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Original Article

Genetic diversity and biofilm formation analysis of *Staphylococcus aureus* causing urinary tract infections in Tehran, Iran

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Abstract

Introduction: Over the past decades, prevalence of biofilm-forming *Staphylococcus aureus* strains has significantly increased in urinary tract infections. The aim of this study was to investigate prevalence of biofilm forming and adhesion encoding genes and to analyze distribution of different *agr* and *spa* types in *S. aureus* isolates.

Methodology: In the present study, 75 *S. aureus* isolates obtained from patients with urinary tract infections were examined for susceptibility to antimicrobial agents. Adhesion, biofilm, and *spa* encoding genes were detected by PCR screening; *agr* types were determined using multiplex PCR.

Results: Among the 75 isolates, 72% were biofilm producers and 28% were non-biofilm producers. Notably, the ability to produce biofilm was higher among MRSA strains ompared to MSSA strains. The most prevalent biofilm forming gene was *icaD* (77.3%), followed by *icaA* (76%), *icaB* (57.3%) and *icaC* (50.7%). Adhesion genes *clfA, clfB*, *fnbB, can, fnbA*, *ebp* and *bap* were detected in 94.7%, 92%, 68%, 64%, 64%, 60% and 5.3% of the isolates, respectively. The *spa* types t426 and t7789 were found among the non-MDR isolates. It was found that t790, t084, t7789 and t325 *spa* types were biofilm producers, while t426 and t1339 *spa* types were non-biofilm producers.

Conclusion: Biofilm encoding genes *icaD* and *spa* type t790 and *agr* type III were the most prevalent factors among MDR biofilm producer isolates. The study emphasized that identification of genes and characterization of molecular types involved in biofilm formation should be considered.

Key words: methicillin-resistant *S. aureus*; multidrug-resistance; biofilm; urinary tract infections.

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Introduction

Urinary tract infections (UTIs) are a significant health problem in both community and health care settings, annually affecting more than 150 million people around the world [1]. Although it is well established that UTIs occur more often in women than in men, it seems that almost all individuals experience at least one episode of UTI during their lifetime [2]. A wide variety of organisms, including Gram-negative and Gram-positive bacteria, as well as certain fungi, are known to cause UTIs. Although the most common causative agents are *Escherichia coli*, *Klebsiella pneumonia* and other *Enterobacteriaceae*, the role of *Staphylococcus aureus* (*S. aureus*) in relation to UTI should not be overlooked [1-3]. Although *S. aureus* is responsible for 0.5-6% of UTI cases, antibiotic therapy in symptomatic UTI related to this species is highly recommended and must be considered [4]. Antimicrobial therapy might lead to emergence of multidrug-resistant (MDR) *S. aureus* which would result in limitations in choice of therapeutic options. Currently, it is documented that *S. aureus* has the ability to adhere and to form a biofilm on biotic and abiotic surfaces [5]. The pathogen could be a great risk to patients with UTI due to its capacity to invade renal tissue by adherence to uroepithelium, resulting in formation of a biofilm. In the biofilm, the proximity of different bacterial populations rasies the possibility of exchange of genetic elements which leads to spread of resistance genes. Hence, biofilm-forming by *S. aureus* strains could play a key role in recurrent UTIs and antimicrobial resistance [6,7].

During the past few decades, the prevalence of biofilm-forming methicillin-resistant *S. aureus*

(MRSA) strains has significantly increased and has received a great deal of attention. *S. aureus* biofilm formation starts with adhesion to surfaces, which is mediated by the expression of different microbial surface components recognizing adhesive matrix molecules (MSCRAMMs), such as fibronectin-binding proteins (Fnb), clumping factors (Clf), and biofilm-associated protein (Bap). Next comes production of polysaccharide intercellular adhesion (PIA), which mediates cell-to-cell adhesion and is encoded by the *ica* operon (*icaABCD*) [8-12].

Although biofilm formation in MRSA causing UTIs is affected by other factors such as phenotypic characteristics, the genetic pattern associated with biofilm formation in these strains and their role in infections caused by MRSA is important. Studies have demonstrated that *icaAD* genes, adhesion genes, activation of the *agr* system and specific *spa* types play a significant role in biofilm formation. Further, understanding the phenotypic and genetic characteristics associated with biofilms can provide critical and timely insights into the prevention, management, and successful treatment of these infections. Therefore, the present research was carried out i) to characterize the antibiotic resistance patterns, ii) to evaluate the biofilm-forming ability and the presence of the *icaABCD* and adhesion genes, and iii) to determine molecular types of MRSA strains using accessory gene regulator (*agr*) and staphylococcal protein A (*spa*) typing in MRSA strains isolated from UTIs.

Methodology

Bacterial isolation

A total of 75 *S. aureus* isolates were collected during the period from May 2017 to August 2018 from four hospitals in Tehran, Iran. Patients signed informed consent was signed before samples were taken. The Ethics Committee of Shahid Beheshti University of Medical Sciences (Tehran, Iran) approved this study (IR.SBMU.MSP.REC.1396.644). Urine samples were immediately inoculated on agar plates and incubated for 24 to 48 hours under aerobic conditions at 37°C, and then the colony count was done. UTI was defined as a positive urine culture for *S. aureus* with a colony count $\geq 10^5$ CFU/mL. Standard biochemical tests were used to identify *S. aureus* isolates. Definite identifications of isolates were based on distinction of polymerase chain reaction (PCR) assay targeting the *S. aureus*-specific *nuc* gene [13].

Phenotypic and genotypic microbiological data

Phenotypic analysis of biofilm formation

Phenotypic biofilm formation was assessed by two *in vitro* methods: i) slime production assay or Congo Red Agar (CRA) method and ii) Microtiter Plate (MtP) assay.

In the MCRA method, briefly, after preparation of CRA by adding 0.8g of CR (Sigma, St. Louis, USA) to 1 liter of brain heart infusion agar (BHI agar, from Merck, St. Louis, Germany) and autoclaving it, filters were used to add saccharose (36 grams) (Sigma, St. Louis, USA) to CRA. Bacteria were inoculated on CRA and incubated at 37°C for 24 hours and then overnight at room temperature. Biofilm formation was categorized in four levels based on colony color of strains that appeared: i) strong biofilm producer strains (dark colonies), ii) moderate biofilm producer strains (black colonies), iii) weak biofilm producer strains (gray colonies), and iv) biofilm non-producer strains (red colonies) [14,15].

The MtP assay is a quantitative method for biofilm detection; it was carried out as described previously [15]. Concisely, overnight cultures of bacterial isolates in Tripticase Soy Broth (TSB, Merck, Darmstadt, Germany) containing 1% glucose were diluted to 1:100 with fresh medium. Sterile, flat-bottomed polystyrene microtiter plates with 96-wells were filled with 200 µL per well of the diluted culture, and incubated for 24 hours at 37 ºC. After incubation, wells were washed 3 times with 200 µL of phosphate buffered saline (PBS; pH 7.2) to remove floating bacteria. Afterwards, wells were fixed by 99% methanol, dried at room temperature, and stained with 0.1% safranin. Safranin dye bound to the adherent cells was dissolved with 1 mL of 95% ethanol per well. As a negative control, 200 µL of TSB-1% glucose was used. The optical density (OD) of each well was measured using an ELISA reader at a wavelength of 490 nm. Optical density cut-off (ODc) was defined as average OD of negative controls + 3×standard deviation (SD) of negative controls. Biofilm formation of strains was analyzed according to the absorbance of the safranin-stained attached cells and interpreted as per the criteria described by Stepanovic *et al.* [15,16]. Accordingly, the degree of biofilm production was categorized into strong, moderate, weak or no biofilm. For quality control, *Staphylococcus epidermidis* ATCC 35984 strain was used in each run.

Antimicrobial susceptibility testing and MRSA screening

Based on the clinical and laboratory standards institute (CLSI) guidelines [17], a standard Kirby-Bauer disk diffusion method was applied for the *in vitro* assessment of antimicrobial susceptibility to antimicrobial agents (Mast Diagnostics Ltd, Merseyside, UK) including tetracycline, erythromycin, clindamycin, nitrofurantoin, chloramphenicol, trimethoprim-sulfamethoxazole, gentamicin, ciprofloxacin, penicillin, and amikacin in the isolates under study. Minimum inhibitory concentration (MIC) value was determined, using the broth microdilution test for vancomycin, according to the CLSI criteria. Inducible ($iMLS_B$) and constitutive ($cMLS_B$) resistance were determined for isolates based on CLSI guidelines [17]. *S. aureus* ATCC 29213 and ATCC 25923 were employed as standard strains. Multidrug resistance (MDR) was defined as resistance of MRSA strains to three or more antimicrobial categories besides beta-lactams,. . For MRSA screening, phenotypic growth was investigated around cefoxitin discs (30 µg) placed on plates of Mueller-Hinton agar (Merck, Germany), containing 4% NaCl. Also, PCR was applied for genotypic amplification of *mecA* genes [13,17].

DNA extraction

Genomic DNA was extracted from overnight pure cultures of *S. aureus* strains on 5% sheep blood agar (BA; Merck, Germany) using phenol-chloroform extraction. If the purity was appropriate, it was used as the template for PCR.

Genetic analysis of biofilm formation and detection of icaABCD and adhesion genes

All of the isolates were screened for the presence of *icaABCD*, *can, ebp, fnbB, fnbA, clfB, clfA* and *bap* genes by conventional PCR [15,18,19].

agr typing

Multiplex PCR amplification was carried out with specific primers for identification of *agr* alleles based on the hyper variable domain of *agr* locus, as suggested by Gilot and colleagues [20]. PCR amplification was performed using a 25 µL reaction mixture composed of 4–17 pmol of each of the following primers: forward primer [Pan (5′-ATG CAC ATG GTG CAC ATG C-3′)] common to all *agr* groups and four reverse primers [*agr1* (5′-GTC ACA AGT ACT ATA AGC TGC GAT-3′)], [*agr2* (5′-TAT TAC TAA TTG AAA AGT GGC CAT AGC-3′)], [*agr3* (5′-GTA ATG TAA TAG CTT GTA TAA TAA TAC CCA G-3′)], and [*agr4* (5′-CGA TAA TGC CGT AAT ACC CG-3′)], each one specific to each *agr* group. The PCR program consisted of an initial denaturation step at 94°C for 5 minutes, denaturation in 26 cycles of 30 seconds at 94°C, annealing at 55°C for 30 seconds, and extension at 72C for 60 seconds; and final extension in 1 cycle of 72°C for 10 minutes. *agr* types I, II, III, and IV were expected to produce 441-bp, 575-bp, 323-bp, and 659-bp fragments respectively.

spa typing

S. aureus isolates underwent *spa* typing as recommended by Harmsen *et al.* [21]. In this method the x region of the *spa* gene consisting of variable numbers of 21- to 30-bp repeats, with the 24-bp repeat being most common, was amplified by PCR with forward (5′-AGACGATCCTTCGGTGAGC-3′) and reverse (5′-GCTTTTGCAATGTCATTTACTG-3′) primers. Purified *spa* PCR products were subjected to sequencing of both strands using an ABI Prism 377 automated sequencer (Applied Biosystems, Perkin-Elmer Co., Foster City, CA). Editing of sequences obtained was performed using the Chromas software (Version 1.45, Australia). Edited sequences were assigned to specific *spa* types according to the guidelines described by a Ridom SpaServer database (http://www.spaserver.ridom.de). In this single-locus typing technique, repeat succession for a given strain determines its *spa* type.

Results

Seventy-five *S. aureus* strains were obtained from patients, 56 (74.7%) isolates belonged to female patients and 19 (25.3%) to males. The patients' average age was 37 years, distributed among three age group; 12 patients ≤ 20 years (16%), 54 patients between 21–45 years (72%) and nine patients between 46-65 years (12%). In the present study, 75 *S. aureus* strains were studied to evaluate biofilm formation, of which 45 (60%) were MRSA and 30 (40%) were methicillin-sensitive *S. aureus* (MSSA). In this survey, antimicrobial susceptibility testing of S. *aureus* isolates indicated that nitrofurantoin (20%) and chloramphenicol (16%) could be used as the most effective antibiotics against *S. aureus* isolated from UTIs. According to our findings, all the isolates were susceptible to vancomycin: 17 (22.6%) isolates had MIC ≥ 0.5 µg/mL, 23 (30.7%) had MIC ≥ 1 µg/mL, and 35 (46.7%) exhibited MIC ≥ 2 µg/mL. Highest and lowest rates of resistance observed were to penicillin (86.7%) and chloramphenicol (16%), respectively. The rrequency of resistance among *S. aureus* isolates and

comparison of resistance patterns between MRSA and MSSA strains to antimicrobial agents are presented in Table 1.

Of the 75 examined isolates, 72 (96%) were identified as MDR. Overall, five different MDR patterns were identified, among which resistance to five antibiotics (52%) was the most frequently identified pattern. Two isolates were found to be resistant only to trimethoprim-sulfamethoxazole (2.7%). The frequency of resistance to multiple antibiotics among *S. aureus* clinical isolates is illustrated in Figure 1. Out of 75 strains tested, MS, $iMLS_B$ and $cMLS_B$ phenotypes were detected in 3 (4%), 8 (10.7%) and 31 (41.3%) isolates, respectively.

The CRA showed that 21 (28%) of the strains were biofilm negative, while 23 (30.7%) were weakly biofilm producers, 21 (28%) were intermediate and 10 (13.3%) were highly positive biofilm producers. The

Figure 1. Summary of resistance to multiple antibiotics simultaneously.

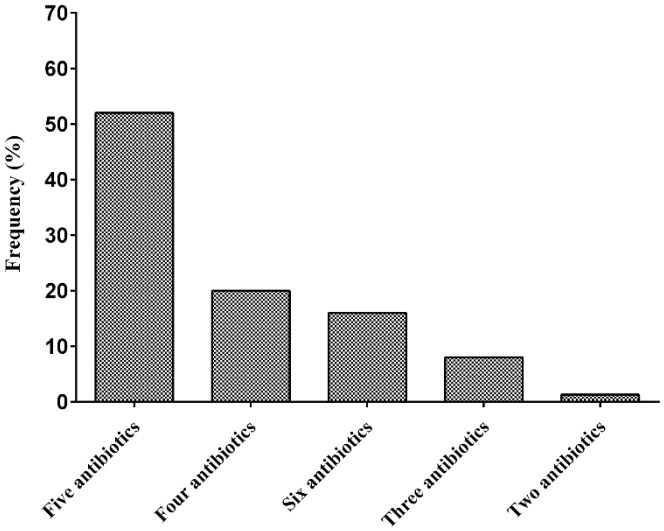


Figure 2. A) Biofilm production of MRSA and MSSA isolates using the Congo Red Agar method. **B)** Biofilm production of MRSA and MSSA isolates using the Microtiter Plate method.

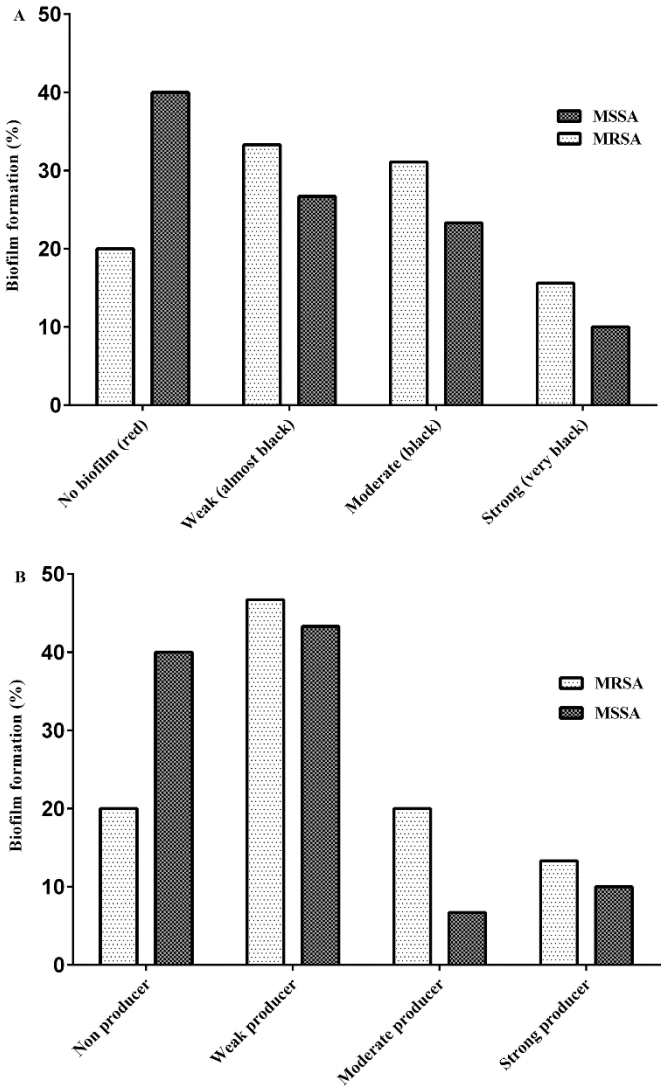


Table 1. Antimicrobial resistance pattern of isolates.

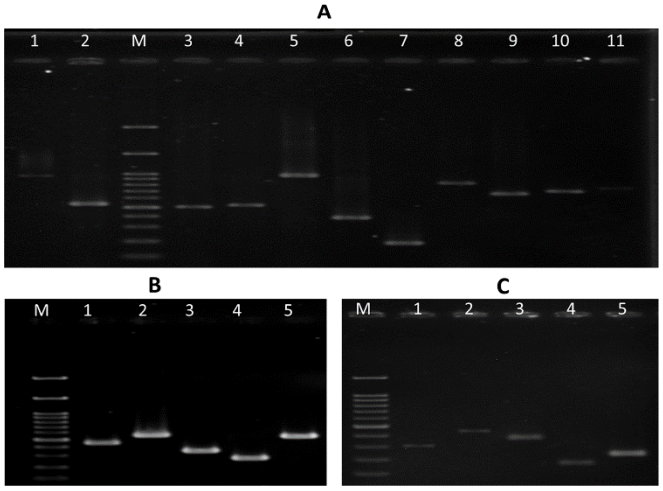
Antibiotic	MSSA N = 30		MRSA N = 45		Total	
	Resistant N (%)	Sensitive N (%)	Resistant N (%)	Sensitive N (%)	Resistant N (%)	Sensitive N (%)
Penicillin	20 (66.7)	10 (33.3)	45 (100)	0 (0)	65 (86.7)	10 (13.3)
Gentamicin	10 (33.3)	20 (66.7)	40 (88.9)	5 (11.1)	50 (66.7)	25 (33.3)
Tetracycline	19 (63.3)	11 (36.7)	30 (66.7)	15 (33.3)	49 (65.3)	26 (34.7)
Ciprofloxacin	16 (53.3)	14 (46.7)	29 (64.4)	16 (35.6)	45 (60)	30 (40)
Erythromycin	14 (46.7)	16 (53.3)	28 (62.2)	17 (37.8)	42 (56)	33 (44)
Clindamycin	5 (16.7)	25 (83.3)	26 (57.8)	19 (42.2)	31 (41.3)	44 (58.7)
Trimethoprim sulfamethoxazole	4 (13.3)	26 (86.7)	17 (37.8)	28 (62.2)	21 (28)	54 (72)
Amikacin	11 (36.7)	19 (63.3)	8 (17.8)	37 (82.2)	19 (25.3)	56 (74.7)
Nitrofurantoin	6 (20)	24 (80)	9 (20)	36 (80)	15 (20)	60 (80)
Chloramphenicol	2 (6.7)	28 (93.3)	10 (22.2)	35 (77.8)	12 (16)	63 (84)

MtP method indicated that of 75 *S. aureus* isolates, attachment abilities in 9 (12%) were strong, 11 (14.7%) were moderate, 34 (45.3%) were weak and 21 (28%) had no attachment and were classified as non-biofilm producers. Overall, use of both methods revealed that 54 (72%) of the *S. aureus* isolates were biofilm producers and 21 (28%) were non-biofilm producers. As presented in Figure 2, 48% of biofilm producers were MRSA, whereas 24% of MSSA isolates were found to be biofilm producers. Our results realed that ability of biofilm formation in MRSA isolates was twice that of MSSA isolates.

Analysis of *icaAD* genes among the *S. aureus* strains tested indicated that the most prevalent gene was *icaD* in 58 strains (77.3%), followed by *icaA* in 57 (76%), *icaB* in 43 (57.3%) and *icaC* in 38 (50.7%). Overall, six different *ica* patterns were identified, wherein *A+B+C+D* (36%, 27/75), was the most frequently identified. Representative results are shown in Figure 3A. The positive results were counted and analyzed statistically. Regarding the presence of adhesion genes, the most prevalent was *clfA* in 71 strains (94.7%) followed by *clfB* in 69 (92%), *fnbB* in 51 (68%), *can* in 48 (64%), *fnbA* in 48 (64%), *ebp* in 45 (60%), and *bap* in 4 (5.3%), respectively. The top three detected genetic adhesion patterns of biofilm producer strains were *clfA+clfB+fnbB+fnbA+ebp+can* (37%, 20/54), *clfA+clfB+fnbA+ebp+can* (27.8%, 15/54) and *clfA+clfB+fnbB* (24.1%, 13/54). Distribution of *icaAD* and adhesion profiles is presented in Table 2.

The *spa* typing was performed following a previously described method [21]. Representative results are shown in Figure 3C. The positive results were counted and analyzed statistically. The *spa* typing discriminated 10 types: t790 (25.3%), t030 (16%), t037 (13.4%), t008 (9.3%), t426 (9.3%), t7580 (8%), t084 (6.7%), t7789 (5.3%), t325 (4%) and t1339 (2.7%). The isolates identified as biofilm producers were assigned to particular t790, t030, t008, t037, t084, t7789, t325 and t7580 *spa* types, whilst non-biofilm producer strains were assigned to *spa* types t030, t037, t008,

Figure 3. Representative amplification of biofilm, *spa* and *agr* encoding genes from *S. aureus* isolates.



A) Lane 1, the 971bp PCR product of *bap* encoding gene; Lane 2, the 526bp PCR product of *ebp* encoding gene; Lane M, 100-bp DNA Ladder (Fermentas, UK); Lane 3, the 509bp PCR product of *icaA* encoding gene; Lane 4, the 526bp PCR product of *icaB* encoding gene; Lane 5, the 989bp PCR product of *icaC* encoding gene; Lane 6, the 371bp PCR product of *icaD* encoding gene; Lane 7, the 191bp PCR product of *fnbA* encoding gene; Lane 8, the 813bp PCR product of *fnbB* encoding gene; Lane 9, the 657bp PCR product of *clfA* encoding gene; Lane 10, the 596bp PCR product of *clfB* encoding gene; and Lane 11, the 744bp PCR product of *can* encoding gene. **B)** Lane M, DNA Ladder; Lane 1, the 441bp PCR product of *agr* type I; Lane 2, the 575bp PCR product of *agr* type II; Lane 3, the 323bp PCR product of *agr* type III, Lane 4, the 270bp PCR product of *nucA* gene; and Lane 5 the 583bp PCR product of *mecA* gene. **C)** Lane M, DNA ladder; Lane 1, the 305bp PCR product of *spa* gene; Lane 2, the 452bp PCR product of *spa* gene; Lane 3, the 389bp PCR product of *spa* gene, Lane 4, the 187bp PCR product of *spa* gene; and Lane 5 the 235bp PCR product of *spa* gene.

Table 2. *icaABCD* and adhesion profiles of 75 *S. aureus* strains investigated in this study.

***icaABCD* profiles**	***icaABCD* pattern**	**N (%)**
Profile I	*A+D*	29 (38.7)
Profile II	*A+B+C+D*	27 (36)
Profile III	*B+C*	11 (14.7)
Profile IV	*B*	5 (6.6)
Profile V	*D*	2 (2.7)
Profile VI	*A*	1 (1.3)
Adhesion profiles	**Adhesion pattern**	**N (%)**
Profile A	*clfA+clfB+fnbB*	25 (33.3)
Profile B	*clfA+clfB+fnbA+ebp+can*	24 (32)
Profile C	*clfA+clfB+fnbB+fnbA+ebp+can*	20 (26.7)
Profile D	*clfA+fnbB+bap*	2 (2.7)
Profile E	*fnbB+fnbA+can*	2 (2.7)
Profile F	*fnbB+fnbA+ebp+can+bap*	1 (1.3)
Profile G	*fnbB+fnbA+can+bap*	1 (1.3)

t426, t1339, and t7580. All t790, t084, t7789 and t325 *spa* types were biofilm producers, while all t426 and t1339 *spa* types were non-biofilm producers. All of the non-biofilm producer strains carried the *bap* gene which was was mainly found among t790 (2 isolates), t030 (1 isolate), and t325 (1 isolate) strains. The results revealed that *icaABCD* genes was present in all of the t790 biofilm producer isolates, whilst *icaAD* was present in all of the t030 (4 isolates), t008 (1 isolate), and t7580 (2 isolates) which were the non-biofilm producer isolates. Characteristics related to the various *spa* types are presented in Table 3.

The *agr* typing was performed following a previously described method [20]. Representative results are shown in Figure 3B. The positive results were counted and analyzed statistically. Multiplex-PCR analysis permitted classification of the strains into *agr* type III with 33 strains (44%), I with 29 (38.7%) and II with 13 (17.3%). Out of 54 biofilm producer strains, 33 and 21 isolates showed *agr* type III (61.1%) and type I (38.9) respectively. The *agr* typing for non-biofilm producer isolates indicated that type II was the predominant *agr* type (61.9%, 13/21), followed by *agr* II (38.1%, 8/21).

Discussion

Biofilm formation has been considered a virulence factor contributing to *S. aureus* infections and has

Table 3. Molecular characterization of biofilm producer and non-biofilm non-producer strains.

Type of strains	*spa* type	*icaABCD* profile[a] (N;%)	Adhesion profile[a] (N;%)	Drug resistance profile (N;%)	N (%)
Biofilm producer (n = 54; 72%)	t790	Profile II (19; 100)	Profiles B (2; 10.5), C (15;79), and D (2; 10.5)	PG, CIP, E, CD, GM (5; 26.3) PG, E, TS, NI, C, GM (4; 21.1) PG, T, CIP, GM (5; 26.3) PG, T, E, CD, GM (5; 26.3)	19 (25.3)
	t030	Profiles I (5; 62.5), and II (3; 37.5)	Profiles B (2; 25), C (3; 37.5), E (2; 25), and G (1; 12.5)	PG, T, E, CD, CIP (3; 37.5) G, CIP, E, CD, GM (3; 37.5) CIP, E, T (2; 25)	8 (10.7)
	t037	Profiles I (3; 60), II (1; 20), and III (1; 20)	Profiles B (5; 100)	PG, T, E, CD, GM (3; 60) PG, T, CIP, GM (2; 40)	5 (6.7)
	t008	Profiles I (2; 33.3), II (2; 33.3), and V (2; 33.3)	Profiles A (4; 66.7), and B (2; 33.3)	PG, CIP, E, CD, GM (2; 33.3) PG, T, GM, AK, C, CIP (2; 33.3) E, NI, GM, TS (1; 16.7)	6 (8)
	t084	Profiles I (1; 20), II (2; 40), and IV (2; 40)	Profile A (5; 100)	CIP, GM, TS (1; 16.7) PG, T, TS, AK, NI (4; 80) PG, T, E, CD, CIP (1; 20)	5 (6.7)
	t7789	Profiles I (4; 100)	Profiles B (2; 50), and C (2; 50)	PG, T, GM, AK, C, CIP (2; 50) CIP, AK, NI (1; 25) E, TS (1; 25)	4 (5.3)
	t325	Profiles I (2; 66.7), and III (1; 33.3)	Profiles B (2; 66.7), and F (1; 33.3)	PG, T, GM, AK, C, CIP (3; 100)	3 (4)
	t7580	Profiles III (3; 75), and IV (1; 25)	Profile A (4; 100)	PG, T, TS, AK, NI (2; 50) PG, T, E, CD, CIP (2; 50)	4 (5.3)
Non-biofilm producer (n = 21; 28%)	t030	Profile I (4; 100)	Profiles A (2; 50), and B (2; 50)	PG, T, CIP, GM (3; 75) PG, E, TS, NI, C, GM (1; 25)	4 (5.3)
	t037	Profiles I (2; 40), III (2; 40), and VI (1; 20)	Profiles A (3; 60), and B (2; 40)	PG, E, CD, AK, TS (3; 60) PG, T, E, CD, GM (2; 40)	5 (6.7)
	t008	Profile I (1; 100)	Profile A (1; 100)	PG, T, CIP, GM (1; 100)	1 (1.3)
	t426	Profiles I (3; 42.9), III (3; 42.9), and VI (1; 14.2)	Profiles A (2; 28.6), and B (5; 71.4)	PG, T, CIP, GM (3; 42.9) PG, CIP, E, CD, GM (1; 14.2) PG, T, TS, AK, NI (1; 14.2) TS (2; 28.6)	7 (9.3)
	t1339	Profile III (1; 50), and IV (1; 50)	Profile A (2; 100)	CIP, E, T (1; 50) PG, CIP, E, CD, GM (1; 50)	2 (2.7)
	t7580	Profile I (2; 100)	Profile A (2; 100)	PG, T, TS, AK, NI (1; 50) CIP, E, T (1; 50)	2 (2.7)

[a] *icaABCD* and adhesion profile are presented in Table 3; PG, Penicillin; GM, Gentamicin; AK, Amikacin; E, Erythromycin; T, Tetracycline; CD, Clindamycin; CIP, Ciprofloxacin; TS, Trimethoprim-sulfamethoxazole, NI, Nitrofurantoin; C, Chloramphenicol.

become a special concern due to increasing resistance to antibiotics which often leads to treatment failures and persistent infections [5]. Previously published data indicated that the prevalence of biofilm formation among *S. aureus* can vary, ranging from 43-88% [10]. In this study, i*n vitro* evaluation of biofilm formation in *S. aureus* strains revealed a prevalence of 72%, which is higher than the previously reported rate in Iran (38.7%) [22] and lower than the reported rate from Egypt (83.3%) [23]. The frequency of biofilm formation among MRSA isolates (48%) was higher than among MSSA isolates (24%). Our data regarding the ability of biofilm production in MRSA isolates, using CRA and MtP methods, are similar to reported rate in previous studies from India (57.6%) [24], China (66%) [25] and South Africa (37.8%) [26].

Biofilm producer strains were resistant to penicillin (86.7%), gentamicin (66.7%), tetracycline (65.3%), and ciprofloxacin (60%), whereas the rate of resistance to these antibiotics in biofilm non-producers was much lower, consistent with previous reports [15,27,28]. In line with other performed studies, the antimicrobial resistance rate was higher in biofilm-producing *S. aureus* isolates than in non-biofilm producers [9,12,15,29,30].

Based on the literature, many factors, such as environmental conditions, surface adhesion characteristics, and genetic makeup of the organism may influence the development of biofilm in *S. aureus* [5]. Although the presence of the biofilm-related genes did not always correlate with actual biofilm production, there are many reports that biofilm formation ability in *S.aureus* causing UTIs is associated with the presence of *ica ABCD* genes [11,12,25,29,30]. According to our data, *icaD* (77.3%) and *icaA* (76%) were the most prevalent genes. Similarly, Mirzaee *et al.* [22], investigated biofilm formation in *S. aureus* isolates from clinical blood cultures and reported that 38.7% isolates were strong biofilm producers and that *icaD*, *icaA*, *icaB* and *icaC* genes were found in 80.6%, 51.6%, 45.1%, and 77.4%, respectively. Yousefi *et al*. reported that out of 39 isolates of *S. aureus* obtained from patients with UTI, 69.2% were biofilm producers and all of the investigated isolates were positive for the presence of *icaA*, *fnbA* and *clfA* genes [15]. In concordance with Mirzaee's report on the rate of 38.7% for strains carrying *ica* genes, we found a high percentage of *icaABCD* genes among tested isolates (36%) [22]. Although previously published data suggest that biofilm formation, despite the presence of the *ica* genes, may not occur under *in vitro* conditions, our findings indicated that isolates having more adhesion and *ica* encoding genes were strictly associated with biofilm formation. We did identify strains that were unable to form biofilms while they were positive for the presence of *ica* genes, which may have resulted from negative regulatory systems acting on *ica* genes and its inactivation. However, our results confirmed the important role of *ica* genes as biofilm producer markers and as a determining factor for the level of biofilm production in *S. aureus*, especially in MRSA strains.

It is notable that adhesion related genes also play a key role in biofilm formation by *S. aureus* strains [5,8]. In our study, *clfA* (94.7%) and *clfB* (92%) were the most prevalent adhesion genes. Ghasemian *et al*. reported a high prevalence of *clfA*, *clfB* genes in comparison to other examined adhesion genes, in relative agreement with the findings of this research [31], while Nourbakhsh *et al*. reported 38.1%, 46.6%, 41.4%, 44.1%, 26.5% and 18.3% for *fnbA*, *fnbB, clfA*, *clfB*, *ebps*, and *can* genes respectively [12]. In contrast, Ghasemian *et al*. reported the frequency rate of 78% and 7%, for *can* and *ebps* genes respectively [31]. In line with the studies which have displayed the role of *bap* gene in biofilm production rarely [32], in our current study the *bap* gene was detected in 5.3% of isolates.

In our study the ten different *spa* types detected had variable biofilm formation abilities. In agreement with data which indicated t790 as the most common *spa* type in Iran [33], the present research reports a prevalence of this *spa* type in 25.3% of isolates. We also found that 790 isolates had a greater propensity to form biofilm compared to other *spa* types. This would suggest that the ability to form biofilm may be an important virulence factor in t790, and may explain the high dissemination of this *spa* type in hospitals.

We found that t790, t084, t7789 and t325 *spa* types were predictive for the biofilm phenotype, while non-biofilm producing isolates were associated with t426 and t1339 *spa* types. Overall, isolates belonging to the same *spa* types exhibited a similarity in biofilm formation and adherence pattern (see Table 3). This is in line with study of Croes *et al*. [34] that indicated that ability to produce biofilms is dependent on a certain genetic lineage and especially the genetic background of protein A.

A study conducted by Croes *et al*. in the Netherlands noted similar strain distributions in biofilm formation [34]. They revealed that strains associated with clonal complex 1 (CC1), CC5, CC8, CC22, CC30 and CC45 were strong biofilm formers. Their results showed that biofilm formation on polystyrene surfaces

was associated with CC8 strains. In a study published in 2012 by Atshan *et al*. comparing phenotypic and genotypic characteristics of biofilm producing *S. aureus* strains, the most common biofilm producers belonged to CC8 (53.3%) followed by CC1 (20%), CC22 (16.7%), and CC7 (10%) [35]. Their results also showed that all 30 MRSA isolates carried out *icaADBC*, *fnbA*, *eno*, *ebps*, *clfA*, and *clfB* genes. In accordance with the results of previous studies evaluating genotypically different clones of MRSA and their production of a biofilm [34,35], we found that *spa* types t030, t037, t008, and t7580 were isolated from both producing and non-producing biofilm strains. Atshan *et al*. also reported enhanced ability to adhere among t037 isolates associated with ST-239- CC8-IIIA, compared to t932 isolates belonging to the same ST-239- CC8-IIIA clone, which may be attributed to heterogeneity in genetic background [35].

Although there are conflicts in reported data regarding *agr* types and ability to form biofilm (*in vivo* or *in vitro*), different studies revealed a relationship between biofilm formation and specific *agr* genotype(s) in *S. aureus*. In contrast to Cafiso *et al*. [36] and Ćirković and colleagues [37] who found that *agr* type II strains were significantly stronger biofilm formers than other *agr* types, our results demonstrated a linkage between *agr* type III and the capacity to form biofilm.

Conclusion

The present study revealed that different isolates of *S. aureus* had diverse abilities to form biofilm. Our screening for the presence of biofilm related genes and specific types of *S. aureus* indicated key roles for *icaAD*, *clfA*, *clfB*, *agrIII* and *spa* type t790 in biofilm formation. As we observed, biofilm formation is very complex. Therefore, it is of great importance to identify genotypes and do biofilm quantification in different clonal lineages to develop effective antimicrobial policy and for biofilm management upon infection.

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Authors' Contributions

Conceived and designed the experiments: MG HG. Performed the experiments: MF MG AM. Analyzed the data: AA MG MJN AH. Contributed reagents/materials/analysis tools: MG HG. All authors read and approved the final manuscript.

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