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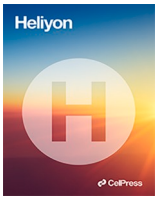

Research article

Assessment of biofilm formation, antibiotic resistance patterns, and the prevalence of adhesion-related genes in clinical *Staphylococcus aureus* isolates

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ABSTRACT

Background: This study aimed to evaluate the biofilm formation abilities of clinical *Staphylococcus aureus* strains, assess their antibiotic susceptibility patterns, and identify the prevalence of adhesion-associated genes.

Methodology: In this study, a total of 60 *S. aureus* strains were collected from urine, pus, wounds, blood, body fluid, and sputum in health centers affiliated with Abadan University of Medical Sciences, Iran. Strains were identified via microbiological methods and polymerase chain reaction (PCR) to target the *nuc* gene. Antibiotic susceptibility testing (AST) was conducted via the disc diffusion method. Methicillin-resistant *S. aureus* (MRSA) strains were identified by cefoxitin disc diffusion and PCR targeting the *mecA* gene. Biofilm formation was assessed via a microtiter plate assay, and the prevalence of adhesion-encoding genes was evaluated via PCR. The data were analyzed in Excel and SPSS via statistical methods, with *P*-values <0.05 considered significant.

Results: Using AST, daptomycin and linezolid were the most effective antibiotics (100 % susceptibility rate). According to the results of the cefoxitin disc test, 48.3 % (n = 29/60) of the strains were MRSA. All the MRSA strains harbored the *mecA* gene. In total, 32 % of the strains were biofilm producers. Moreover, 56.2 %, 28.1 %, and 15.6 % of the strains produced weak, moderate, and strong biofilms, respectively. There were no significant differences between the MRSA and MSSA strains in terms of the association of biofilm formation with antibiotic resistance except for erythromycin (*P*-value = 0.0087), gentamicin (*P*-value = 0.0009), and penicillin (*P*-value = 0.0009). The most prevalent biofilm-encoding genes were *icaA* (76.7 %), followed by *icaD* (70 %), *clfA* (65.0 %), and *fnbA* (53.3 %).

Conclusion: This study identified MRSA strains with biofilm-forming abilities that possess adhesion-associated genes. The most prevalent biofilm-encoding gene was *icaA*. To prevent further spread of these strains, regional preventive measures are needed.

1. Introduction

The increasing prevalence of antibiotic resistance and biofilm formation among *Staphylococcus aureus* is a serious public health concern that complicates the treatment of infections caused by these bacteria [1,2]. This issue is particularly significant in clinical

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settings and poses a significant challenge to healthcare professionals worldwide. Effective management of *S. aureus* infections requires a comprehensive understanding of the mechanisms underlying antibiotic resistance and biofilm formation in these bacteria and the development of innovative therapeutic approaches to address these issues [2,3].

Biofilms are complex, three-dimensional structures formed by bacteria that adhere to surfaces encased in a self-produced extracellular matrix. This matrix not only provides a physical barrier against antibiotics and the host immune system, but also promotes the survival and persistence of bacteria within the biofilm [4,5]. Additionally, biofilms structure allows the formation of tiny, nutrient-rich channels that facilitate bacterial communication and exchange of genetic material, leading to the development of antibiotic resistance [4–6]. The initial stage of biofilm formation by *S. aureus* involves adhering to surfaces and colonizing host tissues.

In the context of host defense against hostile immune responses such as opsonization and phagocytosis, bacteria develop biofilms. However, this growth mode can lead to chronic tissue infections and device-related infections, particularly in orthopedic alloplastic devices, endotracheal tubes, and catheters [4].

Biofilm formation is a multifaceted process that requires the activation of various genes, including the intercellular adhesion A and D (*icaA* and *icaD*) genes [7]. These genes are responsible for producing polysaccharide intercellular adhesion (PIA), which consists mainly of N-acetyl glucosamine. The PIA forms a protective matrix of exopolysaccharides that surrounds the bacterial cells within the biofilm [7,8]. Furthermore, *S. aureus* expresses a family of adhesion molecules known as microbial surface components, that recognize adhesion matrix molecules (MSCRAMM). These molecules are encoded by various genes, including *bap* (biofilm-associated protein), *cna* (collagen-binding protein), *fnbA* and *fnbB* (fibronectin-binding protein A/B), *clfA* and *clfB* (clumping factor A/B), and *fib* (fibrinogen-binding protein) [9,10].

S. aureus is well-known for developing a resistance phenotype against various antibiotics and its high pathogenicity in humans. The widespread distribution of methicillin-resistant *S. aureus* (MRSA) strains has led the increasing complexity of hospital- and community-acquired infections worldwide [11]. Multidrug resistance has increased worldwide and which is considered a public health threat. Several recent investigations reported the emergence of MDR bacterial pathogens from different origins, increasing the necessity for the proper use of antibiotics. In addition, antimicrobial susceptibility testing is routinely used to detect the antibiotic of choice, are the screening of emerging MDR strains [12–19].

This study aimed to assess biofilm formation, antibiotic susceptibility patterns, and the prevalence of adhesion-associated genes in clinical *S. aureus* strains in Abadan, southwest Iran.

2. Materials and methods

2.1. Isolation and identification of S. aureus strains

From September 2021 to June 2022, a total of 60 *S. aureus* strains were obtained from the clinical samples of patients referred to diagnostic laboratories and treatment centers affiliated with the Abadan University of Medical Sciences. For the isolation of *S. aureus* strains, the samples were initially subjected to cultivation on blood agar and mannitol salt agar (MSA) plates (Biolife, Milan, Italy). The plates were incubated overnight at 37 °C. The grown colonies were subjected to phenotypic and molecular identification tests. For primary observation, colonies with a golden color, round, and convex appearance, beta hemolysis on blood agar, and fermentation of mannitol on MSA were selected for further investigation [20]. In the next step, more specific microbiological tests for the identification

Table 1
Primers used in the study.

Primer	Primer Sequence (5′→3′)	Product Size (bp)	Annealing temperature	Reference
nuc	F: GCGATTGATGGTGATACGGTT R: AGCCAAGCCTTGACGAACTAAAGC	270	55 °C	[21]
mecA	F: GATATCGAGGCCCGTGGATT R: ACGTCGAACTTGAGCTGTTA	642	57 °C	[21]
icaA	F: TCTCTTGCAGGAGCAATCAA R: TCAGGCACTAACATCCAGCA	188	56 °C	[21]
icaD	F: ATGGTCAAGCCCAGACAGAG R: CGTGTTTTCAACATTTAATGCAA	198	56 °C	[21]
bap	F: CCCTATATCGAAGGTGTAGAATTG R: GCTGTTGAAGTTAATACTGTACCTGC	971	62 °C	[21]
fib	F: CGTCAACAGCAGATGCGAGCG R: TGCATCAGTTTTCGCTGCTGGTTT	239	61 °C	[27]
fnbA	F: CGACACAACCTCAAGACAATAGCGG R: CGTGGCTTACTTTCTGATGCCGTTC	133	60 °C	[27]
fnbB	F: ACGCTCAAGGCGACGGCAAAG R: ACCTTCTGCATGACCTTCTGCACCT	197	59 °C	[27]
cna	F: AATAGAGGCGCCACGACCGT R: GTGCCTTCCCAAACCTTTTGAGCA	156	61 °C	[27]
clfA	F: TTACGAATCAGTTGACGAATGTG R: AGGCACTGAAAAACCATAATTCA	104	55 °C	[27]
clfB	F: TGCAAGTGCAGATTCCGAAAAAAAC R: CCGTCGGTTGAGGTGTTTCATTTG	194	60 °C	[27]

of *S. aureus* including catalase, slide and tube coagulase, and DNase, were used [20]. All phenotypic test kits were prepared from Baharafshan Co., Tehran, Iran. Finally, species identification was confirmed via polymerase chain reaction (PCR) of the *nuc* gene encoding the *S. aureus* nuclease enzyme [21]. The primers used were prepared by SinaClon BioScience Co., Tehran, Iran.

2.2. Antibiotic susceptibility

The antimicrobial susceptibility of the clinical strains was assessed via the disc diffusion technique on Muller–Hinton agar, following the Clinical Laboratory Standards Institute (CLSI) 2023 guidelines [22]. The following antibiotic categories were used: lipopeptides: daptomycin (30 μg); penicillins: penicillin (10 units), and methicillin (5 μg); cephem: cefoxitin (30 μg); macrolides: erythromycin (15 μg); glycylcycline: tigecycline (15 μg); lincosamides: clindamycin (2 μg); aminoglycoside: gentamicin (10 μg); phenicols: chloramphenicol (30 μg); quinolones: ciprofloxacin (5 μg); tetracyclines: doxycycline (30 μg); oxazolidinones: linezolid (30 μg); ansamycin: rifampin (5 μg); and folate pathway antagonist: trimethoprim-sulfamethoxazole (1.25/23.75 μg) (Roscoe, Albertslund, Denmark). *S. aureus* ATCC 25923 was used as the control in the antibiogram test. According to guidelines recommended by the Centre for Disease Prevention and Control (CDC), multidrug-resistant (MDR) bacteria have acquired nonsusceptibility to at least one agent in three or more antimicrobial categories. On the other hand, extensively drug-resistant (XDR) bacteria are considered non-susceptible to at least one agent in all but two or fewer antimicrobial categories [23]. All *S. aureus* isolates were screened for methicillin resistance with a cefoxitin disc (30 μg) (≤21 mm) following the CLSI guidelines [22]. As described previously [21], PCR amplification of the *mecA* gene was used for more accurate confirmation of the MRSA strains (Table 1). The PCR mixture included 12.5 μl of master mix (SinaClon BioScience Co., Tehran, Iran), 1 μl of genomic DNA, 1 μl of each specific primer (10 p.m./μl), and 9.5 μl of distilled water. The amplification program included: 5 min at 96 °C, followed by 35 cycles of 30 s at 96 °C, annealing at 57 °C for 30 s, 45 min at 72 °C, and 5 min at 72 °C for the final extension. The PCR products were separated on a 1.5 % agarose gel containing 0.5 mg/mL ethidium bromide for 1 h at 100 V. As determined by Krumperman's protocol [24], the MAR index was calculated as the ratio of antibiotics to which a bacterial isolate displays resistance (a) to the total number of antibiotics tested (b).

2.3. Biofilm production

Biofilm production was assessed by measuring adherence to a polystyrene microtiter plate [25]. The overnight blood agar bacterial suspension was standardized to a 0.5 McFarland using sterile tryptic soy broth (TSB; Biolife, Milan, Italy). To assess the biofilm, 300 μl of the bacterial suspension was introduced into three wells of a 96-well microtiter plate. Moreover, the remaining three wells were filled with uninoculated sterile TSB medium, and were considered negative controls [25]. Following a 4-h incubation period, the supernatant was discarded, and each well was washed with 300 μl of phosphate-buffered saline (PBS; 7 mM Na2HPO4, 3 mM NaH2PO4, pH 7.4). Fresh TSB medium was added to each well, and after 24 h, the process was repeated. Fixation was performed by incubating the wells with 300 μl of pure methanol for 15 min [25]. Following the staining of the wells with 0.1 % w/v crystal violet solution (Sigma–Aldrich, Steinheim, Germany) for approximately 10–15 min, the dyes were resolubilized in 30 % acetic acid. The staining intensity was subsequently measured at 590 nm via a spectrophotometer. The assay was conducted three times, and the average of the results was calculated. Biofilm production can be categorized into four groups on the basis of optical density (OD) measurements [26]. ODs ≤ ODc: nonproducer; ODc ≤ ODs ≤2 × ODc: weak producer; 2 × ODc ≤ ODs ≤4 × ODc: moderate producer; ODs >4 × ODc: strong producer. ODc represents the OD of the negative control, and ODs represent the OD of the experimental samples. The capacity for biofilm formation was evaluated in the strains and classified according to the absorbance of the crystal violet-stained adherent cells. To assess biofilm formation, *S. aureus* ATCC 25923 was used as a quality control.

2.4. PCR assay

Bacterial DNA was extracted via a commercial kit following the manufacturer's instructions (SinaClon BioScience Co., Tehran, Iran). The purified DNA was stored at −20 °C for PCR. Various virulence and biofilm formation genes, including *icaA*, *icaD*, *fnbA*, *fnbB*, *clfA*, *clfB*, *fib*, *bap*, and *can*, were assessed via separate standard PCRs. The primer sequences, PCR product sizes, and corresponding references are listed in Table 1 [27]. The PCR amplification mixture included 12.5 μl of master mix (SinaClon BioScience Co., Tehran, Iran), 2 μl of genomic DNA, 1 μl of each specific primer (10 p.m./μl), and 8.5 μl of nuclease-free distilled water. PCR amplification was performed via a C1000 Bio-Rad Thermal Cycler (Bio-Rad Laboratories, Inc.). The amplification conditions included the following thermal cycling profile: 5 min at 96 °C, followed by 30 cycles of 30 s at 96 °C, annealing (Table 1) for 30 s, 1 min at 72 °C, and 5 min at 72 °C for the final extension. The PCR amplicons were electrophoresed on a 1.5 % agarose gel containing 0.5 mg/mL ethidium bromide for 1 h at 100 V.

2.5. Statistical analysis

The data collected during the study and the laboratory investigation results were entered into an Excel spreadsheet version 2021 (Microsoft, Redmond WA, USA) and then analyzed via SPSS software version 25 (IBM, Armonk, USA). Appropriate tests including chi-square tests, Fisher's exact tests, and other statistical methods were used for statistical analysis of parametric or nonparametric data. P-values less than 0.05 were considered statistically significant.

3. Results

3.1. Phenotypic characteristics of the S. aureus isolates

In this study, a total of 60 *S. aureus* strains were identified via phenotypic tests. The majority of strains were collected from urine samples (n = 26, 43.3 %), followed by pus (n = 18, 30 %), wounds (n = 5, 8.3 %), blood (n = 4, 6.7 %), body fluid (n = 4, 6.7 %), and sputum (n = 3, 5 %).

3.2. Antibiotic resistance pattern

Antibiotic susceptibility testing revealed that daptomycin and linezolid were effective against all 60 (100.0 %) strains, whereas penicillin was ineffective (n = 60, 100.0 %) (Table 2).

Different resistance levels were observed for other antibiotics, including trimethoprim-sulfamethoxazole (78.3 %, n = 47), cefoxitin (48.3 %, n = 29), erythromycin (45 %, n = 27), and gentamicin (43.3 %, n = 26) (Table 2).

Moreover, 27 (45 %) and 2 (3.3 %) of the strains presented MDR and XDR phenotypes, respectively. A total of 29 (48.3 %) strains were identified as MRSA via the cefoxitin disc-diffusion test and confirmed by PCR for the *mecA* gene (Fig. 1). In total, 19 antibiotypes including 17 MDR strains and 2 XDR strains were detected in these strains (Table 3). The most prevalent antibiotype was SA2 (n = 7, 11.6 %) with the following resistance pattern: erythromycin, gentamicin, trimethoprim-sulfamethoxazole, cefoxitin, and penicillin.

3.3. Biofilm formation

Among the strains, biofilm formation was observed in 32 (53.3 %) strains via the microtiter plate approach. Among them, 5 (15.6 %) had strong biofilm production, 9 (28.1 %) had moderate biofilm production, and 18 (56.2 %) had weak biofilm production. The correlation between biofilm formation and antibiotic resistance was also evaluated. Significant differences in susceptibility rates to commonly used antibiotics were detected between biofilm-forming and nonbiofilm-forming strains (clindamycin: $P = 0.029$; gentamicin: $P = 0.031$; cefoxitin: $P = 0.001$; ciprofloxacin: $P = 0.020$) (Table 2). However, this association was not demonstrated for other antibiotics. Notably, 22 (68.7 %) and 10 (31.3 %) of the biofilm producers were MRSA and MSSA, respectively. There was a correlation between methicillin resistance and biofilm formation, as MRSA strains had significantly greater biofilm production ability than MSSA strains did ($P < 0.05$; Fig. 1).

3.4. Association of biofilm formation with antibiotic resistance in MRSA and MSSA strains

The results revealed there were no significant differences between the MRSA and MSSA strains in terms of the association of biofilm formation with antibiotic resistance, with the exceptions of erythromycin (*P*-value = 0.0087), gentamicin (*P*-value = 0.0009), and penicillin (*P*-value = 0.0009) (Table 4).

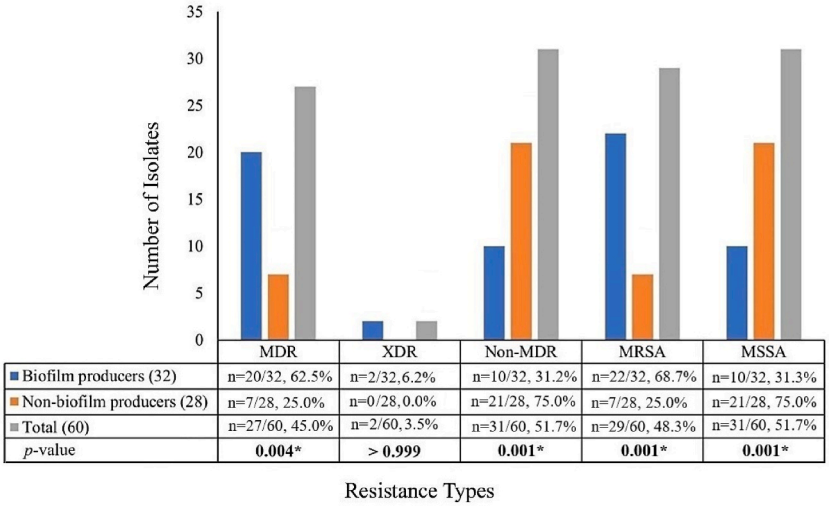
3.5. Detection of adhesion-associated genes by PCR

The molecular analysis identified *icaA* and *icaD* as the predominant genes present in 46 (76.7 %) and 42 (70 %) of the isolates, respectively. The prevalence of the *fnbA*, *fnbB*, *clfA*, *clfB*, *fnbA*, *fib*, and *cna* genes was 53.3 %, 36.7 %, 65 %, 45 %, 43.3 %, and 51.7 %, respectively. Notably, none of the studied strains were found to harbor the bap gene. As shown in Table 5, the *fib*, *clfB*, and *cna* genes were detected in 22 (68.7 %), 21 (65.6 %), and 27 (84.4 %) of the biofilm-forming isolates, respectively, whereas 9 (32.1 %), 5 (17.8

Table 2
Total antibiotic resistance rate and the correlation between biofilm production and antimicrobial resistance.

Antimicrobials	Biofilm		Total (60) n (%)	*P*-value
	Formers (32) n (%)	Non-formers (28) n (%)		
Clindamycin	8 (25.0)	1 (3.6)	9 (15.0)	**0.029***
Erythromycin	16 (50.0)	11 (39.3)	27 (45.0)	0.405
Chloramphenicol	5 (15.6)	0 (0.0)	5 (8.3)	0.055
Gentamicin	18 (56.2)	8 (28.6)	26 (43.3)	**0.031***
Daptomycin	0 (0.0)	0 (0.0)	0 (0.0)	>0.999
Doxycycline	1 (3.1)	0 (0.0)	1 (1.6)	>0.999
Trimethoprim-sulfamethoxazole	27 (84.3)	20 (71.4)	47 (78.3)	0.558
Cefoxitin	22 (68.7)	7 (25.0)	29 (48.3)	**0.001***
Rifampin	2 (6.2)	0 (0.0)	2 (3.3)	>0.999
Ciprofloxacin	10 (31.2)	2 (7.1)	12 (20.0)	**0.020***
Tigecycline	1 (3.1)	0 (0.0)	1 (1.6)	>0.999
Penicillin	32 (100.0)	28 (100.0)	60 (100.0)	>0.999
Linezolid	0 (0.0)	0 (0.0)	0 (0.0)	>0.999

*Chi-square test or Fisher's Exact Test; $P < 0.05$ is statistically significant.



	MDR	XDR	Non-MDR	MRSA	MSSA
Biofilm producers (32)	n=20/32, 62.5%	n=2/32,6.2%	n=10/32, 31.2%	n=22/32, 68.7%	n=10/32, 31.3%
Non-biofilm producers (28)	n=7/28, 25.0%	n=0/28,0.0%	n=21/28, 75.0%	n=7/28, 25.0%	n=21/28, 75.0%
Total (60)	n=27/60, 45.0%	n=2/60,3.5%	n=31/60, 51.7%	n=29/60, 48.3%	n=31/60, 51.7%
p-value	**0.004***	**> 0.999**	**0.001***	**0.001***	**0.001***

Fig. 1. Comparison of MDR, XDR, and MRSA in biofilm producers and non-biofilm producers. * Chi-square test or Fisher's Exact Test; $P < 0.05$ is statistically significant.

Table 3
Antibiotypes, resistance patterns, and MAR index of methicillin-resistant *Staphylococcus aureus* (MRSA) strains.

Type of resistance	Antibiotypes	Antimicrobial classes	Resistance Pattern	Occurrence N (%)	MAR Index
MDR	SA1	Macrolides, Folate pathway inhibitors, Cephems, Ansamycins, Penicillins	E-SXT-FOX-RA-P	1 (1.6)	0.3
	SA2	Macrolides, Aminoglycosides, Folate pathway inhibitors, Cephems, Penicillins	E-GM-SXT-FOX-P	7 (11.6)	0.3
	SA3	Lincosamides, Aminoglycosides, Folate pathway inhibitors, Cephems, Penicillins	CD-GM-SXT-FOX-P	2 (3.3)	0.3
	SA4	Macrolides, Folate pathway inhibitors, Cephems, Quinolones, Penicillins	E-SXT-FOX-CIP-P	2 (3.3)	0.3
	SA5	Macrolides, Phenicols, Aminoglycosides, Quinolones, Penicillins	E-C-GM-CIP-P	1 (1.6)	0.3
	SA6	Macrolides, Aminoglycosides, Folate pathway inhibitors, Penicillins	E-GM-SXT-P	2 (3.3)	0.2
	SA7	Aminoglycosides, Folate pathway inhibitors, Cephems, Quinolones, Penicillins	GM-SXT-FOX-CIP-P	2 (3.3)	0.3
	SA8	Lincosamides, Phenicols, Aminoglycosides, Cephems, Penicillins	CD-C-GM-FOX-P	1 (1.6)	0.3
	SA10	Macrolides, Folate pathway inhibitors, Quinolones, Penicillins	E-SXT-CIP-P	1 (1.6)	0.2
	SA11	Lincosamides, Phenicols, Aminoglycosides, Quinolones, Penicillins	CD-C-GM-CIP-P	1 (1.6)	0.3
	SA12	Lincosamides, Folate pathway inhibitors, Cephems, Penicillins	CD-SXT-FOX-P	1 (1.6)	0.2
	SA13	Macrolides, Aminoglycosides, Folate pathway inhibitors, Quinolones, Penicillins	E-GM-FOX-CIP-P	1 (1.6)	0.3
	SA14	Lincosamides, Macrolides, Aminoglycosides, Folate pathway inhibitors, Penicillins	CD-E-GM-SXT-P	2 (3.3)	0.3
	SA15	Lincosamides, Macrolides, Folate pathway inhibitors, Cephems, Penicillins	CD-E-SXT-FOX-P	1 (1.6)	0.3
	SA16	Aminoglycosides, Folate pathway inhibitors, Quinolones, Glycylcycline, Penicillins	GM-SXT-CIP-TGC-P	1 (1.6)	0.3
	SA17	Macrolides, Aminoglycosides, Folate pathway inhibitors, Quinolones, Penicillins	E-GM-SXT-CIP-P	1 (1.6)	0.3
XDR	SA18	Macrolides, Phenicols, Aminoglycosides, Tetracyclines, Folate pathway inhibitors, Cephems, Ansamycins, Quinolones, Penicillins	E-C-GM-DOX-SXT-FOX-RA-CIP-P	1 (1.6)	0.6
	SA19	Lincosamides, Macrolides, Phenicols, Aminoglycosides, Folate pathway inhibitors, Cephems, Ansamycins, Quinolones, Penicillins	CD-E-C-GM-SXT-FOX-RA-CIP-P	1 (1.6)	0.6

Clindamycin, CD; Erythromycin, E; Chloramphenicol, C; Gentamicin, GM; Daptomycin, DPT; Doxycycline, DOX; Trimethoprim-sulfamethoxazole, SXT; Cefoxitin, FOX; Rifampin, RA; Ciprofloxacin, CIP; Tigecycline, TGC; Penicillin, P.

%), and 4 (14.3 %) of the nonforming isolates were detected, respectively. This suggests a statistically significant difference in the prevalence of these genes concerning biofilm formation (p-value ≤ 0.05). Conversely, there was no statistically significant difference (p-value > 0.05) in the occurrence of other adhesion-related genes across the various categories of biofilm-producing isolates. The least frequent gene was *fnbB* (39.9 %, n = 14), the occurrence of which included 7 (38.9 %) weak biofilm producers, 5 (55.5 %) moderate biofilm producers, and 2 (40 %) strong biofilm producers. The detailed prevalence of each gene in biofilm-forming and nonforming

Table 4
Association of biofilm formation with antibiotic resistance in MRSA and MSSA strains.

Antimicrobials	MRSA (n = 29)		MSSA (n = 31)		*p*-value
	Biofilm Positive n (%)	Biofilm Negative n (%)	Biofilm Positive n (%)	Biofilm Negative n (%)	
Clindamycin	8 (27.6)	1 (3.4)	0 (0.0)	0 (0.0)	>0.999
Erythromycin	15 (51.7)	5 (17.2)	1 (3.2)	6 (19.3)	**0.0087***
Chloramphenicol	5 (17.2)	0 (0.0)	0 (0.0)	0 (0.0)	>0.999
Gentamicin	18 (62.0)	3 (10.3)	0 (0.0)	5 (16.1)	**0.0009***
Daptomycin	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	>0.999
Doxycycline	1 (3.4)	0 (0.0)	0 (0.0)	0 (0.0)	>0.999
Trimethoprim-sulfamethoxazole	18 (62)	13 (44.8)	9 (29.0)	7 (22.6)	>0.999
Rifampin	2 (6.9)	0 (0.0)	0 (0.0)	0 (0.0)	>0.999
Ciprofloxacin	10 (34.5)	2 (6.9)	0 (0.0)	0 (0.0)	>0.999
Tigecycline	1 (3.4)	0 (0.0)	0 (0.0)	0 (0.0)	>0.999
Penicillin	22 (75.8)	7 (24.1)	10 (32.2)	21 (67.7)	**0.0009***
Linezolid	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	>0.999

Table 5
Correlation between biofilm formation and distribution of biofilm-associated genes.

Adhesion Genes	Biofilm positive (n = 32)				Biofilm negative (n = 28)	Total (n = 60) n (%)	*P*-value
	Weak (18) n (%)	Moderate (9) n (%)	Strong (5) n (%)	Total n (%)			
IcaA	12 (66.7)	7 (77.8)	5 (100)	24 (75.0)	22 (78.6)	46 (76.7)	0.744
IcaD	12 (66.7)	7 (77.8)	5 (100)	24 (75.0)	18 (64.3)	42 (70.0)	0.366
fnbA	10 (55.5)	6 (66.7)	4 (80.0)	20 (62.5)	12 (42.8)	32 (53.3)	0.128
fnbB	7 (38.9)	5 (55.5)	2 (40.0)	14 (43.7)	8 (28.6)	22 (36.7)	0.224
clfA	9 (50.0)	7 (77.8)	4 (80.0)	20 (62.5)	19 (67.8)	39 (65.0)	0.667
clfB	11 (61.1)	8 (88.9)	3 (60.0)	22 (68.7)	9 (32.1)	31 (51.7)	**0.005***
cna	7 (38.9)	9 (100)	5 (100)	21 (65.6)	5 (17.8)	26 (43.3)	**<0.001***
bap	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	>0.999
fib	14 (83.3)	8 (88.9)	5 (100)	27 (84.4)	4 (14.3)	31 (51.7)	**<0.001***

*Chi-square test or Fisher's Exact Test; $P < 0.05$ is statistically significant.

strains is summarized in Table 5. The *IcaD* gene was predominant present in 26 (83.8 %) MSSA strains and 25 (86.2 %) MRSA strains. The frequency rates of other studied genes in MRSA were slightly higher than those in MSSA strains: *icaA* (75.8 %, 64.5 %); *fnbA* (62.0 %, 45.1 %); *fnbB* (51.7 %, 19.3 %); *clfA* (79.3 %, 54.8 %); *clfB* (65.5 %, 19.3 %); *cna* (55.1 %, 19.3 %); and *fib* (65.5 %, 16.1 %) (Fig. 2).

4. Discussion

S. aureus biofilm production is a key factor in pathogenesis, protects against the immune system and antibiotics, and is associated with chronic or persistent infectious diseases such as septicemia, endocarditis, and osteomyelitis [6]. Various studies have reported different rates of biofilm formation, ranging from less than 50 % to more than 70 % [28,29]. In our study, the prevalence of biofilm formation was found to be 53.3 %, which falls within this range. However, our findings were significantly lower than those of previous reports from Morocco [30], Iraq [31], and India [32], where all strains were found to be biofilm formers. According to a study conducted in Nepal [4], 77.8 % of *S. aureus* strains were identified as biofilm producers, indicating a higher prevalence than the results observed in our study. The development of biofilms depends on various environmental conditions, such as temperature, pH, oxygen

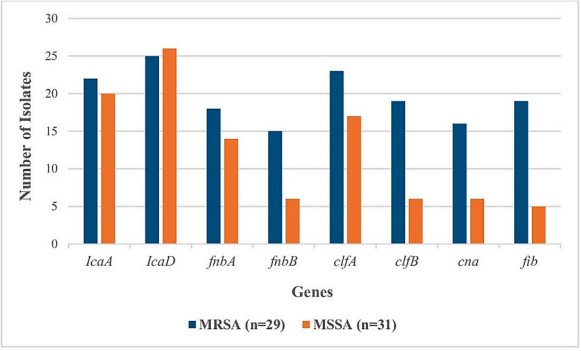


Fig. 2. Distribution of biofilm-associated genes in MRSA and MSSA strains.

concentration, and nutrient level [12]. These factors may have affected the data and led to the lower prevalence observed in this study, although their specific role is not yet understood.

The virulence of *S. aureus* is closely associated with its ability to adhere to surfaces, and the adherence of biofilm producers can be classified as strong, moderate, or weak [33]. In our study, most (56.2 %) strains were weak biofilm producers, whereas only 15.6 % were highly virulent and strongly adhered. Our results align with those of Kadkhoda et al.'s study, which reported that 48.2 % of *S. aureus* strains are weak biofilm producers [34]. Antimicrobial resistance is more prevalent among *S. aureus* biofilm producers than among nonproducers. High concentrations of antimicrobials may be needed to eradicate biofilm producers, but this approach could be impractical in vivo because of toxicity and side effects [35,36]. It might be possible to eradicate biofilm-associated staphylococcal infections, including MRSA, with combination therapies at low concentrations. However, early detection of biofilm-producing strains and antimicrobial susceptibility tests are essential for selecting the appropriate antimicrobial agent [37].

In our study, the biofilm-producing strains presented higher resistance rates to all the tested antibiotics than did the nonproducer strains. However, the resistance rates between the two groups were significantly different only for clindamycin ($P = 0.029$), gentamicin ($P = 0.031$), cefoxitin ($P = 0.039$), and ciprofloxacin ($P = 0.02$). A relatively high resistance rate to the aforementioned antibiotics in biofilm-producing *S. aureus* strains has been reported previously [28]. These findings align with previous studies conducted by Banerjee et al. [38], Boles et al. [39], and Nourbakhsh et al. [40]. The present findings suggest that biofilm development might be a key contributing factor to the emergence of resistance to commonly prescribed antibiotics. Additional studies must be performed to confirm this finding. Gene knockout experiments and expression analyses might be performed for verification. Interestingly, none of the strains tested were resistant to daptomycin or linezolid, both of which are newer antimicrobials commonly used to treat staphylococcal infections. These findings are encouraging and suggest that these antimicrobial agents may effectively treat staphylococcal infections caused by biofilm-forming and nonbiofilm-forming strains. The lower resistance rate to daptomycin and linezolid may be attributed to their unique mechanism of action, which eliminates cross-resistance between these antimicrobials and other antibiotics [1].

The increasing multidrug resistance of *S. aureus* to most antimicrobials for treating various infections has become a significant concern. To address this challenge effectively, close monitoring of the antimicrobial resistance pattern of this bacterium is crucial to successfully manage infections [41,42]. Our findings revealed that 62.5 % of the biofilm-producing *S. aureus* strains were MDR, which was a statistically significant difference from the nonproducer strains ($P < 0.05$). This finding is consistent with previous reports from Morocco (55 %) [21] and Nepal (86.7 %) [28]. Agarwal and Jain reported that biofilm-forming *S. aureus* strains were more likely to be MDR, regardless of their source [43]. These findings underscore the potential role of biofilm formation in the spread of drug-resistant strains of *S. aureus.* The predominant mechanism of antibiotic resistance in *S. aureus* involves the acquisition of resistance genes, such as the *mecA* gene, which imparts methicillin resistance. Other mechanisms include enzymatic modifications leading to aminoglycoside resistance, mutations or efflux pumps causing fluoroquinolone resistance, and the formation of biofilms that decrease antibiotic efficacy [11,44].

The incidence of MRSA has increased alarmingly over the past decade. MRSA strains are considered MDR organisms that can cause community- and hospital-acquired infections. The resistance of MRSA to commonly prescribed antibiotics poses a significant challenge to healthcare providers. In our study, almost half of the strains (48.3 %) were MRSA, which is higher than that reported in other studies performed in Iran. According to a systematic review and meta-analysis conducted in Iran, the prevalence rate of MRSA was reported to be 43 % [45], whereas Poland reported the highest prevalence rate of 56.1 % [46]. In line with previous research conducted by Aniba et al. [21] and Dash et al. [32], our study indicated that most MRSA strains were biofilm formers. In this regard, we observed a significant association between methicillin resistance and the formation of biofilms. Numerous other studies from Iran [47], China [48], India [49], and Korea [50] reported a similarly high prevalence of biofilm formation among MRSA isolates.

The presence of nine selected genes associated with biofilm production was assessed to enhance our understanding of the molecular process of biofilm production by *S. aureus* strains. Several studies have shown a correlation between the *ica* operon and biofilm formation [40,48,51]. According to our data, the majority of strains, including biofilm and nonbiofilm producers, were found to harbor the *icaD* and *icaA* genes, which was in agreement with the findings of Goudarzi et al. [52]. Interestingly, all strong biofilm-forming strains in our study contained both the *icaA* and *icaD* genes. Fowler et al. reported similar findings, noting that all *S. aureus* biofilm-forming strains possessed the *icaD* and *icaA* genes [53]. The reported frequency of *ica* genes has varied considerably across studies. Several researchers, including Azmi et al. [7], have demonstrated that *S. aureus* strains with the ability to form biofilms contain both the *icaD* and *icaA* genes. In contrast, Nasr et al. [54] reported that 46 % of staphylococcal clinical strains were biofilm former strains, with only 17 % carrying the *icaA* or *icaD* genes. Although the genes described above were found in staphylococcal strains, no significant correlation was detected between their presence and biofilm formation in vitro. Additionally, some biofilm producers do not possess *icaA/icaD* genes. Similarly, Fitzpatrick et al. reported that biofilm formation in clinical strains of *S. aureus* occurred independently of the presence of *icaADBC* genes [55]. Considering the aforementioned findings, it can be proposed that factors other than the *ica* operon also play a significant role in biofilm formation. The environmental conditions, surface adhesion characteristics, and genetic content of bacteria are among the factors to be considered [56,57].

In the present study, we also detected MSCRAMM genes, which play crucial roles in biofilm formation by *S. aureus* strains [10]. The results revealed that the most prevalent adhesion genes among the biofilm-producing strains were *fib* (84.4 %), *clfB* (68.7 %), and *cna* (65.6 %). There was a significant difference between biofilm-positive and biofilm-negative strains regarding the presence of the *fib*, *clfB*, and *can* genes. Pourzal et al. [58] reported a high prevalence of the *fib* and *clfB* genes in clinical strains of *S. aureus*, which is consistent with the findings of our study. Moreover, Mir et al. [59] reported that the frequencies of the *fib*, *clfB*, and the *cna* genes were 71.8 %, 70 %, and 59.2 %, respectively. In contrast, Serray et al. [30] reported a lower prevalence rate of the *clfA/clfB* (43.39 %), *cna* (11.32 %), and *fib* (5.6 %) genes. The role of *cna* adhesin in the pathogenesis of staphylococcal infections is well-documented as an

essential virulence determinant [30]. In line with our findings, Goudarzi et al. [56] reported that the *cna* gene was prevalent in 64 % of *S. aureus* strains isolated from patients with urinary tract infections. In another study conducted in Iran on 123 MRSA clinical strains, Motamedi et al. [60] reported a *cna* prevalence of only 4.8 %, which was lower than our study's reported frequency.

Both the *fnbA* and *fnbB* genes, which are associated with bacterial invasion, adhesion, and biofilm production [30], were present in considerable percentages in this study. However, no significant difference was found in the distribution and biofilm formation ability of these genes. Various studies have reported different frequencies of the *fnbA* and *fnbB* genes in *S. aureus*, as reported for other genes involved in adhesion. Sharma et al. [61] reported 97 % and 80 % frequencies for the *fnbA* and *fnbB* encoding genes, respectively, whereas Mohammadi et al. [62] reported a lower frequency of these genes. Discrepancies in the occurrence of MSCRAMMs and *icaADBC* genes across various studies may stem from epidemiological differences and the collection periods of the isolates. Methodological differences may also contribute to the observed variation, including the use of different primer pairs to amplify different locus regions.

4.1. Strengths and limitations

In this study, the distribution of biofilm-associated genes was investigated among clinical *S. aureus* strains in Abadan, southwest Iran. Additionally, we included the MRSA strains for phenotypic and genotypic biofilm formation experiments. However, this study has several limitations, including a small sample size and a lack of investigations of clonal relatedness via concise methods such as multilocus sequence typing (MLST).

5. Conclusions

Our study revealed that daptomycin and linezolid are the most effective antibiotics and can be used for the treatment of MDR strains in the studied region. Additionally, the predominant adhesin-associated gene was *icaA*. On the basis of the high resistance rate and presence of biofilm formation genes in circulating MRSA strains in the studied region, effective prevention and management strategies should consider genetic factors and biofilm formation to control the distribution of resistant strains and improve treatment outcomes.

CRediT authorship contribution statement

Nabi Jomehzadeh: Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Software, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Sogol Seif Emrani:** Writing – original draft, Methodology.

Ethics approval and consent to participate

The research was approved by the local research ethics committee of the Abadan University of Medical Sciences (IR.ABADANUMS.REC.1400.066), Abadan, Iran. Verbal or informed consent was unnecessary as the study solely concentrated on the microorganisms obtained from the clinical specimens.

Consent for publication

Not Applicable.

Data availability statement

All data would be fully available without restriction as per request via the corresponding author's email.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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