and V. SCCmec type III was present in 64% (N = 22) of the strains (Table 3). Additionally, 24% (N = 8) and 76% (N = 26) of the strains were classified as community-acquired MRSA (CA-MRSA) and hospital-acquired MRSA (HA-MRSA), respectively. Out of the three strong biofilm-forming MRSA strains, two (67%) harbored SCCmec type III and one (33%) harbored SCCmec type IVc. SCCmec type III (65%, N = 20) was the most prevalent among moderate

biofilm-producing strains, followed by types V (13%, N = 4), I (13%, N = 4), IVa (6%, N = 2), and IVc (3%, N = 1).

All four *agr* types were successfully identified in 47 strains (60%), with *agr* type I being the dominant type among all biofilm-producing strains (Table 3). Furthermore, 31 (40%) strains were negative for all *agr* types and classified as non-typeable. *agr* type I (47%) was the predominant type among moderate biofilm-forming strains, followed by *agr* types III (7%), II (3%), and IV (1%). Molecular detection of 10 strong biofilm-forming *S. aureus* isolates indicated that *agr* types I (30%) and III (30%) were the most prevalent, followed by *agr* type II (10%).

4.7. Clonality of Bacterial Strains

Phene plate typing of bacterial strains revealed the presence of 22 PhP types, consisting of 8 common types (CTs) and 14 single types (STs). CT2 contained the highest number of bacterial strains (N = 31, 40%), followed by CT3 (N = 13, 17%) and CT4 (N = 8, 10%) (Table 4 and Appendix 2 in Supplementary File). While the prevalence of strong biofilm-producing strains was limited to CTs 1, 2, 3, and 5, CTs 4, 6, 7, and 8 were only common among moderate biofilm-forming *S. aureus* strains. Among the 14 STs detected, STs 2, 4, 5, and 7 belonged to strong biofilm

Table 2 . Antibiotic Susceptibility Patterns Among Biofilm-Producing Strains (N = 78) ^a

Antibiotics	Pattern	Strong Biofilm Producing	Moderate Biofilm Producing	Total
No antibiotic				
0	1	-	4 (6)	4 (5)
One antibiotic				
NA	2	-	5 (7)	5 (6)
S	3	1 (10)	5 (7)	6 (8)
AZM	4	-	1 (1.45)	1 (1.2)
TE	5	1 (10)	-	1 (1.2)
Two antibiotics				
NA, TE	6	1 (10)	-	1 (1.2)
NA, S	7	2 (20)	9 (13)	11 (14)
NA, FOX	8	-	1 (1.45)	1 (1.2)
NA, TOB	9	-	1 (1.45)	1 (1.2)
S, FOX	10	-	1 (1.45)	1 (1.2)
TE, FOX	11	-	1 (1.45)	1 (1.2)
Three antibiotics				
NA, S, TE	12	-	2 (3)	2 (3)
NA, S, AZM	13	1 (10)	-	1 (1.2)
NA, AZM, E	14	-	1 (1.45)	1 (1.2)
NA, S, TOB	15	1 (10)	1 (1.45)	2 (3)
NA, S, FOX	16	-	1 (1.45)	1 (1.2)
S, AN, TE	17	-	1 (1.45)	1 (1.2)
S, AZM, E	18	-	1 (1.45)	1 (1.2)
TE, D, FOX	19	-	1 (1.45)	1 (1.2)
Four antibiotics				
NA, AZM, TE, E	20	-	1 (1.45)	1 (1.2)
NA, S, TOB, TE	21	-	1 (1.45)	1 (1.2)
Five antibiotics				
NA, S, K, TOB, TE	22	-	1 (1.45)	1 (1.2)
NA, S, AZM, E, FOX	23	-	1 (1.45)	1 (1.2)
Six antibiotics				
NA, S, AZM, E, TE, FOX	24	1 (10)	-	1 (1.2)
Seven antibiotics				
NA, GM, K, TOB, AZM, E, TE	25	-	1 (1.4)	1 (1.2)
Nine antibiotics				
NA, S, GM, K, TOB, AZM, E, TE, FOX	26	-	1 (1.45)	1 (1.2)
Ten antibiotics				
NA, S, LEV, CIP, OFX, NOR, AZM, E, TE, FOX	27	-	1 (1.45)	1 (1.2)
NA, S, LEV, CIP, OFX, NOR, AZM, E, TE, D	28	-	1 (1.45)	1 (1.2)
Eleven antibiotics				
NA, S, LEV, CIP, OFX, NOR, AZM, E, TE, D, FOX	29	-	1 (1.45)	1 (1.2)
Twelve antibiotics				
NA, S, LEV, CIP, OFX, NOR, AZM, E, GM, K, TOB, FOX	30	-	1 (1.45)	1 (1.2)
NA, S, LEV, CIP, OFX, NOR, GM, AN, TE, K, TOB, FOX	31	-	1 (1.45)	1 (1.2)
NA, LEV, CIP, OFX, NOR, GM, K, AN, TOB, AZM, E, FOX	32	-	1 (1.45)	1 (1.2)
LEV, CIP, OFX, NOR, GM, K, AN, TOB, AZM, E, TE, FOX	33	-	1 (1.45)	1 (1.2)
Thirteen antibiotics				
NA, S, LEV, CIP, OFX, NOR, GM, AN, AZM, K, E, TE, TOB	34	-	1 (1.45)	1 (1.2)
Fourteen antibiotics				
NA, S, LEV, CIP, OFX, NOR, GM, K, AN, TOB, AZM, R, TE, FOX	35	2 (20)	17 (25)	19 (24)
Fifteen antibiotics				
NA, S, LEV, CIP, OFX, NOR, GM, K, AN, TOB, AZM, R, TE, D, FOX	36	-	1 (1.45)	1 (1.2)

Abbreviations: AN, amikacin; AZM, azithromycin; CIP, ciprofloxacin; D, doxycycline; E, erythromycin; FOX, ceftiofur; GM, gentamicin; K, kanamycin; LEV, levofloxacin; NA, naldixic acid; NOR, norfloxacin; OFX, ofloxacin; S, streptomycin; TE, tetracycline; TOB, tobramycin; VAN, vancomycin.

^a Values are expressed as No. (%).

producers, and the remaining STs were limited to moderate biofilm formers.

5. Discussion

Staphylococcus aureus is considered a significant threat to patients with UTIs due to its potential to invade renal tissues by adhering to the urothelium as a result of biofilm formation (23). Therefore, reliable, convenient, accessible, and inexpensive methods are needed to identify biofilm-forming strains (24). In this study, biofilm-producing strains were detected using a combination of the Congo-red agar plate test as a primary screening method and the MTP assay as the

gold standard test for quantitative evaluation of biofilm formation. Our results showed that 76% of isolates were biofilm producers, which is consistent with the study carried out by Yousefi et al. in Iran (1).

To understand the molecular mechanism of biofilm formation in *S. aureus*, we aimed to detect five key genes involved in this process. According to our results, *icaD* and *icaA* were the most prevalent genes. Several reports have indicated that the biofilm formation capacity in *S. aureus* causing UTIs is associated with the presence of *icaABCD* genes (23, 25), mentioning these genes as essential factors for intercellular adhesion and multilayer bacterial biofilm production (26). Moreover,

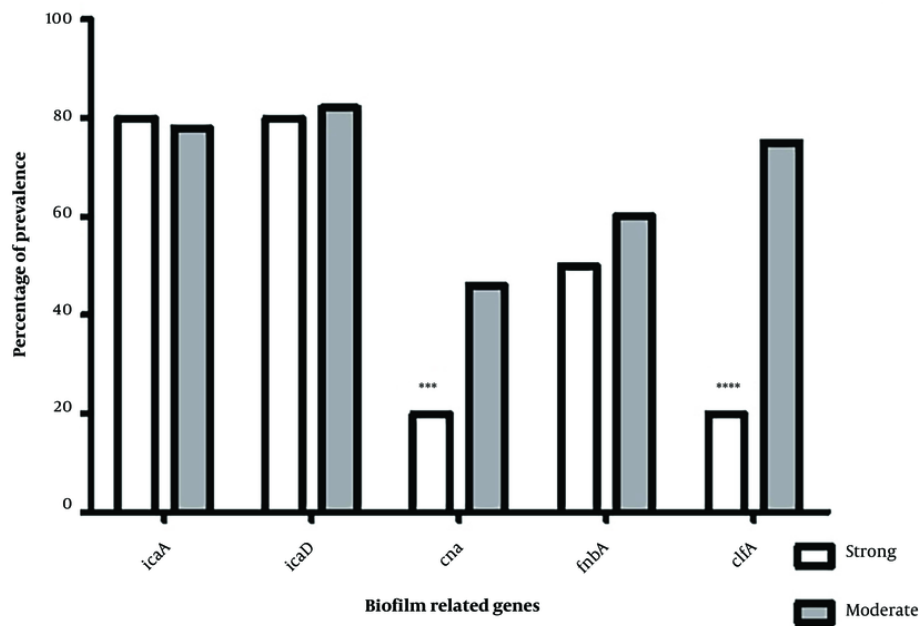


Figure 1. The frequency of genes involved in biofilm formation in the studied *Staphylococcus aureus* strains (****, $P < 0.0001$ and ***, $P = 0.0001$).

in a study in China, the *ica*ABCD genes were detected in all *S. aureus* strains (27). The biofilm-forming capacity of some isolates in the absence of the *ica*AD genes indicated the importance of further genetic investigations into *ica*-independent biofilm formation mechanisms. In the *ica*-independent pathway, different proteins including FnbpA, ClfA/B, and collagen-binding adhesin (CNA) may be involved in biofilm formation, as detected in this study.

In this study, 44% of biofilm-producing strains isolated from patients with UTIs were categorized as MRSA. In similar surveys conducted in Iraq and Australia, the prevalence of MRSA strains among urine specimens was 7.7% and 4.06%, respectively (28, 29). Different frequencies of MRSA strains have been reported from various sources in Iran, ranging from 19% to 70%, indicating the dissemination and persistence of different MRSA clone types in the country (30, 31). The increase in resistance to ceftaxime in the present study could be attributed to bacterial evolution, excessive antibiotic consumption, antibiotic abuse, self-treatment, and the transmission of resistant strains

from the environment, animals, foods, and between individuals.

Our results revealed that the highest antibiotic resistance rates were against nalidixic acid and streptomycin. This finding contrasts with previous findings by Khaleel et al. in Iraq, who reported that *S. aureus* strains from UTIs were highly resistant to penicillin (100%), ceftazidime (100%), and gentamicin (87.2%) (28). Another study conducted in Iran showed high resistance of *S. aureus* to tetracycline, ciprofloxacin, and erythromycin (1). Despite the high resistance rates to different antibiotics tested, resistance to doxycycline was relatively low and uncommon among biofilm-producing isolates in this study. Therefore, this antibiotic can be used as an appropriate treatment for UTIs caused by biofilm-forming *S. aureus* strains.

Previous studies conducted in Nigeria showed that ciprofloxacin was the most effective antibiotic against *S. aureus* strains isolated from UTIs (32). Alshomrani et al. reported that 91% of *S. aureus* isolated from urine in Saudi Arabia were susceptible to trimethoprim-sulfamethoxazole (33). Treatment of UTIs is usually started empirically before the preparation of laboratory

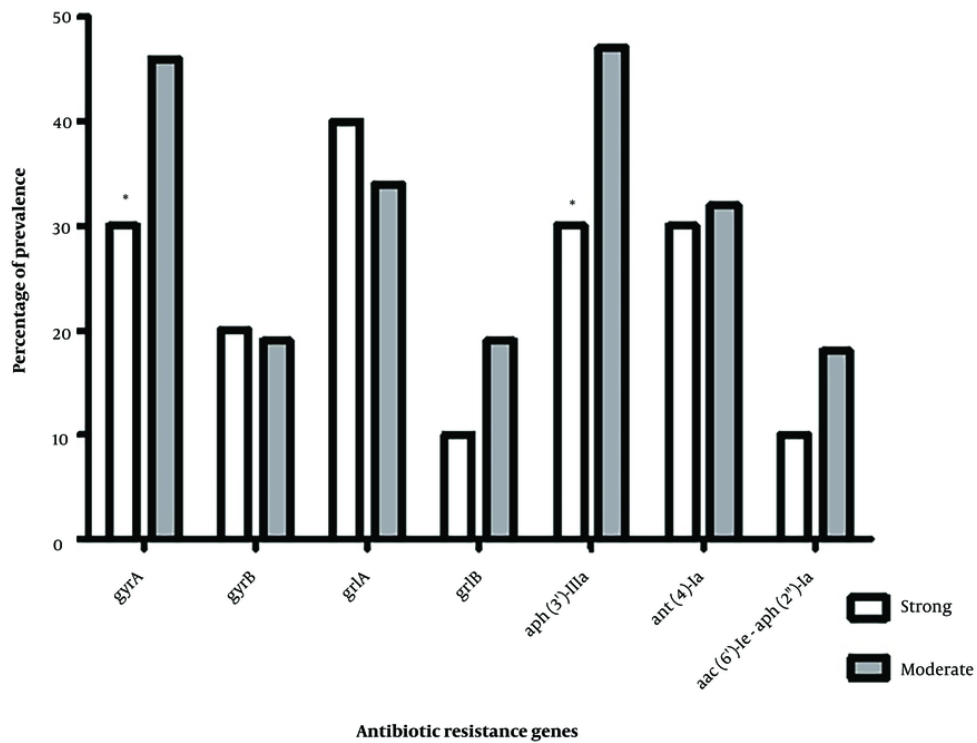


Figure 2. Molecular diversity of genes encoding aminoglycoside-modifying enzymes and fluoroquinolone resistance among biofilm-forming *Staphylococcus aureus* strains isolated from urinary tract infection (UTI) (*, P = 0.0286).

Table 3. Frequency of SCCmec and agr Types Among Biofilm-Producing Strains^a

Variables and Types	Strong Biofilm Producing	Moderate Biofilm Producing	Total	P-Value
SCCmec				
Type I	-	4 (13)	4 (12)	0.0002
Type III	2 (67)	20 (65)	22 (64)	0.8814
Type IVa	-	2 (6)	2 (6)	0.0289
Type IVc	1 (33)	1 (3)	2 (6)	< 0.0001
Type V	-	4 (13)	4 (12)	0.0002
agr				
Type I	3 (30)	32 (47)	35 (45)	0.0198
Type II	1 (10)	2 (3)	3 (4)	0.0818
Type III	3 (30)	5 (7)	8 (10)	< 0.0001
Type IV	-	1 (1)	1 (1)	1.0000
Non typeable	3 (30)	28 (42)	31 (40)	0.1048

^a Values are expressed as No. (%) unless otherwise indicated.

results of urine culture. However, the prevalent causes of the infection and their antibiotic susceptibility patterns show wide geographic variation. Therefore, we

recommend that empirical antibiotic selection be based on knowledge of local patterns of urinary pathogens and their antibiotic sensitivities.

Table 4. Phene Plate Typing, Antimicrobial Resistance Profiles, SCCmec and agr Types of Biofilm-Forming *Staphylococcus aureus* Strains (N = 78)

No.	Phene plate Type	Antibiotype	Biofilm Formation	agr Type	mecA	SCCmec Type
1	CT1	5	Strong	-	-	-
2	CT1	4	Moderate	4	-	-
3	CT1	1	Moderate	-	-	-
4	CT1	35	Moderate	1	+	III
5	ST1	14	Moderate	-	-	-
6	CT2	33	Moderate	-	+	V
7	CT3	7	Strong	1	-	-
8	ST2	13	Strong	3	-	-
9	CT2	7	Moderate	-	-	-
10	CT3	7	Moderate	1	-	-
11	CT2	3	Moderate	1	-	-
12	CT2	29	Moderate	1	+	III
13	CT3	35	Moderate	1	+	III
14	CT2	27	Moderate	1	+	III
15	CT2	2	Moderate	-	-	-
16	CT2	7	Moderate	1	-	-
17	CT3	2	Moderate	-	-	-
18	CT3	28	Moderate	1	-	-
19	CT3	30	Moderate	-	+	V
20	CT3	31	Moderate	1	+	I
21	ST3	1	Moderate	-	-	-
22	CT2	35	Moderate	1	+	III
23	CT3	35	Moderate	1	+	III
24	CT2	35	Moderate	-	+	III
25	ST4	24	Strong	3	+	IVc
26	ST5	3	Strong	3	-	-
27	ST6	2	Moderate	1	-	-
28	CT2	11	Moderate	1	+	V
29	CT3	3	Moderate	3	-	-
30	CT2	6	Strong	1	-	-
31	ST7	7	Strong	2	-	-
32	CT2	34	Moderate	-	-	-
33	CT4	35	Moderate	1	+	III
34	CT2	25	Moderate	3	-	-
35	CT2	2	Moderate	3	-	-
36	CT2	1	Moderate	1	-	-
37	CT2	20	Moderate	1	-	-
38	CT4	19	Moderate	3	+	I
39	CT3	7	Moderate	-	-	-
40	CT5	35	Strong	-	+	III
41	CT2	35	Moderate	1	+	III
42	CT3	35	Strong	1	+	III
43	CT6	35	Moderate	1	+	III
44	CT6	2	Moderate	-	-	-
45	CT3	1	Moderate	-	-	-
46	CT2	8	Moderate	1	+	IVa
47	CT2	3	Moderate	-	-	-
48	CT3	3	Moderate	1	-	-
49	CT2	7	Moderate	1	-	-
50	CT7	3	Moderate	-	-	-
51	CT8	7	Moderate	1	-	-
52	CT2	35	Moderate	1	+	III
53	CT2	35	Moderate	1	+	IVc
54	CT4	35	Moderate	1	+	III
55	CT8	16	Moderate	1	+	I
56	ST8	10	Moderate	-	+	III
57	ST9	22	Moderate	-	-	-
58	CT2	35	Moderate	1	+	III
59	ST10	35	Moderate	1	+	III
60	CT2	7	Moderate	3	-	-
61	CT2	7	Moderate	1	-	-
62	CT2	35	Moderate	-	+	III
63	ST11	35	Moderate	1	+	III
64	CT2	35	Moderate	1	+	III
65	CT4	15	Moderate	2	-	-
66	CT7	9	Moderate	2	-	-
67	CT4	18	Moderate	-	-	-
68	CT2	12	Moderate	-	-	-
69	CT2	17	Moderate	-	-	-
70	CT4	21	Moderate	-	-	-
71	CT2	15	Strong	-	-	-
72	CT2	35	Moderate	1	+	III
73	CT4	32	Moderate	-	+	IVa
74	ST12	36	Moderate	-	+	III
75	CT5	23	Moderate	-	+	V
76	ST13	7	Moderate	-	-	-
77	ST14	12	Moderate	-	-	-
78	CT4	26	Moderate	-	+	I

The most common resistance mechanism against aminoglycosides in staphylococci is drug inactivation

by cellular aminoglycoside-modifying enzymes, including acetyltransferase (AAC), adenylyltransferase, and phosphotransferase (APH) (12). Unlike other studies (14, 34, 35), the *aac(6')-Ie-aph(2'')-Ia* gene had the lowest prevalence among aminoglycoside-resistant (AMR) strains in our study, indicating the emergence of different clone types in the studied hospital that are distinct from previous clones circulating in Iran and other countries.

Mechanisms of fluoroquinolone resistance in *S. aureus* are mostly related to mutations in drug targets, including DNA topoisomerase IV (encoded by *griA* and *griB*) and DNA gyrase (encoded by *gyrA* and *gyrB*). Our results showed that *gyrA* and *griA* were the most prevalent genes among quinolone-resistant strains. Compared to previous reports from Iran, the frequency of these genes was higher among biofilm-producing strains (34, 36). This genetic diversity may be due to the presence of multiple sources of resistant bacteria or the possible exchange of genes among different strains through the transfer of mobile genetic elements (34).

The presence of an antibiotic resistance phenotype is mostly associated with the presence of corresponding antibiotic resistance genes (36). Our results indicated that the incidence of a phenotypic profile of quinolone resistance (36%) is almost equal to the incidence of a genotypic profile (*gyrA*; 44% and *griA* 35%). Thus, the expression of such genes under suitable conditions may potentially lead to therapeutic failure.

Few studies describe the prevalence and dissemination of various clonal groups of biofilm-forming *S. aureus* strains among patients with UTIs in Iran. In this study, the majority of bacterial strains harbored *SCCmec* type III. Previous reports have supported our findings, indicating that *SCCmec* type III is the predominant type in Iran (5, 22, 37), suggesting the hospital origin of such strains. However, other studies have shown that biofilm-producing *S. aureus* strains were mostly *SCCmec* type IV (11, 38). In the present research, *agr* type I was the dominant type among all biofilm-producing strains. These results contrast with previous studies indicating that isolates with *agr* types II and III produced large amounts of biofilm due to a deficiency in *icaR* transcription, which acts as a repressor of the *icaADBC* operon (5, 39). Although our findings align with other research showing that isolates belonging to *agrI* had a great ability to form strong biofilms (40), some studies have indicated the absence

of an association between specific *agr* genotypes and enhanced ability to form biofilm (41).

Compared to genotyping methods such as pulsed-field gel electrophoresis (PFGE) and multilocus sequence typing (MLST), the PhP system is specifically designed for each bacterial species to yield the highest discrimination among the strains of that species (14). Our results showed the presence of diverse PhP types (22 types) among biofilm-producing strains, consisting of 8 CTs and 14 STs, indicating high diversity in the kinetics of metabolism of strains. CT2 was the predominant type, and its dissemination is consistent with previous studies in Iran (5, 14). The presence of different antibiograms and biofilm formation abilities among the strains with the identical PhP type indicated no relatedness between their clonal dissemination, biofilm production, and antibiotic resistance profiles.

Despite the valuable insights provided by this study, several limitations may impact the interpretation of our results. First, there were relatively long time intervals between sample collection, data analysis, and reporting of results. Second, the study focused on a particular geographical zone for the selection of patients. Therefore, these results should be interpreted with caution, as they may not fully represent the prevalence, diversity, and antimicrobial susceptibility profiles of *S. aureus* strains among patients with UTIs in this country at the present time.

5.1. Conclusions

Our results revealed the presence of various biofilm production capacities, antimicrobial resistance profiles, and clonal lineages in *S. aureus* isolated from patients with UTIs. Considering the significant role of *S. aureus* in human medicine, the carriage of biofilm-related and antibiotic resistance genes provides evolutionary benefits to this microorganism, potentially leading to the selection of more resistant strains in the future. The biofilm-forming capacity of both MRSA and MSSA strains indicates a high ability of these strains to persist in hospital environments, increasing the risk of disease development in hospitalized patients. Therefore, continuous tracking of epidemic strains and adopting novel approaches for inhibiting biofilm formation are recommended to control the dissemination of bacteria among inpatients effectively. Additionally, our results suggest that doxycycline can be prescribed in the

empirical therapy of UTIs caused by biofilm-forming *S. aureus* strains. We recommend future research to determine the accurate role of other virulence factors and their correlation to the pathogenic potential of *S. aureus* and the development of drug resistance to develop better therapeutic measures against *S. aureus* infections.

Supplementary Material

Supplementary material(s) is available [here](#) [To read supplementary materials, please refer to the journal website and open PDF/HTML].

Footnotes

Authors' Contribution: Conceptualization, F. R.; data curation and formal analysis, N. S. M, and F. R.; investigation, F. R.; methodology and project administration, F. R.; supervision, F. R.; validation, F. R.; writing of original draft, S. K.; writing, reviewing, and editing, F. R.

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