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Contribution of *icaADBC* genes in biofilm production ability of *Staphylococcus aureus* clinical isolates collected from hospitalized patients at a burn center in North of Iran

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Abstract

Introduction The pathogenicity of *Staphylococcus aureus* is significantly attributed to its capacity to produce biofilms, which bolster bacterial resistance against antibiotics and host immune responses. This study aimed to explore the involvement of *icaABCD* genes in biofilm formation ability of *S. aureus* clinical isolates.

Materials and methods One hundred clinical *S. aureus* isolates were collected from hospitalized patients at a burn center in North of Iran. The isolates were identified using standard biochemical tests and confirmed by the presence of the *nuc* gene. Antibiotic susceptibility profiles were determined through the disk agar diffusion method. Biofilm formation capacity was determined using microtiter plate assay. PCR test was conducted to detect the presence of *icaABCD* genes.

Results Penicillin exhibited the highest resistance rate (94%), while vancomycin was most effective antibiotic with 6% resistance. Besides, 32% of the isolates demonstrated as multidrug resistant (MDR) and 29% were Methicillin-resistant *S. aureus* (MRSA). Notably, 89% of the isolates were identified as biofilm produces, while 54 (60.67%), 28 (31.46%), and 7 (7.86%) isolates exhibited strong, moderate, and weakly biofilm production ability, respectively. PCR results revealed a prevalence of 90%, 92%, 92%, and 94% for the *icaA*, *icaB*, *icaC*, and *icaD* genes, respectively. Intriguingly, the MDR isolates exhibited a 100% prevalence of these genes. Similarly, 96.55%, 89.65%, 89.65% and 96.55% of the MRSA isolates were carrying the *icaA*, *icaB*, *icaC*, and *icaD* genes, respectively.

Conclusion This study revealed a noteworthy prevalence of biofilm-producing strains of *S. aureus*. High prevalence of *icaADBC* genes as well as highlighted capacity of the biofilm formation in MRSA and MDR strains exhibited a potential correlation between biofilm and antibiotic resistance patterns. Given the enhanced resilience of bacteria within biofilms against antibiotics, addressing biofilm production is imperative alongside antibiotic treatments for effective control and eradication of infections.

Keywords *Staphylococcus aureus*, Biofilm, *IcaA*, *IcaB*, *IcaC*, *IcaD*, *IcaADBC*

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Introduction

Staphylococcus aureus, as a gram-positive coccus, is one of the most widespread and significant bacterial pathogens, and considered as a major global health threat due to its high mortality rates [1, 2]. This bacterium has the ability to form biofilm as well as to producing numerous toxins, and can cause various infections, such as bacteremia, endocarditis, pulmonary, bone, skin and soft tissue infections [3]. One of the main mechanisms contributing to the development of hospital-acquired infections caused by *S. aureus* is the ability to form biofilms [2]. Biofilm is a collection of bacterial structures enclosed by an extracellular matrix (ECM) outside the cell, composed of extracellular polymeric substances (EPS), enabling bacteria to adhere to various surfaces [4, 5]. Adhesion to surfaces provides significant advantages, such as acquiring new genetic traits and relative antibiotic resistance [6]. There is a concern regarding biofilm formation as it can lead to the spread of infections caused by these bacteria, resulting in increased mortality, prolonged hospitalization, and higher treatment costs [6]. This issue presents significant challenges to physicians in treating infections caused by *S. aureus* [6]. So, the presence of genes involved in biofilm production is considered as one of the important factors of pathogenesis, and their disquisition is veritably important [7].

The polysaccharide intercellular adhesion molecule (PIA) that plays a significant role in the adhesion and aggregation steps, is an important ECM in the formation of *S. aureus* biofilms [8]. The genes involved in the cellular aggregation and biofilm formation of *S. aureus* include the *fib*, *fnbA*, *fnbB*, *eno*, *icaADBC*, *sasG* & *C*, and *pls* genes, as well as the *agr* system [9]. The biofilm production in *S. aureus* is mediated by the activity of *icaADBC* operon, which is the major factor in the formation of the extracellular polysaccharide matrix [10]. In this system, the *icaA* gene is responsible for the production of the N-acetylamino-glucosamine transferase enzyme, and the *icaD* gene is responsible for the production of a chaperone protein for correct folding of the *icaA*. The *icaC* gene product is also involved in the transport of PIA to the cell surface, while the *icaB* gene product is responsible for the deacetylation of mature PIA resulting in its cell surface and intercellular adhesion [9]. The *icaA*, *icaB*, *icaC*, and *icaD* genes are regulated by various regulatory systems, including Staphylococcal accessory regulator (*SarA*) and *sigB* [11, 12]. In recent decades, there has been a significant increase in the emergence of multidrug resistant (MDR) and Methicillin-resistant *S. aureus* (MRSA) strains due to increased antibiotic prescription [13, 14]. Therefore, the ability to produce biofilm is considered an important factor in treatment unresponsiveness in human diseases and infections. It seems that the number of biofilm-related diseases is increasing, and understanding the developmental characteristics of biofilm and its various aspects is crucial for successful treatment. On the other hand, burns are a major global public health crisis that leads to a reduction in local and systemic immune responses, and burn wounds become a suitable place for microbial growth [15]. Microbial infections, especially those caused by MDR bacteria, including *S. aureus*, *Pseudomonas aeruginosa*, and *Acinetobacter baumannii*, are a major cause of increased mortality in burn patients [16]. Given the high prevalence of hospital infections caused by *S. aureus* and the expansion of resistance-enhancing factors, the present study aimed to investigate the biofilm-forming ability and prevalence of *icaADBC* genes in *S. aureus* clinical isolates collected from hospitalized patients in a burn center.

Materials and methods

Ethical approval statement

This study was approved by the Ethics Committee of Mazandaran University of Medical Science (MAZUMS), Iran) with the ethics number IR.MAZUMS.REC.1403.504. This study was engaged according to the Declaration of Helsinki. However, a written informed agreement form was delivered by the patients or close relatives. Also, the categorizing data of patients was kept secret.

Sample collection

In this study, 100 non-repeated clinical isolates of *S. aureus* were collected from hospitalized patients in a burn center (Zare Hospital) in Sari city, North Iran. The isolates were collected during March to December 2024. The isolates were obtained from various clinical sources, including Wound, blood, urine, Respiratory, and ascites. Subsequently, the isolates were identified using common microbiological and biochemical tests, such as gram staining, catalase, coagulase, mannitol fermentation, and DNase assay [17]. Then, the isolates were confirmed by PCR test using the thermonuclease encoding gene (*nuc*) specific primers shown in Table 1. Also, *S. aureus* ATCC 25,923 was used as a control strain for diagnostic tests.

Antimicrobial susceptibility testing (AST)

The antibiotic susceptibility pattern of the *S. aureus* isolates was determined using the disk agar diffusion (Kirby-Bauer) method. The antibiotics tested in this study included Penicillin (10 unites), Cefoxitin (30 µg), Ceftaroline (30 µg), Gentamicin (10 µg), Erythromycin (15 µg), Tetracycline (30 µg), Ciprofloxacin (5 µg), and Clindamycin (2 µg) (Roscoe, Denmark), following the Clinical and Laboratory Standards Institute (CLSI) guidelines [18]. The *S. aureus* ATCC 29,213 was used as the control strain. Additionally, the minimum inhibitory concentration (MIC) of Vancomycin was evaluated using the micro

Table 1 Sequences of primers and PCR conditions used to amplify the *nuc*, and *IcaABCD* genes

Genes	Sequences (5′ to 3′)	Denaturation temperature and time	Annealing temperature and time	Extension temperature and time	Final Extension temperature and time	Amplicon Size (bp)	Reference
nuc- F	AGCTCAGCAAATGCATCACA	95 °C for 25 s	53˚C for 30 s	72˚C for 30 s	72˚C for 5 min	400	[20]
nuc- R	TAGCCAAGCCTTGACGAACT						
icaA- F	GAGGTAAAGCCAACGCACTC	95 °C for 25 s	53˚C for 30 s	72˚C for 30 s	72˚C for 5 min	151	[21]
icaA- R	CCTGTAACCGCACCAAGTTT						
icaB- F	ATACCGGCAACTGGGTTTAT	95 °C for 25 s	52˚C for 30 s	72˚C for 30 s	72˚C for 5 min	141	
icaB- R	ATGCAAATCGTGGGTATGTGT						
icaC- F	CTTGGGTATTTGCACGCATT	95 °C for 25 s	53˚C for 30 s	72˚C for 30 s	72˚C for 5 min	209	
icaC- R	GCAATATCATGCCGACACCT						
icaD- F	ACCCAACGCTAAAATCATCG	95 °C for 25 s	52˚C for 30 s	72˚C for 30 s	72˚C for 5 min	211	
icaD- R	GCGAAAATGCCCATAGTTTC						

broth dilution method, following CLSI guidelines [18], and the *S. aureus* ATCC 29,213 was used as the standard strain in this test, too.

Phenotypic evaluation of biofilm production by clinical isolates of *S. aureus*

The ability to produce biofilm in the studied strains was investigated using the micro titer plate method [19]. Briefly, 180 µl of Trypticase Soy Broth (TSB) containing 1% glucose was added to each well of a Flat bottom 96 well micro plate. Then, 20 µl of bacterial suspension equivalent to a 0.5 McFarland standard was added to each well. To reduce errors and ensure reliable analysis, three wells were considered for each bacterial isolate. Next, the micro plates were incubated at 37 °C for 20 h. Then, the wells contents were removed and washed three times with 0.15 M phosphate-buffered saline (PBS) and the microplates were air-dried completely. Subsequently, the well contents were stained with 0.1% crystal violet for 30 min and washed three times with distilled water. Next, 200 µl of a 33% acetic acid glacial was added to the wells and their optical density (OD) were measured at a wavelength of 590 nm, using an ELISA reader (Biotech, USA) [19]. Also, three wells were used as negative control that were contained just TSB with 1% glucose, and their mean OD was considered as OD cut off (ODC). Then, the sample's ODs were compared with the ODC. The bacteria with an $OD \leq ODC$ were considered as biofilm-negative. Besides, the bacteria with an $ODC < OD \leq 2\times ODC$ were weak biofilm producers, and the bacteria with a $2\times ODC < OD \leq 4\times ODC$ were defined as moderate biofilm producers, while the bacteria with a $4\times ODC < OD$ were considered as strong biofilm-producer organisms. Also, the *S. aureus* ATCC 35,556 that is a strong biofilm-producer, was used as the positive control in this test [19].

Identification of the *nuc*, and *IcaABCD* genes by PCR

The DNA extraction process was performed using a standard DNA extraction kit (Poya Gene Azma, Iran) according to the instructions of the manufacturer. To confirm the purity and the quality of the extracted DNAs, their ODs were measured at 260 and 280 nm by a NanoDrop (Thermofisher, USA), and the DNAs were electrophoresed on a 1.5% agarose gel (SinaClon, Iran). The PCR assays were conducted using the primers listed in Table 1. The PCR reactions were carried out in a 25 µl final volume, including 12.5 µl Master Mix (Ampliqon, Denmark), 10 pmol (1 µl) of each primer (Metabion, Germany), 500 ng (2 µl) DNA template. and 8.5 µl RNase-free distilled water. The amplification of the target genes was performed under standard conditions using a thermal cycler (BioRad, USA). All reactions were performed for 35 cycles with an initial denaturation at 95˚C for 5 min. The PCR conditions for all genes are shown in Table 1. The PCR products were electrophoresed on 1.5% agarose gel (SinaClon).

Statistical analysis

The results obtained from the data of this study were analyzed using SPSS software (version 22). The quantitative data were analyzed using the Descriptive program and presented as Mean ± SD. The Crosstabs program was used to determine the percentage and number of certain parameters. Chi-Square was used to compare the number or percentage of some parameters between two groups, and a P-*value* less than 0.05 was considered statistically significant. Also, in cases where one of the samples compared was less than or equal to 9 the Fisher exact test was applied.

Results

In this descriptive-analytical study, 100 clinical *S. aureus* isolates were included based on the presence of the *nuc* gene. The mean age of patients was 42.59 ± 24.59 years, ranging from 8 months to 88 years. Besides, 50% of the participants were male. The frequency of clinical samples obtained from different hospital departments is summarized in Fig. 1. Most isolates (50%) were obtained from

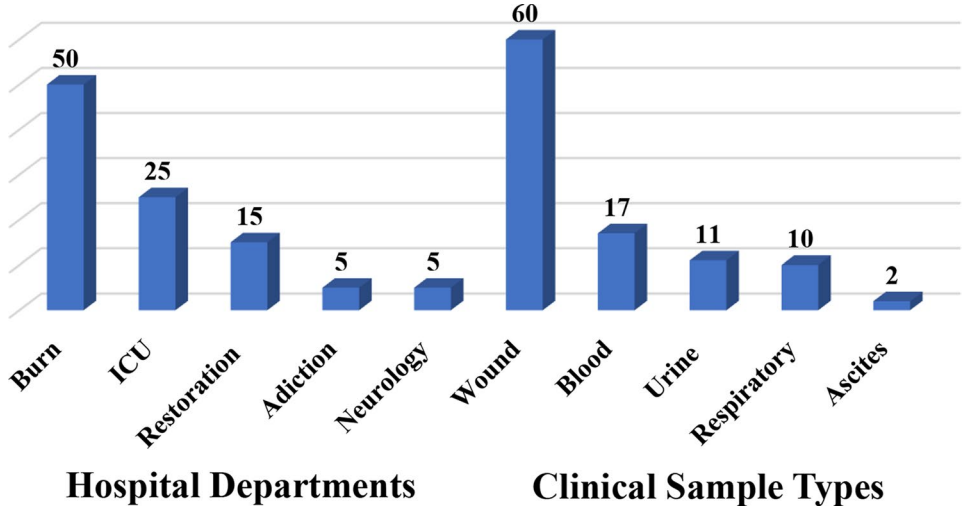

Fig. 1 Frequency of isolates collected from different hospital departments and various clinical samples. Abbreviations: ICU; Intensive Care Unit

Table 2 Antibiotic resistance pattern of 100 *S. aureus* clinical isolates

Antibiotics	Percentage of the isolates which were		
	Resistant	Intermediate Resistant	Susceptible
Cefoxitin	29	-	71
Penicillin	94	-	6
Ceftaroline	7	7	86
Ciprofloxacin	37	16	47
Gentamicin	24	9	67
Tetracycline	50	40	10
Erythromycin	42	24	34
Clindamycin	35	16	49
Vancomycin	6	-	94

the burn units, while 5% were collected from the neurology and 5% from the addiction departments. Moreover, the frequency of clinical specimen types is shown in Fig. 1. Most isolates (60%) were collected from wound culture and the lowest (2%) was from ascites fluid.

Antibiotic susceptibility pattern of *S. aureus* clinical isolates

The antibiotic resistance profile of the *S. aureus* clinical isolates is shown in Table 2. The highest bacterial resistance was observed against penicillin (94%) and the highest susceptibility was observed against vancomycin (94%). Besides, 32 isolates (32%) were defined as MDR. The MIC ranges of vancomycin against the isolates were as follows: 0.25 µg/ml for 4 isolates, 0.5 µg/ml for 11 isolates, 1 µg/ml for 42 isolates, 2 µg/ml for 37 isolates, and 16 µg/ml for 6 isolates.

Also, the antibiotic resistance pattern of the *S. aureus* isolates in different hospital wards is summarized in Table 3. A significant difference was observed between the resistance pattern of bacteria to most antibiotics tested and the inpatient wards.

Besides, the antibiotic resistance pattern of *S. aureus* clinical isolates based on the clinical samples is shown in Table 4. Except for penicillin, tetracycline, and erythromycin, no statistically significant difference was observed between the antibiotic resistance pattern of the bacteria and the type of clinical specimen.

The antibiotic resistance pattern of MDR and Non-MDR *S. aureus* isolates

The antibiotic resistance pattern of MDR and non-MDR *S. aureus* is reported in Fig. 2. Overall, MDR isolates had significantly higher resistance compared to non-MDR strains against ciprofloxacin (100% vs. 7.35%), gentamicin (75% vs. 0%), tetracycline (100% vs. 26.47%), cefoxitin (68.75% vs. 10.29%), clindamycin (87.5% vs. 10.29%), and erythromycin (100% vs. 14.7%). There was a statistically significant difference in antibiotic resistance between MDR and non-MDR isolates (P-*value* < 0.001).

The antibiotic resistance pattern of MRSA and MSSA

Overall, 29% of *S. aureus* clinical isolates were MRSA. The antibiotic resistance pattern of MRSA and methicillin-susceptible *S. aureus* (MSSA) is shown in Fig. 3. Significant differences were observed in antibiotic resistance patterns of MRSA and MSSA isolates against ciprofloxacin (82.75% vs. 18.3%), gentamicin (55.17% vs. 11.26%), tetracycline (82.75% vs. 36.61%), clindamycin (86.2% vs. 14.08%), erythromycin (93.1% vs. 21.12%), and ceftaroline (17.24% vs. 2.81%) ($p < 0.001$).

Biofilm production ability of *S. aureus* isolates

Overall, 89 isolates (89%) were capable to produce biofilms, from which biofilm production was strong in 54 isolates (60.67%), moderate in 28 isolates (31.46%), and weak in 7 isolates (86.7%). The frequency of biofilm production in MDR and non-MDR *S. aureus* clinical isolates

Table 3 Antibiotic resistance pattern of *S. aureus* isolates based on hospital wards

Antibiotics	Resistance pattern	No. (%) of isolates with different susceptibility pattern					P-value
		Burn ($n=50$)	ICU ($n=25$)	Restoration ($n=15$)	Addiction ($n=5$)	Neurology ($n=5$)	
Penicillin	NS	50 (100)	25 (100)	15 (100)	2 (40)	2 (40)	0.018
	S	-	-	-	3 (60)	3 (60)	
Cefoxitin	NS	15 (30)	9 (36)	5 (33.33)	-	-	0.189
	S	35 (70)	16 (64)	10 (66.66)	5 (100)	5 (100)	
Ceftaroline	NS	8 (16)	4 (16)	2 (13.33)	-	-	0.238
	S	42 (84)	21 (84)	13 (86.66)	5 (100)	5 (100)	
Ciprofloxacin	NS	37 (74)	13 (52)	3 (20)	-	-	0.047
	S	13 (26)	12 (48)	12 (80)	5 (100)	5 (100)	
Gentamicin	NS	20 (40)	9 (36)	4 (26.66)	-	-	0.128
	S	30 (60)	16 (64)	11 (6.66)	5 (100)	5 (100)	
Tetracycline	NS	50 (100)	25 (100)	15 (100)	-	-	0.00
	S	-	-	-	5 (100)	5 (100)	
Erythromycin	NS	43 (86)	18 (72)	7 (46.66)	-	-	0.038
	S	7 (14)	7 (28)	8 (53.33)	5 (100)	5 (100)	
Clindamycin	NS	35 (70)	13 (52)	3 (20)	-	-	0.044
	S	15 (30)	12 (48)	12 (80)	5 (100)	5 (100)	

Abbreviations: NS: Non-susceptible that means resistant and intermediate resistant; S: Susceptible

Table 4 Antibiotic resistance pattern of *S. aureus* clinical isolates in terms of clinical samples

Antibiotics	Resistance pattern	No. (%) of isolates with different susceptibility pattern					P-value
		Wound ($n=60$)	Blood ($n=17$)	Urine ($n=11$)	Respiratory ($n=10$)	Ascites ($n=2$)	
Penicillin	NS	60 (100)	15 (88.23)	9 (81.81)	2 (20)	-	0.015
	S	-	2 (11.76)	2 (18.18)	8 (80)	2 (100)	
Cefoxitin	NS	21 (35)	3 (17.64)	2 (18.18)	3 (30)	-	0.387
	S	39 (65)	14 (82.35)	9 (81.81)	7 (70)	2 (100)	
Ceftaroline	NS	11 (18.33)	2 (11.76)	-	1 (10)	-	0.568
	S	49 (81.66)	15 (88.23)	11 (100)	9 (90)	2 (100)	
Ciprofloxacin	NS	40 (66.66)	7 (41.17)	3 (27.27)	3 (30)	-	0.156
	S	20 (33.33)	10 (58.82)	8 (72.72)	7 (70)	2 (100)	
Gentamicin	NS	29 (48.33)	3 (17.64)	-	1 (10)	-	0.178
	S	31 (51.66)	14 (82.35)	11 (100)	9 (90)	2 (100)	
Tetracycline	NS	60 (100)	15 (88.23)	8 (72.72)	7 (70)	-	0.045
	S	-	2 (11.76)	3 (27.27)	3 (30)	2 (100)	
Erythromycin	NS	50 (83.33)	11 (64.70)	-	5 (50)	-	0.038
	S	10 (16.66)	6 (35.29)	11 (100)	5 (50)	2 (100)	
Clindamycin	NS	41 (68.33)	5 (29.41)	-	5 (50)	-	0.058
	S	19 (31.66)	12 (70.58)	11 (100)	5 (50)	2 (100)	

Abbreviations: NS: Non-susceptible that means resistant and intermediate resistant; S: Susceptible

is shown in Fig. 4. A significant difference was observed between MDR and non-MDR isolates in the frequency of biofilm production ($p=0.016$). Also, the frequency of biofilm production in MRSA and MSSA isolates is shown in Fig. 4. No significant difference was observed between these isolates in the frequency of biofilm production ($p=0.45$).

Prevalence of *IcaABCD* genes in *S. aureus* isolates

Based on the results of PCR test, the prevalence of *icaA*, *icaB*, *icaC*, and *icaD* genes among all isolates was found to be 90%, 92%, 92%, and 94%, respectively. The frequencies of *icaA*, *icaB*, *icaC*, and *icaD* genes in MDR and non-MDR *S. aureus* isolates are shown in Fig. 5. A significant difference was observed between MDR and non-MDR isolates in the frequencies of *icaA* ($p=0.022$), *icaB* ($p=0.043$), and *icaC* ($p=0.043$) genes, while no significant difference was detected in the frequency of *icaD* gene between two groups ($p=0.083$).

The frequency of *icaA*, *icaB*, *icaC* and *icaD* genes in MRSA and MSSA isolates is shown in Fig. 6. No significant difference was observed in the frequency of *icaA* ($p=0.16$), *icaB* ($p=0.58$), *icaC* ($p=0.58$) and *icaD* ($p=0.49$) genes between MRSA and MSSA isolates.

On the other hand, a significant difference was observed between biofilm-positive and biofilm-negative

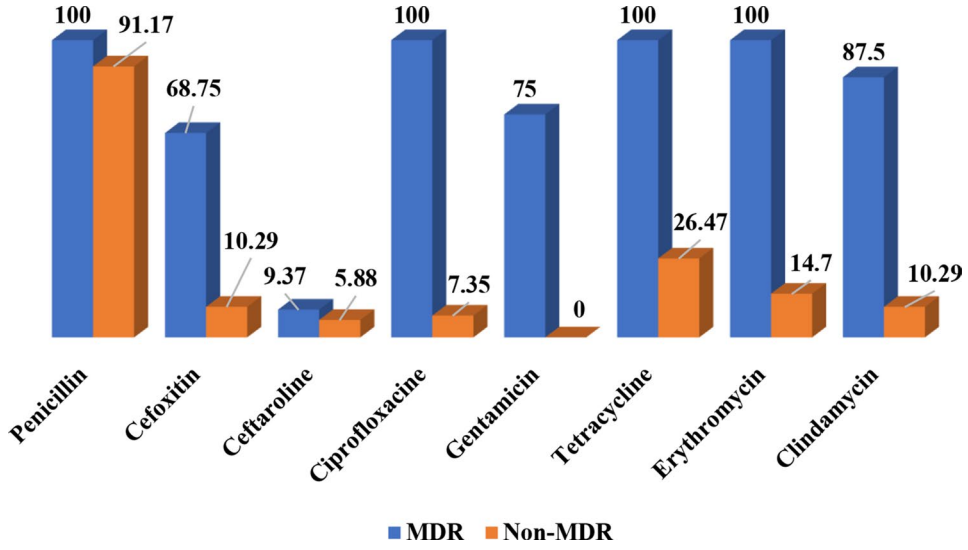

Fig. 2 Comparison of antibiotic resistance patterns of MDR and non-MDR isolates

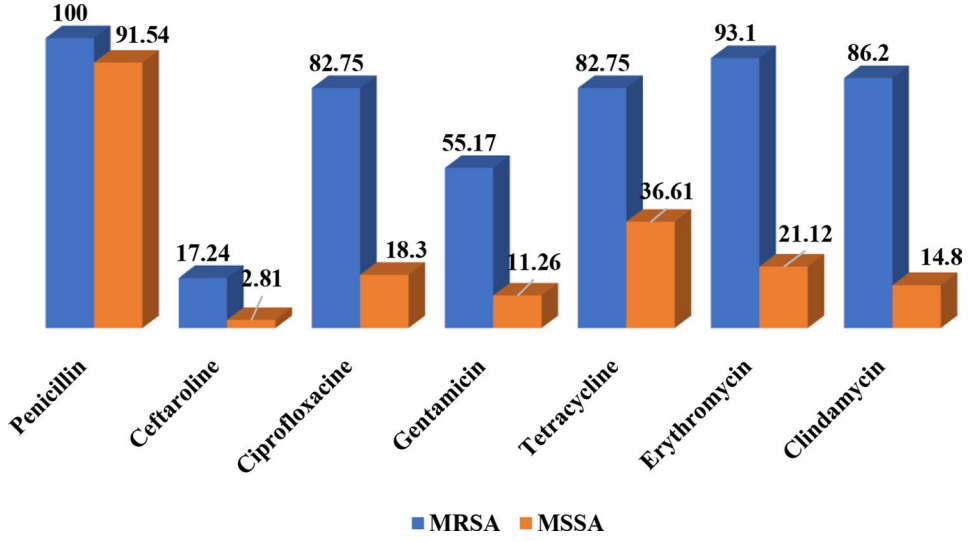

Fig. 3 Comparison of antibiotic resistance patterns of MRSA and MSSA isolates

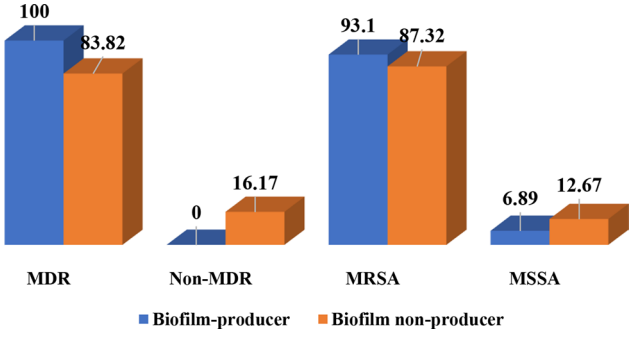

Fig. 4 Biofilm production ability in MDR, non-MDR, MRSA and MSSA isolates

isolates in the frequency of *icaA*, *icaB*, *icaC* and *icaD* genes ($p = 0.000$). Almost more than 95% of the biofilm-producing isolates had all genes. The frequencies of *icaA*, *icaB*, *icaC* and *icaD* genes in biofilm-producing *S. aureus* isolates were 95.5%, 98.87%, 98.87% and 100%, respectively, while they detected in 45.45%, 36.36%, 36.36% and 45.45% of biofilm-negative isolates, respectively.

Discussion

Due to its strong biofilm production and antibiotic resistance, *S. aureus* is a significant organism in the occurrence of hospital-acquired infections [1]. The ability to form biofilms leads to increased antibiotic resistance and mortality rates [6]. This study aimed to investigate the biofilm-forming ability and related genes, as well as the antibiotic resistance pattern of the *S. aureus* clinical

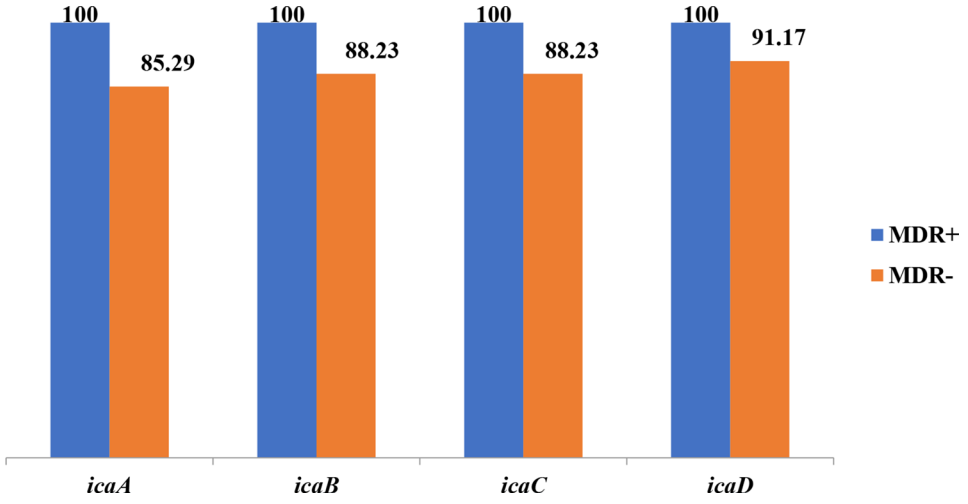

Fig. 5 Frequency of *icaA*, *icaB*, *icaC*, and *icaD* genes in MDR and non-MDR *S. aureus* isolates

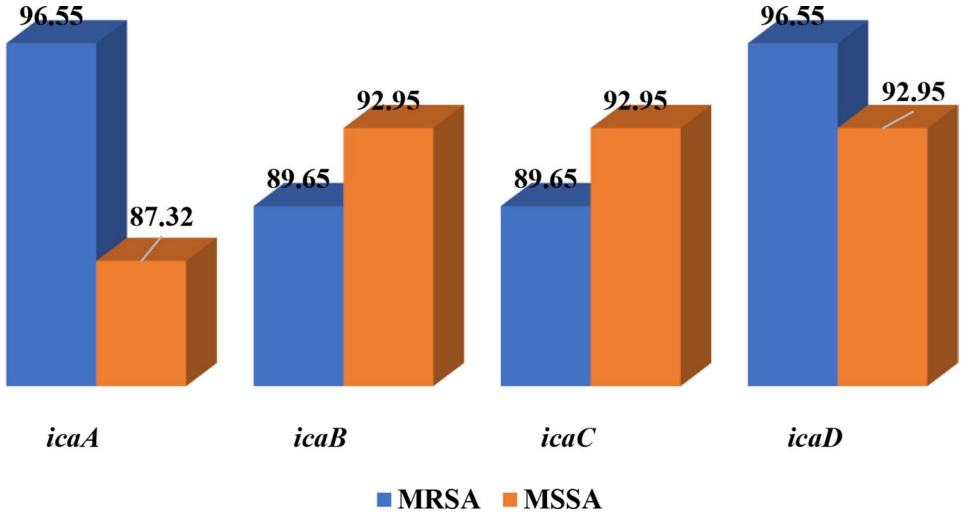

Fig. 6 Frequency of *icaA*, *icaB*, *icaC* and *icaD* genes in MRSA and MSSA isolates

isolates. The results of this study showed that the ability to form biofilm is associated with the development of antibiotic resistance, especially in MRSA and MDR isolates. Considering the formation of multilayer units in biofilm structures, the development of biofilm can be considered as a key step and indicator in the development of infection and antibiotic resistance [22]. In this study, the highest antibiotic resistance was observed to penicillin (94%), followed by tetracycline (50%) and erythromycin (42%). On the other hand, the highest sensitivity was seen against vancomycin (94%) and ceftaroline (86%). Also, 32% and 29% of the isolates were MDR and MRSA, respectively. Also, among MRSA isolates, 3 (10.34%) were detected as vancomycin-resistant *S. aureus* (VRSA), while among 32 MDR isolates, 22 (68.75%) were MRSA but no VRSA isolates were detected among the MDRs. Poli et al. (2025) indicated that 18.64% of *S. aureus* isolated from milk samples in Italy were MRSA [23], indicating differences between clinical isolates and animal origin foods. However, foods can also be a potential source to transmit these strains to humans. Umar et al. studied 14 *S. aureus* isolates in Nigeria (2024) and reported that 50% and 35.7% of the isolates were MRSA and MDR, respectively. Also, the antibiotic resistance patterns of the isolates were as follows: penicillin (100%), levofloxacin (64.3%), doxycycline (50%), tetracycline (28.6%), and erythromycin (21.4%) [24]. Their higher prevalence of MRSA and the same prevalence of MDR strains may be due to the fact that 9/14 (64.28%) isolates were obtained from burn wounds, and 60% of our isolates were from the wound samples. Abd et al. conducted a study in Iraq (2022) on 50 *S. aureus* clinical isolates and showed that all (100%) isolates were resistant to cefoxitin (MRSA), penicillin, and ceftazidime, while 88%, 46%, 50%, and 50% were resistant to vancomycin, azithromycin, erythromycin, and tetracycline, respectively [25].

This could be due to differences in antibiotic use in the two regions. Anzabi et al. (2021) conducted a study on human and animal strains and reported the 64.1% and 36.1% frequency of the *mecA* gene in human and animal isolates, respectively, indicating differences between human and animal strains [26]. Kim et al. in South Korea reported a 51% prevalence of MRSA [27], and Tyagi et al. reported a 44% prevalence of MRSA strains in India [28]. The variation in the prevalence of MRSA strains could be due to differences in the pattern of beta-lactam consumption in hospitals and community. On the other hand, studies have shown that the spread of MRSA isolates is strongly associated with geographical regions and biological patterns [29]. In a study conducted by Ghasemian et al., among 29 MRSA isolates, 56% were MDR [30]. We found a significant difference in the antibiotic resistance pattern between MDR and Non-MDR *S. aureus* as well as MRSA and MSSA isolates ($p < 0.001$), demonstrating arbitrary use of antibiotics by people, inappropriate antibiotic prescriptions, as well as long-term and improper usage of broad-spectrum antibiotics.

On the other hand, Ali et al. in Pakistan showed that 28%, 40%, and 22.7% of MRSA isolates were strong, moderate, and weak biofilm producers, respectively. They found that antibiotic resistance was more prevalent among biofilm-forming isolates, while ceftaroline was effective regardless of biofilm-forming ability [31]. These results were similar to our study, where 100% and 93.10% of our MDR and MRSA isolates were biofilm-positive, respectively. Umar et al. in Nigeria showed that all *S. aureus* isolates collected from burn wounds and skin of healthcare workers were moderate biofilm producers [24]. The variation in biofilm production ability in different studies could be attributed to the genetic characteristics and features of bacteria in different geographical regions. Also, differences in hygiene and infection control practices in hospitals can affect the prevalence of biofilm production, because by properly disinfecting hospital surfaces with appropriate disinfectants, the formation of biofilm by bacteria on non-living surfaces can be prevented [32]. The present study is consistent with most studies showing the high ability of *S. aureus* to produce biofilms. This factor can cause the bacteria to escape from the host immune system, make it more difficult for drugs penetration, increase bacterial survival, and consequently increase its pathogenicity [22]. In our study, there was no significant difference in the prevalence of biofilm production between MRSA and MSSA strains ($p = 0.45$), but the MRSA strains demonstrated a slightly higher ability (93% vs. 87%) to form biofilms. In a study in western China, Wu et al. showed that MRSA increases biofilm structure and adhesion ability [33]. This phenomenon might be due to an increased chance of transferring drug resistance genes within the biofilm structure and the role of the *mecA* gene [34]. Furthermore, a significant difference in the prevalence of biofilm production was observed between MDR and Non-MDR *S. aureus* isolates in our study ($p = 0.016$). On the other hand, 75.43% of MDR isolates in our study were strong biofilm producers. Bacteria in biofilms can spread antibiotic resistance in different parts of healthcare facilities through various mechanisms. This phenomenon can also be a serious threat to the care of patients hospitalized in healthcare settings [33].

Biofilm production in *S. aureus* is mediated by intercellular adhesive polysaccharide (PIA), which is produced by the intercellular adhesion proteins, including IcaA, IcaB, IcaC, and IcaD [35]. Molecular analysis in this study demonstrated that the prevalence of the *icaA*, *icaB*, *icaC*, and *icaD* genes was 90%, 92%, 92%, and 94%, respectively. The frequencies of *icaA*, *icaB*, *icaC* and *icaD* genes in biofilm-producing strains were 95.5%, 98.87%, 98.87% and 100%, respectively. It is obvious that the presence of the mentioned genes is significantly associated with biofilm production. Many reports have been published on the existence of this association in *S. aureus* isolates. In a study in Portugal, Silva et al. investigated the biofilm formation of multidrug-resistant MRSA strains isolated from bacteremia, osteomyelitis, and diabetic foot ulcers [36]. The average biofilm formation for all isolates of bacteremia, diabetic foot infection and osteomyelitis was 80.5%, 77.6% and 58.3%, respectively. Besides, *icaA* was detected in 85%, 77.4% and 53.7%, *icaD* in 89.1%, 74.1% and 59.65%, *icaB* in 73%, 64.1% and 44.5%, and *icaC* in 75.5%, 68% and 47.8% of bacteremia, diabetic foot and osteomyelitis isolates, respectively [36]. These results show the significant role of biofilm formation ability in bacteremia and wound infection. Piechota et al. also showed that 99.2% of their isolates were biofilm producers, while 39.7% and 36.8% of MRSA and MSSA isolates were strong biofilm producers [37]. Also, 66.7% of sputum and tracheostomy tube isolates, 50% of nasal and catheter isolates, 44.4% of throat isolates, and 43.8% of bronchoalveolar lavage isolates were strong biofilm producers, while fecal isolates had much lower biofilm-forming capacity [37]. Besides, MRSA isolates had a higher biofilm-forming capacity than MSSA strains, and isolates with *icaABCD* and *icaABD* produced significantly more biofilm than strains with *icaAD* [37]. Biofilm formation by both MRSA and MSSA strains indicates the high ability of these strains to persist in the hospital environment, which increases the risk of disease in hospitalized patients. Poli et al. showed that all MRSA isolates collected from milk samples had the *icaA*, *icaB*, *icaC*, and *icaD* genes, indicating the potential of these strains to form biofilms [23]. Ali et al. studied a total of 150 MRSA isolates in Pakistan and showed that the *icaA* and *icaD* genes were detected in 85.3% and 86.7% of the

isolates, respectively [31]. Umar et al. showed that 14.7% and 28.64% of the *S. aureus* isolates carried the *icaB* and *icaD* genes, respectively [24]. Capri et al. investigated 18 coagulase-negative staphylococci isolated from the milk of sheep with subclinical mastitis and identified the *ica* genes in 27.7% of the isolates, indicating that the coagulase-negative staphylococci can acquire virulence genes as pathogens in subclinical mastitis [38]. Abd et al. in Iraq showed that all 50 isolates were MRSA and all carried *icaABCD* genes [25]. Ghaioumy et al. in Iran observed that all *S. aureus* isolates collected from adenoid samples of patients under 15 years-old were biofilm-positive, while 6.3% and 59.4% of the isolates had *icaA* and *icaD* genes, respectively, and all were *icaC*- and *icaB*-negative [39]. Anzabi et al. by assessing 39 human and 35 animal *S. aureus* isolates showed that 64.1%, 64.1%, 30.8%, and 64.1% of human isolates and 36.8%, 31.6%, 26.3%, and 36.8% of animal isolates carried *icaA*, *icaB*, *icaC*, and *icaD* genes, respectively, and there was a significant relationship between *mecA* and *icaAD* genes in human isolates [26]. Azmi et al. in Palestine showed that 21%, 46.4%, 32.6% of *S. aureus* isolates were strong, moderate, and weak biofilm producers. Also, all isolates contained *icaA* and *icaD* genes, and 26.6% were MDR [40].

Previous studies had demonstrated that the presence of the icaADBC operon alone might not necessarily indicate biofilm production, and other factors are also significant [41]. Similarly, despite the presence of the *ica* genes, biofilm formation did not occur in some isolates of our study, as well as the study conducted by Dadgar et al. that showed no biofilm formation ability in some *icaA*- and *icaD*-positive isolates [42]. The phenotypic variations may be due to deletions or insertions in *ica* operon [43]. Bi et al. reported that the silencing of the *icaA* and *icaB* genes in *S. aureus* ATCC 25,923 reduces the sensitivity to linezolid, decrease in biofilm formation, and alterations in the surface structures [44]. Overall, these findings highlight the potential efficacy of targeting Ica proteins as a strategy for reducing biofilm formation in *S. aureus*. However, further research is needed to validate the practical applications of this approach in preventing biofilm-related infections.

Conclusions

The emergence of strains with high biofilm formation capacity in hospital environments is a serious health threat, especially for immunocompromised patients and patients connected to artificial devices. The results of this study showed that there is higher antibiotic resistance and biofilm formation ability in MRSA and MDR strains, indicating the significant role of antibiotic consumption patterns in each region. Bacteria in biofilms are able to spread drug resistance genes in hospitals and emerging challenges in antimicrobial treatments. On the other hand, the strong presence of *icaABCD* operon genes in these isolates indicates that the Ica proteins may be suitable targets for future research on how to control the pathogenicity of this bacteria in hospitals.

Limitations

The limitation of this study was no investigation of the expression levels of the *icaABCD* genes. Another limitation of this study was that we did not investigate other virulence factors related to biofilm production.

Abbreviations

MDR	Multidrug Resistant
MRSA	Methicillin-resistant *S. aureus*
PCR	Polymerase Chain Reaction
ATCC	American Type Culture Collection
CLSI	Clinical and Laboratory Standards Institute
TSB	Trypticase Soy Broth
PBS	Phosphate-buffered Saline
OD	Optical Density
ELISA	Enzyme-linked Immunosorbent Assay
ODC	OD Cut Off
DNA	Deoxyribonucleic Acid
ICU	Intensive Care Unit
MSSA	Methicillin-susceptible *S. aureus*

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Author contributions

Conceptualization: HRG; Data curation: HRG, AB, AF, HJS; Formal analysis: MA, PNG, HJS; Investigation: HRG, MA, AB, AF; Methodology: HRG, MA, AB; Project administration: HRG; Software: HRG, AF, PNG; Supervision: HRG; Validation: HRG; Visualization: HRG, MA, AB, PNG; Writing - original draft: MA; Writing - review & editing: HRG, MA, AB, AF, PNG, HJS.

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Data availability

All data generated or analyzed during this study are included in this published article.

Declarations

Ethics approval

This study was approved by the Ethics Committee of Mazandaran University of Medical Science (MAZUMS), Iran) with the ethics number IR.MAZUMS.REC.1403.504. This study was engaged according to the Declaration of Helsinki. However, a written informed agreement form was delivered by the patients or close relatives. Also, the categorizing data of patients was kept secret.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

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