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ORIGINAL ARTICLE

Relationship between biofilm gene expression with antimicrobial resistance pattern and clinical specimen type based on sequence types (STs) of methicillin-resistant *S. aureus*

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Abstract

The *ica* genes in methicillin-resistant *Staphylococcus aureus* (MRSA) play an important role in biofilm formation. The aim of this study is to define effect of antibiotic resistance and clinical specimens to the expression of *ica* genes based on their sequence types (STs) and clonal complex (CC). One-hundred (100) *S. aureus* strain were collected from two teaching therapeutic centers in Hamedan, Iran. Then, the PCR, qPCR, and MLST were used to characterize strains. The results indicated that 29 (29%), 15 (15%), and 5 (5%) strain were strong, mediate, weak biofilm producer, respectively, and the *icaA* (17%) and *icaC* (14%) genes were the most abundant. However, two unique STs (3667, 491) in Iran were reported and ST30 and ST11 were the most abundant STs and CC30 and CC5 were observed among MRSA and MSSA strains. High activity in *ica* locus was observed among strains collected from wound and catheter strains. Also, expression level of *icaA* gene increased in all strains except ST30 and ST491. Moreover, the highest expression level was observed in CC1, CC7, and CC11. Likewise, activity of the *icaC* gene was only observed in CC5. Furthermore, the expression of all ica genes in CC5 was significantly correlated with the type of biofilm and the clinical sample. In this study demonstrated that the frequency distribution of STs and CCs in different strains of MRSA was higher than methicillin-sensitive strains. Also, the type of clinical specimen and expression of ica genes played an important role in this abundance.

Keywords Methicillin resistant *S. aureus* · Virulence factors · Biofilm · Antibiotic resistance · Gene expression

Introduction

Antimicrobial resistance in *Staphylococcus aureus* was first identified in the 1940s with the isolation of penicillin-resistant strains [1]. One year after introducing Methicillin- a synthetic derivative of penicillin- in 1959, first strains of methicillin-resistant *S. aureus* (MRSA) have been emerged [2]. Nowadays, MRSA remains a global health care issue empowered through acquiring resistance against multiple classes of antibiotics [3]. Living in a biofilm gives the bacteria the advantage of a better adaptation to environmental factors and increased resistance to hostile conditions [4]. It is becoming increasingly more difficult to treat bacteria with antimicrobial agents due to an increase in the resistance to antimicrobial compounds which occurs either through the spread of resistance genes, generalized stress response mechanisms, or by the formation of a biofilm [5, 6].

Biofilms can provide protection in a number of different ways. The exopolysaccharide present in the biofilm can act as a physical barrier inhibiting the entry of antimicrobial agents and antibodies into the biofilms [7]. There are two major ways in which biofilm forms, one relies on the *ica* operon and poly-*N*-acetyl-β-(1–6)-glucosamine (PNAG) production, while the second is *ica* independent. The *ica*ADBC encodes four genes including *icaA, icaB, icaC*, and *icaD* [8]. *icaA* and *icaD*, which collectively produce PIA, facilitate the cells binding together and forming into biofilms. The majority of *S.aureus* strains contain the *icaADBC* operon which is upregulated under in vivo conditions [9]. The process of *S. aureus* biofilm formation is controlled by quorum sensing which is a system used by bacteria for cell–cell

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communication to regulate gene expression in response to the cell density. The staphylococcal accessory gene regulator (*agr*) system plays an important role in QS [10], by activating some PIA (polysaccharide intercellular adhesin) dependent surface factors, the system can increase the pathogenicity of *S. aureus*. In several studies, this system plays the role of downregulation in bacterial colonization and upregulation in host disease [9]. The downregulation and upregulation of the genes involved in the described processes promote the establishment and development of MRSA infections. In addition, these genes play an important role in MRSA biofilm formation which, in turn, leads to a more aggressive infection giving the patient a poor prognosis [11, 12].

Staphylococcus aureus lineages are commonly described by their clonal complex (CC) or sequence type (ST), as determined by molecular typing method. Molecular epidemiology analyses of globally derived *S. aureus* strain indicate the most prevalent lineages in CC1, CC5, CC8, CC9, CC12, CC15, CC22, CC25, CC30, CC45 and CC51 [1]. Although all of the described lineages are common among MSSA strain, their distribution is more limited among the MRSA clones [13].

Hence, the aim of this study is to define effect of antibiotic resistance and clinical specimens to the expression of *ica* genes based on their sequence types (STs) and clonal complex (CC). Also, for investigate the relationship between different variables, STs and CCs were identified in different strains of *S. aureus* based on MLST typing.

Material and methods

Study design and sampling

In this cross-sectional study, 100 strain of *S. aureus* collected from 492 different clinical samples (including blood, urine, wound infection and catheter) of Hamadan's Hospitals, Hamadan, Iran between Jun 2017 and Oct 2018 (In this case, the patient was not directly sampled and the strain were collected from the hospital laboratory). Sample size was selected using a simple random sampling technique based on inclusion and exclusion criteria [14]. All strain were transferred to the Microbiology Laboratory of Hamedan University of Medical Sciences for differential tests.

Detection of *S. aureus*

Conventional identification of *S. aureus* from human infection is based on microbial culture of clinical samples, including examination of colony morphology and hemolysis type on blood agar after incubation for 18-24 h at 37 °C, Gram stain morphology, catalase reaction, and coagulase reaction. All samples were inoculated onto 5% sheep blood agar base (Merck, Darmstadt, Germany) and mannitol salt agar (Merck, Darmstadt, Germany) and incubated for 18–24 h at 37 °C and finally confirmed as *S. aureus* on DNase agar (Merck, Darmstadt, Germany) [15]. Based on our observation, some phenotypic tests were optimized based on workflow and laboratory conditions. In this case, we used high salt concentration (10%NaCl) for inhibits the growth of all bacteria except *S. aureus*. Also, due to contamination of urine samples with *Proteus mirabilis*, Blood Agar medium with 5% agar was used for urine samples. Finally, *ITS* gene was used for molecular confirmation of *S. aureus* strains. All strain were stored in Brain heart infusion broth (Merck, Germany) medium containing 15% glycerol at − 20 °C. This study was approved by the Ethics Committee of Hamadan University of Medical Sciences (Code No: IR.UMSHA.REC.1395.757).

Antimicrobial susceptibility testing and MRSA detection

Antimicrobial susceptibility test was investigated by the Kirby Bauer disc diffusion method according to Clinical Laboratory Standards Institute (CLSI) guidelines. The following drugs and concentrations were used to determine the antibiotic resistance of the strains: penicillin (10U), tetracycline (30 µg), clindamycin (30 µg), gentamicin (30 µg), ciprofloxacin (30 µg), erythromycin (15 µg), rifampin (5 µg) and linezolid (30 µg). All antibiotic disks were obtained from MAST® Company, United Kingdom. MRSA strains was determined using th cefoxitin E-test (Liofilchem, Italy) for Minimum Inhibitory Concentration (MIC) determination of strains. *S. aureus*ATCC25923 and *S. aureus*ATCC43300 were used as the negative and positive control, respectively [16].

Detection of biofilm forming strains in *S. aureus*

Biofilm formation was determined via crystal violet staining method according to the procedure described by Côrtes et al. [17]. A biofilm unit (BU) was defined as proposed by Côrtes et al. and the strain were classified as non-producers ($BU \leq 0.230$) or as weak ($0.230 > BU \leq 0.460$), moderate ($0.460 > BU \leq 0.920$) or strong producers ($BU > 0.920$).

Extraction of genomic DNA

DNA was extracted from *S. aureus* samples using the Qiagen DNA extraction kit (Qiagen, Germany), with modifications. Briefly, strain were sub-cultured onto Tryptose Agar (Merck, Germany) and incubated overnight at 37 °C. Between four to eight colonies were then inoculated into 10 mL sterile plastic tubes containing a 6 mL LB broth (Merck, Germany) and incubated for a further 18–36 h at

37 °C. Cell pellets were prepared from the broth by centrifuging the tubes at 8000 rpm for 10 min. The supernatant was removed except for 50 µL liquid, then the cell pellet and liquid were transferred to 1.5 mL microcentrifuge tubes before being centrifuged a second time at 8000 rpm for 5 min. Cell pellets were then frozen at − 20 °C. To achieve cell wall lysis, frozen cell pellets were resuspended in 180 µL lysozyme 200 mg/mL (Sigma Aldrich, USA) and incubated at 37 °C for 1 h. DNA extraction then continued following the Qiagen DNA kit protocol. DNA yield and purity (A260/A280) were measured using the spectrophotometer Nanodrop (Hangzhou Allsheng Instruments Co., Ltd, Chaina).

Screening of *ica* genes by PCR method

The *ica* genes were amplified using specific primers and conditions described in Table 1 [18]. For all genes, the PCR was done in 25 µL reactions containing 1 µL of DNA template, 12 µL of 2.5X master mix (Fermentas, United States), 1 µL of each primer and 10 µL of deionised water. The programmable thermal cycler (Eppendorf, Mastercycler® 5332, Germany) PCR device was applied in all PCR reactions. The PCR conditions were modified as follows: denaturation at 94 °C for 10 min, followed by 25 cycles of denaturation at 94 °C for 45 s, annealing at 57 °C for 45 s and extension at 72 °C for 75 s and final extension step at 72 °C for 10 min. PCR products were separated in 1% agarose gel for 95 min at 85 V, stained with GelRed 3X (Biotium, Fremont, CA) and detected by UV trans illumination. *S. aureus*ATCC25923 and *S. aureus*ATCC43300 were used as the negative and positive control, respectively. Finally, among the collected strain of *S. aureus*, 16 strain for *ica* genes with the highest frequency were selected to evaluate the gene expression.

Table 1 Primers used for *ica* genes and *ITS* gene in this stydy

Genes	Primers	Product size	Refs.
icaA	F: ACAGTCGCTACGAAAAGAAA R: GGAAATGCCATAATGACAAC	103	[18]
icaB	F: CTGATCAAGAATTTAAATCAC AAA R: AAAGTCCCATAAGCCTGTTT	302	[18]
icaC	F: TAACTTTAGGCGCATATGTTTT R: TTCCAGTTAGGCTGGTATTG	400	[18]
icaD	F: ATGGTCAACCCAGACAGAG R: AGTATTTTCAATGTTTAA AGCAA	198	[18]
icaR	F: TAATCCCGAATTTTTGTGAA R: AACGCAATAACCTTATTTTCC	469	[18]
ITS	F: GTTAGAGCGCACGCCTGATA R: AATGGTGGAGACTAGCGGGA	155	[1]

Measurement of ica genes expression levels

The total RNA was extracted using RiboEx kit (GeneAll, Korea) and cDNA synthesized by Hyper ScriptTM Reverse Transcriptase kit (GeneAll, Korea). q-PCR experiments were performed in a total volume of 20 µL including 1 µL of target cDNA, 1 µL of each primer (100 nM), and 5 µL fluorescent EvaGreen dye (Solis BioDyne, Estonia) by a StepOne Plus device (ABI, USA). Amplification process was performed as 95 °C for 15 min followed by 95 °C for 30 s, 59–60 °C (according to the melting temperature of the primers) for 30 s, and 72 °C for 30 s, with data collection in each cycle at 72 °C. Relative expression is used in which the expression of a target gene is standardized by a non-regulated reference gene. All the experiments were performed as triplicate.

MLST typing

All MRSA and MSSA biofilm producing strains were further typed by Multi-Locus Sequence Typing (MLST) based on the study of Strommenger et al. [19]. The amplification products of 7 house-keeping genes including *arcC*, *aroE*, *glpF*, *pta*, *gmk*, *tpi* and *yqiL* were purified and sequenced in both the directions. Then, the data were analyzed and assigned to sequence types using the tools on the *S. aureus* MLST webpage.

Statistical analysis

Data were analyzed using Wilcoxon signed rank test, t-test, and Chi-square tests using SPSS version 16(SPSS, Chicago, IL, USA). Statistical significance was defined as the p-value < 0.05.

Results

Prevalence of *S. aureus* in clinical strain

Among 521 different clinical samples, 100 *S. aureus* were collected including 79 (79%) from female and 21 (21%) strain from male patients. Also, out of 100 clinical strain of *S. aureus*, 42(42%) strain from wound, 21(21%) strain from blood culture, 24(24%) strain from urine, and 23(23%) strain from catheter were collected (Table 2). The *ITS* gene was detected in 100 (100%) of the *S. aureus* strain.

Prevalence of antibiotic resistance and MRSA strains

Figure 1a, b shows that strain had a very high level of antimicrobial resistance. Out of 100 *S. aureus*, 12 strain (12%) were resistant to linezolid, 17 strain (17%) were resistant to

Table 2 Prevalence of MRSA and MSSA strains and ica genes in different clinical samples of *S. aureus*

Clinical strain	Positive biofilms ($n=49$)				Negative biofilms ($n=61$)			
	MRSA[a] ($n=39$)		MSSA[b] ($n=10$)		MRSA ($n=5$)		MSSA ($n=56$)	
	Female	Male	Female	Male	Female	Male	Female	Male
Wound	23	6	1	4	0	0	2	8
Blood	10	6	0	0	5	0	0	0
Urine	19	1	0	0	4	0	0	0
Catheter	19	4	0	0	0	0	0	0

Clinical strain	*ica* Genes									
	MRSA ($n=44$)					MSSA ($n=56$)				
	icaA	*icaB*	*icaC*	*icaD*	*icaR*	*icaA*	*icaB*	*icaC*	*icaD*	*icaR*
Wound	5	3	6	3	4	3	4	0	0	0
Blood	0	0	1	0	0	0	0	0	0	2
Urine	1	0	1	1	0	1	0	0	0	3
Catheter	5	1	1	3	1	2	1	2	0	1

[a]MRSA: methicillin-resistant *S. aureus*

[b]MSSA: methicillin-susceptible *S. aureus*

rifampin, 66 strain (66%) were resistant to erythromycin, 12 strain (69%) were resistant to erythromycin, 83 strain (83%) were resistant to tetracycline, 88 strain (88%) were resistant to ciprofloxacin, and 91 strain (91%) were resistant to gentamicin. Also, all strain (100%) resistant were to penicillin and 44(44%) strain were resistant to cefoxitin (MIC ≥ 8 µg/mL) which were considered as MRSA strains. However, 22 MDR strain (22%) and 11 XDR strain (11%) were detected.

Prevalence of biofilm producer strains

According to Tables 2 and 3, from the total number of 100 *S. aureus* strain tested for biofilm formation, strong biofilm producers were 29 (29%), 15 (15%) were moderate and 5 (5%) strain were considered as non or weak biofilm producers. In addition, strong biofilm forming strain were detected in 23 MRSA (52.27%) and 6 MSSA (9.09%) strain.

Prevalence of *ica* genes

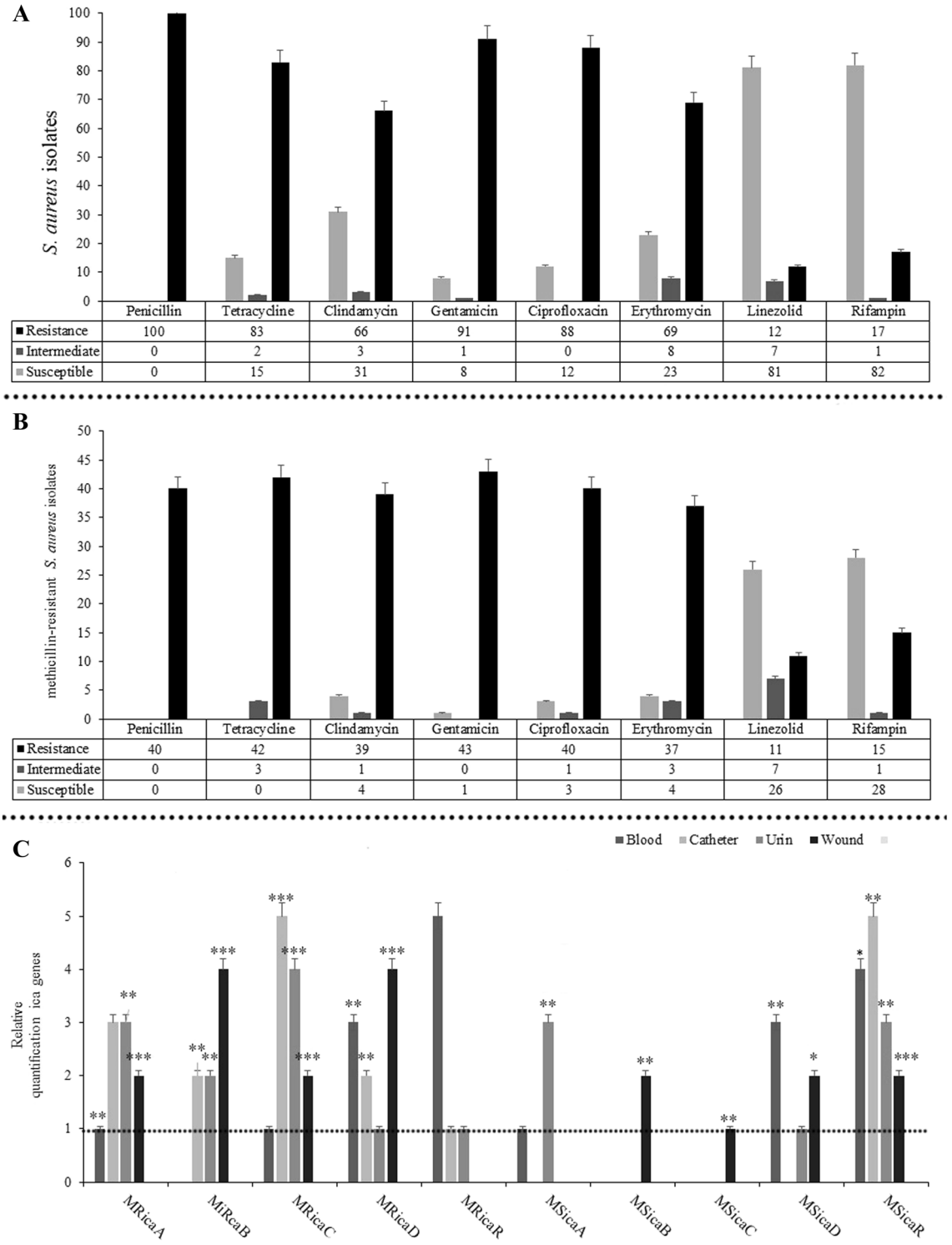
The results of biofilm gene distribution are shown in Tables 2 and 3. Out of 100 strain of *S. aureus*, 17 strain (17%) were positive for *icaA*, 9 strain (9%) were positive for *icaB*, 14 strain (14%) were positive for *icaC*, 7 strain (7%) were positive for *icaD* and 11 strain (11%) were positive for *icaR*.

The frequency of *ica* genes among 17 strong biofilm producing strains was as following: 15 (88.23%) *icaA* gene, 7 (41.11%) *icaB*, 10 (58.88%) *icaC* isolate, and 5 (29.41%) *icaD*. In *S. aureus* with moderate biofilm, 2 strain (13.33%) were positive for *icaA* gene, 1 (6.66%) for *icaB*, 2 (13.33%) for *icaC*, 2 (13.33%) for *icaD, and* 2 (13.33%) for *icaR*. And among weak biofilm formers, 1 isolate (20%) possesses *icaB*, 1 (20%) *icaC*, and 2 (40%) *icaD*.

Analysis of MLST typing of *S. aureus* strain

One-Hundred (100) of *S. aureus* strain was analyzed and typing by MLST and shown in Fig. 3. However, our analysis revealed a broad phylogenetic distribution of the *S. aureus* strain included in this study. We detected 32 different multilocus sequence types (ST), two of which (isolate ST3667 and ST491) has not been described before, indeed, each of them detected in single patients. A unique case of *S. aureus* ST3667 and ST491 harboring all ica genes, except icaR. Further, in *S. aureus* strains, CC30, ST30, ST5 and ST7 and CC5 had the highest distribution, and ninety of the 100 strain were of ST30 origin. Additionally, we identified 11 and 6 strain, belonging to the broadly distributed MDR high-risk clones ST5 and ST30, respectively. The absence of ST30, ST5 and ST7 in female patients more than male potions. The highest frequency of *icaB*, *iacA* and *icaD* genes was reported in ST30 and ST5 and the highest frequency of *icaC* genes was observed in ST30 and ST70. Also, the *ica* genes were most abundant in CC30 and CC5. In addition, high frequency of ST30, ST7 and ST5 was reported in bacteria isolated from wound and blood infection. Prevalence of CC30 and CC5 in bacteria isolated from wound and urinary tract infections was higher than other clinical samples.

Based on Fig. 2, the frequency of STs and CCs in the 44 MRSR strains was as follows: CC30 in 13 strain (29.45%), CC5 in 11 strain (25%), CC7 in 10 strain (22.72%), CC8



	Penicillin	Tetracycline	Clindamycin	Gentamicin	Ciprofloxacin	Erythromycin	Linezolid	Rifampin
Resistance	100	83	66	91	88	69	12	17
Intermediate	0	2	3	1	0	8	7	1
Susceptible	0	15	31	8	12	23	81	82

	Penicillin	Tetracycline	Clindamycin	Gentamicin	Ciprofloxacin	Erythromycin	Linezolid	Rifampin
Resistance	40	42	39	43	40	37	11	15
Intermediate	0	3	1	0	1	3	7	1
Susceptible	0	0	4	1	3	4	26	28

Fig. 1 Antibiotic resistance and *ica* gene expression in different clinical strain of *S. aureus*. **a** Antibiotic Resistance Pattern in Clinical Strain of *S. aureus* strains. **b** Antibiotic Resistance Pattern in Clinical Strain of MRSA strains. **c** The expression of ica genes in MRSA and MSSA strains and clinical samples. Bars represent means ± SD of the results of three independent experiments. Asterisks indicate significant differences in gene expression levels between (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$). Dotted horizontal lines represent limit of detection

Table 3 Prevalence of ica genes and antibiotic resistance in *S. aureus* strain with and without biofilm

Antibiotics	Positive biofilms ($n=49$)						Negative biofilms ($n=61$)					
	MRSA[a] ($n=39$)			MSSA[b] ($n=10$)			MRSA ($n=5$)			MSSA ($n=56$)		
	Rc	Id	Se	R	I	S	R	I	S	R	I	S
Penicillin	37	0	0	10	0	0	7	0	0	56	0	0
Tetracycline	39	0	0	1	0	9	5	0	0	39	9	8
Clindamycin	33	4	2	7	0	3	5	0	0	21	0	35
Gentamicin	39	0	0	5	3	2	5	0	0	47	0	4
Ciprofloxacin	31	2	6	10	0	0	5	0	0	43	2	2
Erythromycin	39	0	0	2	2	8	5	0	0	33	11	9
Linezolid	7	2	30	0	0	18	5	0	0	0	0	70
Rifampicin	13	0	26	0	0	14	4	0	5	0	0	28
Antibiotics	*ica* Genes											
	MRSA ($n=44$)						MSSA ($n=56$)					
	icaA	*icaB*	*icaC*	*icaD*	*icaR*		*icaA*	*icaB*	*icaC*	*icaD*	*icaR*	
Penicillin	8	3	6	4	1		4	5	2	0	7	
Tetracycline	0	1	0	0	1		1	0	0	0	1	
Clindamycin	0	0	1	0	1		1	0	0	0	0	
Gentamicin	0	0	0	0	0		0	0	0	0	0	
Ciprofloxacin	0	0	0	0	0		0	0	0	0	0	
Erythromycin	0	0	0	0	0		0	0	0	0	0	
Linezolid	3	2	2	2	1		0	0	0	0	0	
Rifampicin	0	1	0	1	0		0	0	0	0	0	

[a]MRSA: Methicillin-resistant *S. aureus*

[b]MSSA: Methicillin-Susceptible *S. aureus*

[c]R: Resistance

[d]I: Intermediate

[e]S: Susceptible

in 4 strain (9.09%), CC1 in 3 strain (6.81%) and CC22 in 1 strain (2.27%).

Relative expression report

As illustrated in Figs. 1c, 3, 4, high activity in *ica* locus was observed among strains collected from wound and catheter isolated strains. Furthermore, it is indicated higher expression level of *ica* genes in methicillin-resistant strain than methicillin-sensitive strains. Furthermore, the expression of *icaA* and *icaD* was high in all biofilm-producing bacteria.

As displayed in Fig. 3, *icaA* expression level increased in all investigated strain except for ST30 and ST491. Moreover, the highest expression level was observed in CC1, CC7, and CC11. No activity of *icaB* gene was observed in the weak biofilm producing strain. The *icaC* gene was only active in the CC5. Additionally, the activity of all these genes, along with the *icaR* gene in the CC5 isolate was related to strong, moderate, and weak biofilm with an increasing trend.

Correlation of the ica locus prevalence with MRSA resistance

As shown in Tables 4 and 5, a significant relationship was observed between antibiotic resistance and *ica* genes. In addition, there is a substantial relationship between the MRSA strains and MSSA strains with biofilm production.

Correlation of the ica locus expression with MRSA resistance and clinical specimen

As illustrated in Fig. 3, a noticeable expression in *ica* loci was observed in CC5 and CC1. Based on the results of statistical analysis, a significant relationship was detected between the increased expressions of *ica* genes with methicillin resistance ($p<0.03$). In the other words, MRSA strains have a strong biofilm compared to MSSA strains. Moreover, there was a significant relationship between male and female patients and an increase in the expression of *ica* genes. Also,

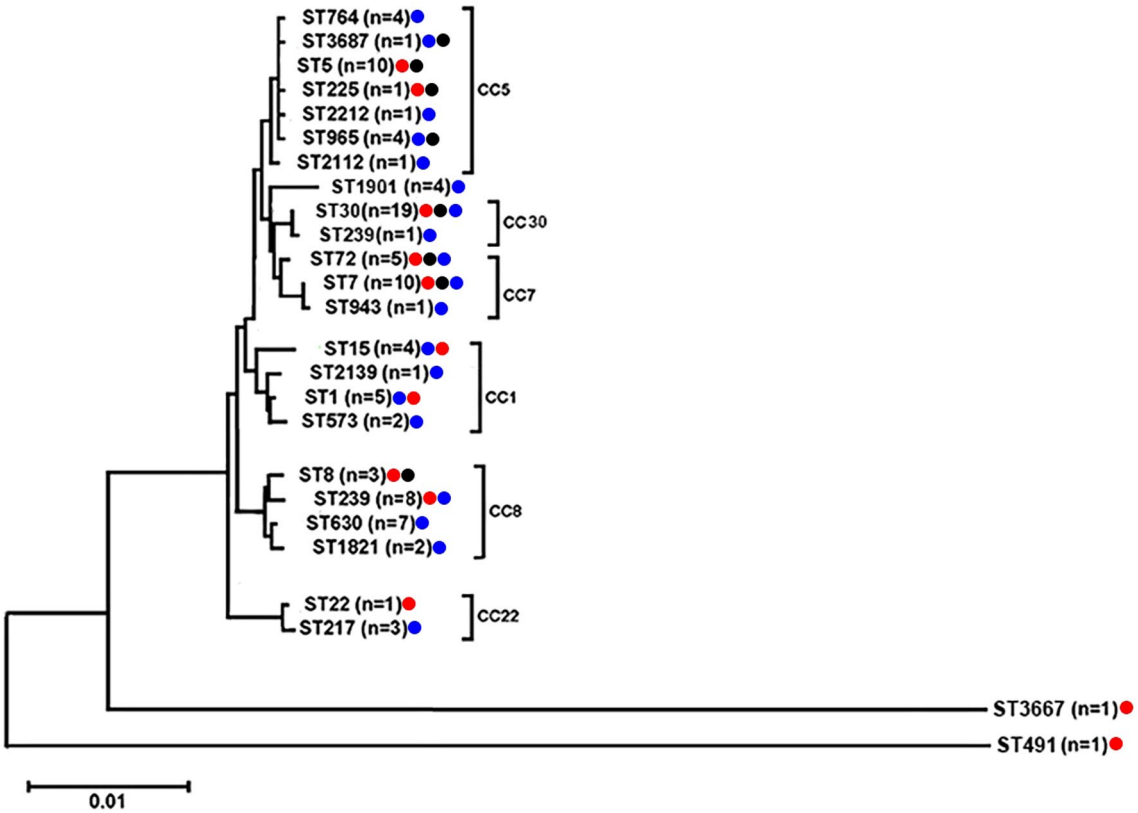


Fig. 2 Phylogenetic analysis of multilocus sequence types (STs) of bacteremia *S. aureus* strain. The neighbor-joining tree was constructed with the concatenated sequences of the seven MLST genes (arcc, aroe, glpf, gmk, pta, tpi, and yqil) based on the distance matrix of pair-wise differences between STs. Blue circle: MSSA; strains; red circle: MRSA strains; and black circle: MDR strains. (Color figure online)

wound and catheter specimens were associated with a rise in the expression of *ica* genes ($p < 0.08$, $p < 0.01$).

Discussion

Based on Fig. 1a, b in the present study, more than 90% of *S. aureus* strain were resistant to penicillin and gentamicin. In addition, the resistance to lienozolide and rifampin was found in less than 20% of strain and most strain with antibiotic resistance were wound and blood samples. These results are consistent with the studies of Abubakar and Sulaiman and Khosravi et al. [20, 21]. In our study, the frequency of MRSA and MSSA strains was 44% and 66%, respectively, which are in line with the results of Sit et al. [22]. However, in some studies in the Netherlands [23], Turkey [24], and UK [25], the results were inconsistent with those of the present study with less than 20% frequency in MRSA strains.

Our analysis of Tables 2 and 3 indicated that biofilm forming *S. aureus* had a high prevalence in MRSA strains. This results agree with Loughman et al. study [26]. Furthermore, the high frequency of MRSA strain with biofilm forming ability in the present study showed that virulence factors are involved in increasing the resistance to methicillin. In line with our findings, Algburi et al. concluded that biofilms play an important role in increasing antibiotic resistance [27]. Jimi et al. reported that there is a significant relationship between methicillin resistance and biofilm formation in *S. aureus* [28].

Present study described that *icaA and icaC* genes with 17% and 14% had the highest frequency, while *icaD* (7%) and *icaB* (9%) genes had the lowest prevalent. Additionally, based on Tables 2 and 3 nine MDR and XDR strains (2.04%) were found among methicillin-resistant *S. aureus* strongly produced biofilm. Therefore, no *icaR* gene was observed in *S. aureus* with a strong biofilm. Cerca et al. indicated that the *icaR* gene plays an important role in inhibiting biofilm production and suppressing *ica* locus, and that *ica*R fails to play a significant effect on the global regulator of virulence genes such as *SarA* and *agr* expression [29]. These results conformed a significant relationship between the frequency of *icaA, icaB, icaC, icaD* and *icaR* genes with resistance to methicillin in *S. aureus*. Further, the extension of these genes was significantly associated with the type of

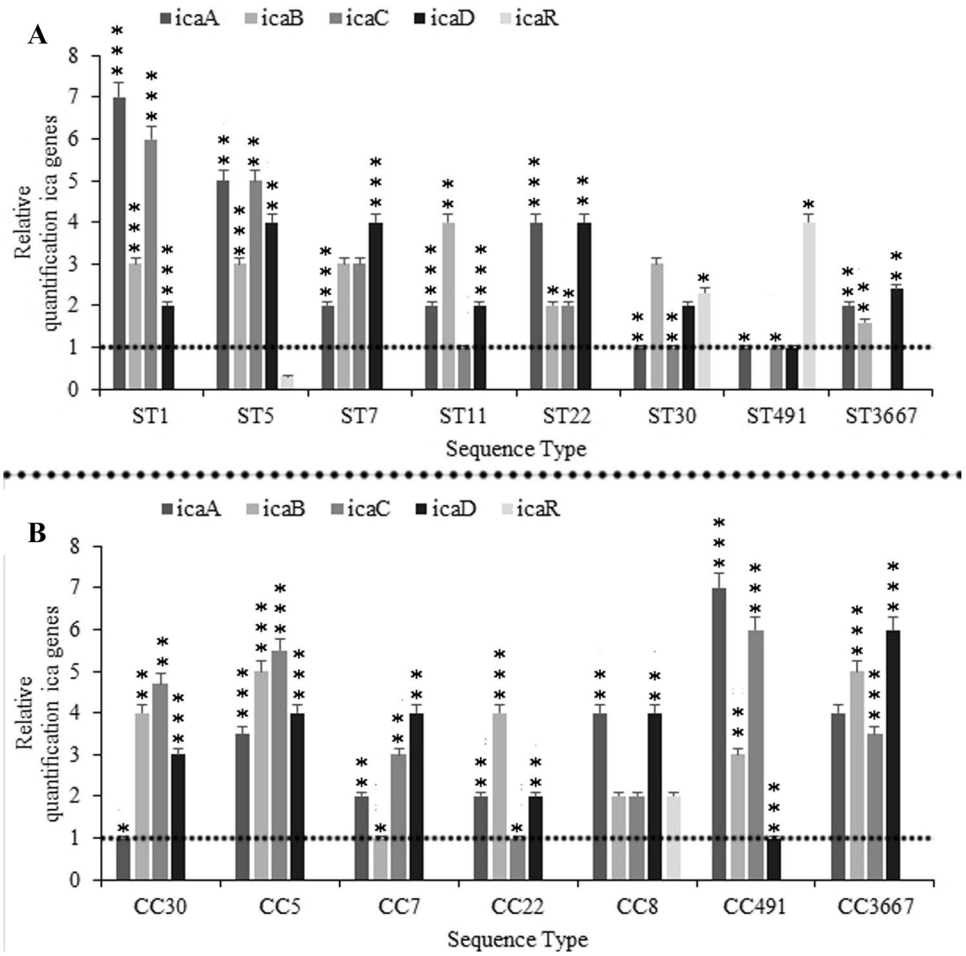


Fig. 3 Differences in the expression levels of the *ica* genes, *icaA, icaB, icaC, icaD* and *icaR*, in MRSA and MSSA strain and different Sequence Types. (MSSA strains: ST30, ST22, ST 491) (MRSA strains: ST1, ST5, ST7, ST11, ST30, ST3667). Bars represent means ± SD of the results of three independent experiments. Asterisks indicate significant differences in gene expression levels between (*P < 0.05, **P < 0.01, ***P < 0.001). Dotted horizontal lines represent limit of detection

clinical specimen, and the highest frequency of *ica* locus was observed in the wound and catheter specimens. In order to confirm the results, Serray et al. [30] reported a significant correlation between the frequency of *ica* genes and antibiotic resistance in *S. aureus*.

As shown in Fig. 1c, it is found that the expression of ica genes increased in MRSA strains. This figure released that gene expression if ica profiles of *S. aureus* in a biofilm differ significantly from those clinical sample source, which contributes to antibiotic resistance, evasion of immune responses and expression of virulence factors. Proteins active within a biofilm population undergo up and down regulation as antibiotics are introduced to the environment to counter the action of the antibiotic. Further, studies by Kot et al., [31] and Piechota et al., [32] demonstrated that in biofilm of *S. aureus*, genes such as ica are actively expressed and can result in β-lactam resistance. Figure 1c also showed that wound and catheter specimens were associated with a rise in the expression of ica genes ($p < 0.08$, $p < 0.01$). Despite the importance of *S. aureus* acquired antibiotic resistance the innate resistance also plays a crucial role in the widespread of antimicrobial resistance. Furthermore, both acquired and innate resistances are closely linked to the increased pathogenesis of device-related biofilm infections [32].

As illustrated in Figs. 3 and 4, high activity in *ica* locus was observed among strains collected from wound and catheter isolated strains. Furthermore, the relationship between biofilm formation and the expression of ica genes was assessed; This result was in agreement with Piechota et al. [32] study.

As displayed in Fig. 3, *icaA* expression level increased in all investigated strain except for ST30 and ST491. Moreover, the highest expression level was observed in CC1, CC7, and CC11. No activity of *icaB* gene was observed in the weak biofilm producing strain and *icaC* gene was only active in the CC5. This is consistence with observation of Tasse et al. [33] study.

In Figs. 2 and 3, we indicated that strong biofilm producing strains of MRSA group abundantly belonged to CC5, CC30, CC22, and CC7. The first reported CC491 and CC3667 were observed among weak and moderate biofilm producers with the lowest frequency in MRSA strains and MSSA. Vanhommerig et al. [34] reported that

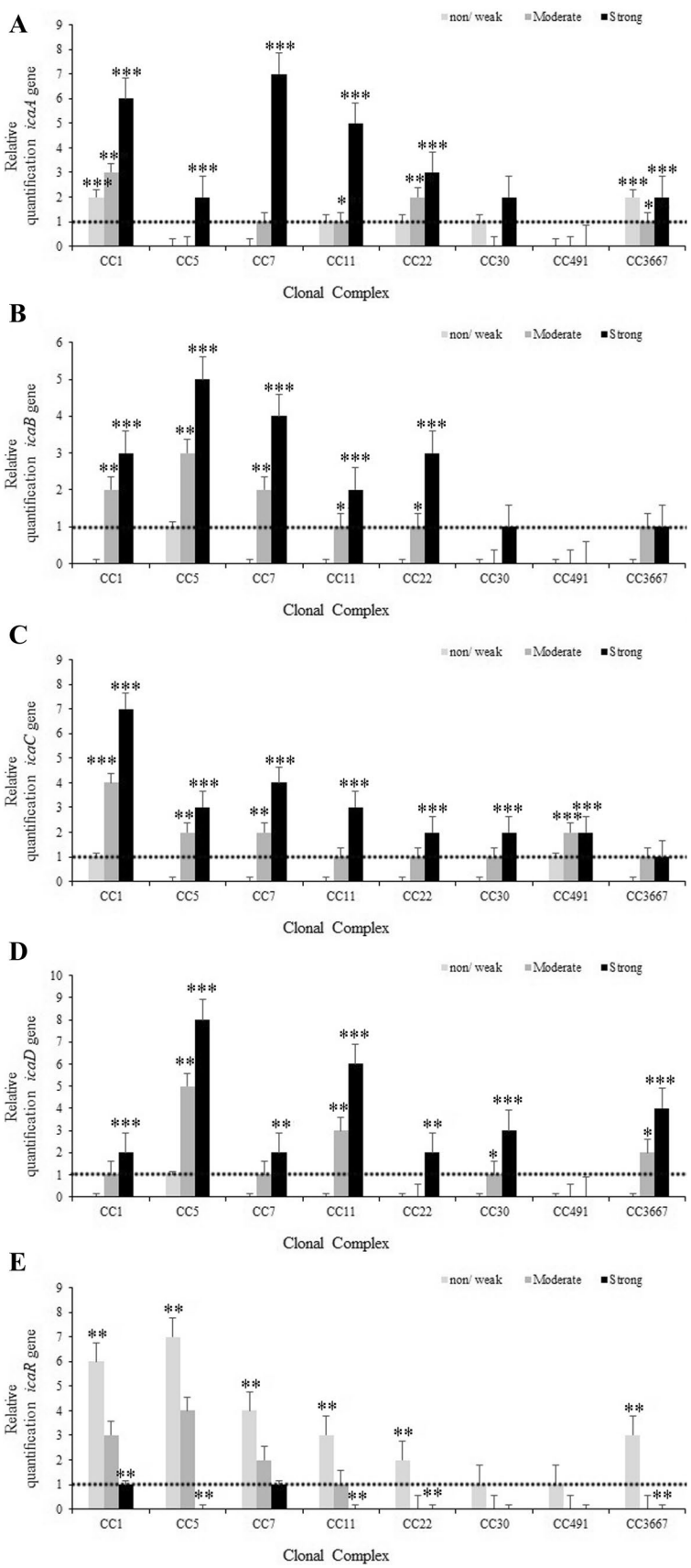


Fig. 4 **a** Relationship between the expression level of *icaA* gene, CC, and biofilm formation. **b** Relationship between the expression level of *icaB* gene, CC, and biofilm formation. **c** Relationship between the expression level of *icaC* gene, CC, and biofilm formation. **d** Relationship between the expression level of *icaD* gene, CC, and biofilm formation. **e** Relationship between the expression level of *icaR* gene, CC, and biofilm formation. Bars represent means ± SD of the results of three independent experiments. Asterisks indicate significant differences in gene expression levels between (*P < 0.05, **P < 0.01, ***P < 0.001). Dotted horizontal lines represent limit of detection

Table 4 Distribution of CC and STs according to genotyping, clinical origin and biofilm formation/accumulation

Predicted CCa	MLST STb	Methicilin resistance		MDRe/XDRf	Clinical origin				Biofilm unit	Biofilm phenotype				*ica* Genes
		MR[c] n = 44	MS[d] n = 56		Blood n = 21	Cath-eter n = 23	Urine n = 24	Wound n = 42		Non n = 51	Weak n = 29	Mod-erate n = 15	Strong n = 5	
1	15	0	2	0	2	0	0	0	0.59 ± 0.04	2	0	0	0	*NA*
	2139	0	1	0	0	0	3	0	0.16 ± 0.09	0	1	0	0	*icaD,icaR*
	1	4	1	1	0	1	0	3	0.89 ± 0.06	4	1	0	0	*icaR*
	573	0	2	0	2	0	0	0	1. 1 ± 0.07	0	1	1	0	*IcaA, icaC,*
5	764	1	3	1	2	1	0	2	0.09 ± 0.09	4	0	0	0	*NA*
	3687	0	1	0	1	1	0	0	1.03 ± 0.05	0	0	1	0	*IcaA,icaC,icaD,*
	5	11	2	4	0	0	0	13	1.0 ± 0.01	6	2	3	2	*IcaA,icaB,icaC,*
	225	0	1	0	1	0	0	0	0.69 ± 0.07	1	0	0	0	*icaR*
	2212	0	1	0	0	0	1	0	1.06 ± 0.06	0	0	1	0	*IcaA,icaB,icaC,icaD,*
	965	1	3	0	1	2	1	0	0.18 ± 0.14	4	0	0	0	*NA*
	2112	0	1	0	0	1	0	0	1.39 ± 0.04	1	0	0	0	*NA*
7	7	8	2	3	2	1	4	3	1.01 ± 0.09	6	0	3	1	*IcaA,icaB,icaC,icaD, icaR*
	72	2	3	1	1	0	1	1	0.11 ± 0.09	5	0	0	0	*NA*
	943	0	1	0	1	0	0	0	0.99 ± 0.06	1	0	0	0	*icaR*
8	8	2	1	1	2	0	0	1	1. 1 ± 0.07	0	1	2	0	*IcaA, icaC,*
	239	0	3	0	1	1	1	0	1.09 ± 0.09	3	0	0	0	*IcaA,icaB,*
	630	2	5	0	3	0	0	4	1.03 ± 0.05	0	7	0	0	*IcaA,*
	1821	0	2	0	1	1	0	0	1.0 ± 0.01	0	2	0	0	*NA*
22	22	2	1	2	1	0	0	2	0.69 ± 0.07	3	0	0	0	*icaR*
	217	3	10	2	5	1	3	2	0.96 ± 0.06	9	3	0	0	*icaD,icaR*
30	1901	1	3	0	0	1	0	3	0.98 ± 0.09	0	3	0	0	*IcaA, icaC,*
	30	6	5	3	3	2	2	3	1.19 ± 0.08	2	7	0	2	*IcaA,icaB,icaC,icaD, icaR*
	239	0	1	0	1	0	0	0	1.1 ± 0.01	0	1	0	0	*IcaA, icaC,*
3667	3667	1	0	1	0	0	0	1	1.18 ± 0.14	0	0	1	0	*IcaA,icaB,icaC,icaD,*
491	491	0	1	0	1	0	0	0	0.39 ± 0.04	1	0	0	0	*NA*

[a]Clonal complex

[b]Sequence types

[c]Methicillin-resistant *S. aureus*

[d]Methicillin- Susceptible *S. aureus*

[e]Multidrug-resistant

[f]Extensively drug-resistan

Table 5 Analysis of variables significantly associated STs, antimicrobial resistance, clinical samples, ica genes and biofilm formation in clinical strain of MRSA and MSSA strains

Antibiotics	MRSA strains							MSSA strains						
	Genotypic ica operon					Biofilm formation	STs	Genotypic ica operon					Biofilm formation	STs
	icaA	*icaB*	*icaC*	*icaD*	*icaR*			*icaA*	*icaB*	*icaC*	*icaD*	*icaR*		
Penicillin	0.047	0.052	0.015	0.029	0.097	0.019	0.025	0.077	0.252	0.005	0.050	0.027	0.079	0.025
Tetracycline	0.041	0.065	0.003	0.001	0.004	0.035	0.045	0.089	0.085	0.063	0.006	0.045	0.035	0.045
Clindamycin	0.093	0.080	0.033	0.033	0.029	0.044	0.050	0.193	0.080	0.073	0.043	0.025	0.044	0.050
Gentamicin	0.069	0.071	0.040	0.832	0.321	0.097	0.097	0.169	0.061	0.340	0.032	0.031	0.097	0.097
Ciprofloxacin	0.046	0.097	0.020	0.065	0.051	0.009	0.012	0.056	0.047	0.080	0.005	0.149	0.064	0.012
Erythromycin	0.017	0.305	0.283	0.895	0.347	0.004	0.024	0.117	0.015	0.211	0.095	0.239	0.034	0.024
Linezolid	0.146	0.597	0.220	0.365	0.051	0.019	0.060	0.146	0.097	0.109	0.165	0.171	0.053	0.060
Rifampicin	0.218	0.824	0.343	0.383	0.647	0.052	0.055	0.088	0.204	0.413	0.683	0.072	0.065	0.055
Clinical samples														
Wound	0.071	0.040	0.832	0.097	0.033	0.001	0.042	0.061	0.340	0.032	0.031	0.073	0.025	0.079
Urine	0.097	0.020	0.065	0.009	0.832	0.009	0.074	0.047	0.080	0.005	0.149	0.169	0.003	0.057
Blood	0.193	0.080	0.073	0.043	0.193	0.003	0.060	0.056	0.047	0.080	0.005	0.056	0.009	0.072
Cathater	0.169	0.061	0.340	0.032	0.169	0.035	0.085	0.061	0.340	0.032	0.031	0.117	0.008	0.012

CC5 and CC22 are more abundant in MRSA strains with strong biofilms. Furthermore, according to Table 5 and Fig. 4 a significant relationship was observed between CCs and resistance to methicillin in *S. aureus*. In another study, Challagundla et al. [35] reported that CC5 and CC22 showed high pathogenicity in MRSA strains and the frequency of antibiotic resistance is higher in these CCs. Additionally, in Figs. 3 and 4, CC30 was observed in non-biofilm forming strains, medium biofilm producers, and strong biofilm forming strains. However, some studies in Ireland [9], Brazil [13], and United Kingdom [36] have shown that the horizontal transfer of virulence and resistance genes between similar CCs and STs might play no role in pathogenicity.

In conclusion, Biofilm formation is different in methicillin-sensitive and methicillin-resistant *S. aureus* which appear in weak, moderate, and strong states. Based on our findings, the formation of biofilms in MRSA strains is much more than MSSA strains, and the frequency of *ica* genes can vary in different antibiotic patterns. Furthermore, the diversity of CCs and STs in strains with moderate and strong biofilms indicates extensive genetic movements in drug-resistant strains and plays an important role in virulence and resistance to antibiotics. Hence, it is concluded that resistance to methicillin is affected by pathogenicity due to the significant relationship between the presence of *ica* genes and antibiotic resistance patterns. Finally, regarding the high expression of the studied genes, biofilm formation in MRSA strains was more dependent on *ica* locus.

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Author contributions MRZ designed research; HT, SD and MJ performed experiments. HT and MRA wrote the manuscript.

Compliance with ethical standards

Conflict of interest All authors declare that they have no conflicts of interest.

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