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Original Article

Characterization of *Staphylococcus aureus* Biofilm Formation in Urinary Tract Infection

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Abstract

Background: The aim of this study was to investigate the antibiotic susceptibility pattern as well as the phenotypic and genotypic biofilm formation ability of *Staphylococcus aureus* isolates from patients with urinary tract infection (UTI).

Methods: A total of 39 isolates of *S. aureus* were collected from patients with UTI. The antibiotic susceptibility patterns of the isolates were determined by the Kirby-Bauer disk-diffusion. We used the Modified Congo red agar (MCRA) and Microtiter plate methods to assess the ability of biofilm formation. All isolates were examined for determination of biofilm related genes, *icaA*, *fnbA*, *clfA* and *bap* using PCR method.

Results: Linezolid, quinupristin/dalfopristin and chloramphenicol were the most effective agents against *S. aureus* isolates. Overall, 69.2% of *S. aureus* isolates were biofilm producers. Resistance to four antibiotics such as nitrofurantoin (71.4% vs. 28.6%, *P*=0.001), tetracycline (57.7% vs. 42.3%, *P*=0.028), erythromycin and ciprofloxacin (56% vs. 44%, *P*=0.017) was higher among biofilm producers than non-biofilm producers. The *icaA*, *fnbA* and *clfA* genes were present in all *S. aureus* isolates. However, *bap* gene was not detected in any of the isolates.

Conclusion: Our findings reinforce the role of biofilm formation in resistance to antimicrobial agents. Trimethoprim-sulfamethoxazole and doxycycline may be used as an effective treatment for UTI caused by biofilm producers *S. aureus*. Our results suggest that biofilm formation is not dependent to just *icaA*, *fnbA*, *clfA* and *bap* genes harbor in *S. aureus* strains.

Keywords: Urinary tract infection, *Staphylococcus aureus*, Biofilm formation, Antibiotic resistance

Introduction

Urinary tract infection (UTI) is one of the most common infectious diseases in humans both in the clinical and community settings. Its global incidence is estimated to be 250 million cases each year (1, 2). *Escherichia coli* is the most prevalent causative organism of UTI, accounting for about 80% of bacterial isolates (3, 4). However, involvement of Gram-positive bacteria cannot be ruled out in relation to UTI. *Staphylococcus aureus* is one of such agents involved in the infection that is capable of invading the urinary tract. Although *S. aureus* accounts to 0.5-6% of UTI, but if leave untreated infection can lead to severe life-threatening condition (5, 6).

Emergence of multidrug-resistant (MDR) *S. aureus* has become an increasing health concern worldwide (7). Currently, it is estimated that biofilms are responsible for more than 65% of all nosocomial infections and 80% bacterial infections. Bacterial biofilms can play an important role in recurrent urinary tract infections and resistance to antimicrobial agents (8, 9). In addition,

the proximity of cells within the biofilm structure can facilitate genetic elements exchange and hence enhance the possible spread of genes responsible for antibiotic resistance (10, 11). *S. aureus* is known to form biofilms on various surfaces. This pathogen, can invade renal tissue causing UTI by adherence to uroepithelium and formation of biofilm. Since the ability of biofilm production in *S. aureus* can increase resistance to commonly used antibiotics, hospitalized patients infected with this organism are at significant risk for treatment failure (12, 13).

S. aureus biofilm formation is regulated by expression of polysaccharide intracellular adhesion (PIA), which mediates cell to cell adhesion and is encoded by the *ica*ADBC operon (14). Moreover, surface-associated proteinaceous adhesins can contribute to the adherence, colonization and biofilm formation of *S. aureus*. This pathogen can express a variety of microbial surface components recognizing adhesive matrix molecules (MSCRAMMs), such as fibronectin-binding proteins A (FnbA), clumping factors A (ClfA) and biofilm-associated protein (Bap) (15, 16). However, the mechanism of biofilm formation and pathogenicity of *S. aureus* infections in the urinary tract is controversial. Recently, the study of genes involved in biofilm formation and their role in infections caused by *S. aureus* have attracted great interest (12).

Given the role of biofilm related genes in biofilm formation and antibiotic resistance, the need for study is more than ever. Therefore, the aim of this study was to evaluate of biofilm formation and antimicrobial resistance in *S. aureus* isolated from urinary tract infection.

Material and Methods

Bacteria isolates

The study was conducted with a total of 39 isolates of *S. aureus* among UTI patients collected at Sina Hospital, Tehran University of Medical Sciences (TUMS). *S. aureus* isolates were confirmed using conventional microbiological methods (Gram's stain, catalase, coagulase, DNase tests, and mannitol fermentation on mannitol salt agar [Merck, Germany]). MRSA strains were identified phenotypically using cefoxitin disk diffusion method (30 µg; MAST, UK). This method was performed according to the Clinical and laboratory standards institute (CLSI) guidelines (17).

Antibiotic susceptibility determination

The antibiotic susceptibility patterns of *S. aureus* isolates were determined by the Kirby-Bauer disk-diffusion method, and the results were interpreted according to CLSI guidelines (17). The antimicrobial agents (MAST, UK) tested in this study included erythromycin (15 µg), tetracycline (30 µg), ciprofloxacin (5 µg), chloramphenicol (30 µg), rifampin (50 µg), nitrofurantoin (300 µg), linezolid (30 µg), quinupristin/dalfopristin (15 µg), clindamycin (2 µg), doxycycline (30 µg), trimethoprim-sulfamethoxazole (25 µg) and gentamicin (10 µg). *S. aureus* ATCC 25923 was used as a standard strain.

Biofilm formation assay

Modified Congo red agar method (MCRA)

Phenotypic production of biofilm in all *S. aureus* isolates was assessed by culture on MCRA plates as previously explained (18). Briefly, CRA plates were prepared adding 0.8 g of Congo red (Merck, Germany) and 36 g of saccharose (Sigma, USA) to 1 liter of brain heart infusion agar (BHI agar, from Merck, Germany). The plates were incubated for 24 h at 37 ºC, and subsequently over night at room temperature. The morphology of colonies was then interpreted based on colony color as red, almost black, black, and very black. Very black and black colonies were considered as strong biofilm producer strains, while almost black colors were indicative of a weak biofilm production activity. Conversely, strains with red colonies were classified as strains unable to produce biofilm.

Microtiter plate assay

Biofilm production was determined quantitatively using a modified Microtiter plate method as described previously (19). Briefly, bacterial isolates were grown in tripticase-soy broth (TSB, from

Merck, Germany) with 0.5% glucose and incubated at 37 ºC for overnight. Cultures were diluted 1:40 in fresh TSB-0.5% glucose. Then 200 µl of the diluted solution was added to wells of a flat-bottomed polystyrene microtitre plate and incubated for 48 hours at 37 ºC. The negative control wells contained 200 µl of TSB-0.5% glucose. Wells were gently washed 3-times with phosphate-buffered saline (PBS; pH 7.2), fixed by methanol for 20 min, dried at room temperature, and then strained with 0.1% safranin. The safranin dye bound to the adherent cells was dissolved with 1 ml of 95% ethanol per well. Finally, the optical density (OD) of each well was measured at 490 nm (A490) using ELISA reader. Optical density cut-off (ODc) defined as average OD of negative control + 3× standard deviation (SD) of negative control. Formation of biofilm by strains was analyzed and categorized based on the absorbance of the safranin-stained attached cells (Table 1). *Staphylococcus epidermidis* ATCC 35984 was used as the biofilm producer control strain.

Table 1: Classification of biofilm formation abilities by microtiter plate method

Cut-off value calculation	Mean of OD values results	Biofilm formation abilities
OD > 4×ODc	OD > 0.236	Strong
2×ODc < OD ≤4×ODc	0.118< OD ≤ 0.236	Moderate
ODc< OD ≤ 2×ODc	0.059< OD ≤ 0.118	Weak
OD ≤ 0.059	OD ≤ 0.059	None

Gene pattern characterization

Genomic DNA was extracted from pure cultures using High Pure PCR Template Preparation Kit (Roche, Germany) according to the manufacturer. The purified DNA was used for PCR.

In this study, *S. aureus* isolates screening for *icaA*, *fnbA*, *clfA* and *bap* genes using PCR and primers described in Table 2. PCR reaction was conducted on the final volume of 25 µl using HotStar Taq Master Mix kit (SinaClon, Iran) containing 12.5 µl of 2x HotStar Taq Master Mix (Containing 3 mM MgCl2, 0.4 mM of each dNTP and 0.08 U/µl Taq DNA polymerase in reaction buffer), 1 µl of the DNA template, 1 µl of each primer (20 pmol) and 9.5 µl of ddH2O. DNA amplification was performed in a thermocycler (Eppendorf, Hamburg, Germany) with an initial denaturation step at 95 ºC for 5 min, 35 amplification cycles each with 1 min at 95 ºC; 30 seconds at different temperatures for different genes (Table 2); and 50 seconds at 72 ºC, followed by an additional extension step of 10 min at 72 ºC. The amplified products were electrophoresed on 1% agarose gel containing 1x GelRed DNA stain (Biotium, Inc., USA).

Table 2: Target genes and their primers used in this study

Primer	Sequence (5'-3')	Products sizes (bp)	Annealing (ºC)	Ref.
icaA	Fw- CAATCAAGGCATTAAACAGGCTTC Rv- ACCTTTTCGTTTTCATTGTGCTAA	509	62	This study
fnbA	Fw- GATACAAACCCAGGTGGTGG Rv- TGTGCTTGACCATGCTCTTC	191	55	(20)
clfA	Fw- ATTCTGCTGTTAAAGGTGACACAT Rv- GTGTTGTAATTTGATCATCAGGCG	657	62	This study
bap	Fw- CCCTATATCGAAGGTGTAGAATTG Rv- GCTGTTGAAGTTAATACTGTACCTGC	971	62	(21)

Statistical analysis

The relationship between biofilm formation, multidrug resistance and presence of the biofilm related genes among *S. aureus* isolates was evaluated by the Pearson Chi-Square test using SPSS version 21. P values less than 0.05 were considered to be significant.

Results

Antibiotic susceptibility

A total of 39 clinical isolates of *S. aureus* were collected, of which 30 and 9 isolates were MRSA and MSSA, respectively. The overall susceptibility of *S. aureus* isolates to antimicrobial agents was 100% for linezolid and quinupristin/dalfopristin; 97.4% for chloramphenicol; 76.9% for trimethoprim-sulfamethoxazole; 64.1% for rifampin; 43.6% for clindamycin; 41% for nitrofurantoin, doxycycline and gentamicin; 35.9% for erythromycin and ciprofloxacin; and 33.3% for tetracycline. It should be noted that 30.8% and 41% of isolates were intermediate to doxycycline and nitrofurantoin, respectively. Linezolid, quinupristin/dalfopristin and chloramphenicol were the most effective agents against *S. aureus* isolates. Comparison of resistance pattern of MRSA and MSSA strains to antimicrobial agents is shown in Fig. 1.

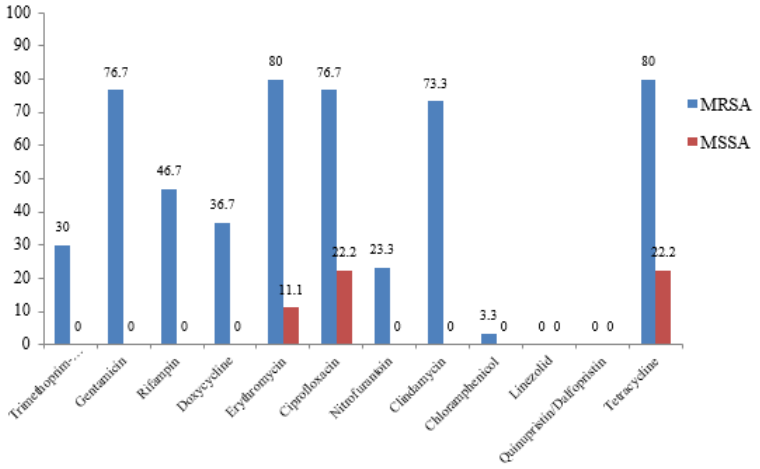

Fig. 1: Antimicrobial resistance pattern of *S. aureus* isolates (MRSA and MSSA).

Biofilm production

Biofilm formation of *S. aureus* isolates were studied by culturing them on Modified Congo red agar (MCRA). The numbers of different biofilm-producing isolates of MSSA and MRSA on MCRA were found to be different with varying degrees like very black, black, weak black, and red colonies (Fig. 2). In this method, 48.7% and 20.5% of *S. aureus* isolates, respectively, showed black/very black colonies (Strong biofilm producers) and almost black (Weak biofilm producers) (Table 3). Moreover, in Microtiter plate method, among the MSSA strains, 33.3% of the strains were strong, 33.3% were moderate, and 22.2% of them were found to be weakly adherent. Whereas among the MRSA strains, 16.6% of the strains were found to be strong, 26.7% were moderate, and 20% were found weakly adherent (Table 3). Overall, in both methods 30.8% and 69.2% of *S. aureus* isolates were non-biofilm and biofilm producers, respectively. Biofilm formation abilities of MSSA strains were found to be slightly higher than those of MRSA strains.

Antimicrobial resistance pattern and phenotypic biofilm formation in *S. aureus* isolates is shown in Table 4. Statistical analysis showed a significant relationship between biofilm formation of *S. aureus* isolates and some antibiotic resistance. Re-

sistance to four antibiotics such as nitrofurantoin (71.4% vs. 28.6%, *P*=0.001), tetracycline (57.7% vs. 42.3%, *P*=0.028), erythromycin and ciprofloxacin (56% vs. 44%, *P*=0.017) was higher among biofilm producers than non-biofilm producers.

Table 3: Biofilm production *S. aureus* isolates in microtiter plate and Congo red agar method

Method	Biofilm formation	MSSA (n = 9)	Percent (%)	MRSA (n = 30)	Percent (%)	Total (n=39)	Percent (%)
Modified Congo red agar	Very black	3	33.3	1	3.3	4	10.2
	Black	4	44.5	11	36.7	15	38.5
	Almost black	1	11.1	7	23.3	8	20.5
	Red	1	11.1	11	36.7	12	30.8
Microtiter plate assay	Strong	3	33.3	5	16.6	8	20.5
	Moderate	3	33.3	8	26.7	11	28.2
	Weak	2	22.2	6	20	8	20.5
	None	1	11.1	11	36.7	12	30.8

Table 4: Antimicrobial resistance pattern and phenotypic biofilm formation in *S. aureus* isolates

Antibiotic	Resistance		Intermediate		Susceptible	
	Biofilm former	Non-biofilm former	Biofilm former	Non-biofilm former	Biofilm former	Non-biofilm former
Erythromycin	14 (56)	11 (44)	0 (0)	0 (0)	13 (92.9)	1 (7.1)
Ciprofloxacin	14 (56)	11 (44)	0 (0)	0 (0)	13 (92.9)	1 (7.1)
Tetracycline	15 (57.7)	11 (42.3)	0 (0)	0 (0)	12 (92.3)	1 (7.7)
Chloramphenicol	0 (0)	1 (100)	0 (0)	0 (0)	27 (71.1)	11 (28.9)
Rifampin	8 (57.1)	6 (42.9)	0 (0)	0 (0)	19 (76)	6 (24)
Nitrofurantoin	5 (71.4)	2 (28.6)	10 (62.5)	6 (37.5)	12 (75)	4 (25)
Linezolid	0 (0)	0 (0)	0 (0)	0 (0)	27 (69.2)	12 (30.8)
Quinupristin/Dalfopristin	0 (0)	0 (0)	0 (0)	0 (0)	27 (69.2)	12 (30.8)
Clindamycin	12 (54.5)	10 (45.5)	0 (0)	0 (0)	15 (88.2)	2 (11.8)
Doxycycline	5 (45.5)	6 (54.5)	7 (58.3)	5 (41.7)	15 (93.8)	1 (6.3)
Trimethoprim-Sulfamethoxazole	4 (44.4)	5 (55.6)	0 (0)	0 (0)	23 (76.7)	7 (23.3)
Gentamicin	12 (52.2)	11 (47.8)	0 (0)	0 (0)	15 (93.8)	1 (6.3)

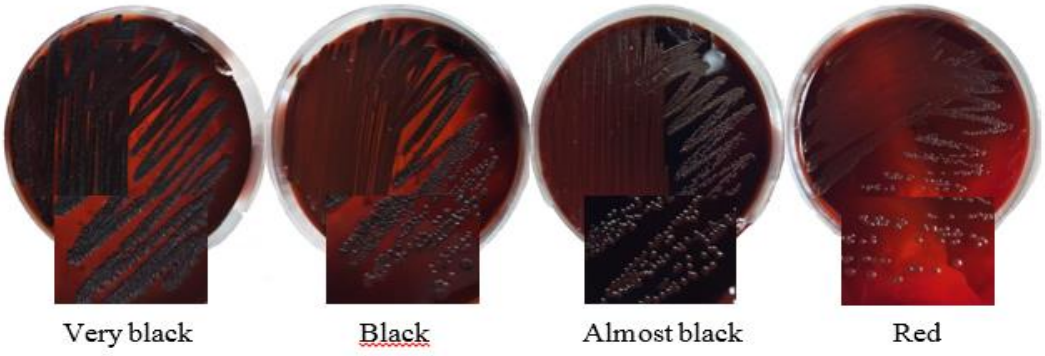

Fig. 2: Colony morphologies of *S. aureus* isolates on the modified Congo red agar medium

Gene pattern characterization

In this study, the presence of biofilm related genes were evaluated in *S. aureus* isolates by PCR method. All isolates were investigated for *icaA*, *fnbA*, *clfA* and *bap* genes. The *icaA*, *fnbA* and *clfA* genes were present in all *S. aureus* isolates (100%). However, *bap* gene was not detected in any of the isolates. PCR- product of *icaA*, *fnbA* and *clfA* genes from *S. aureus* isolates is shown in Fig. 3. Overall, *icaA*, *fnbA* and *clfA* genes were detected in all isolates, and *bap* gene was not found in any of the *S. aureus* isolates from UTI patients; therefore, we were not able to investigate relationship between biofilm formation and the presence of these genes in isolates.

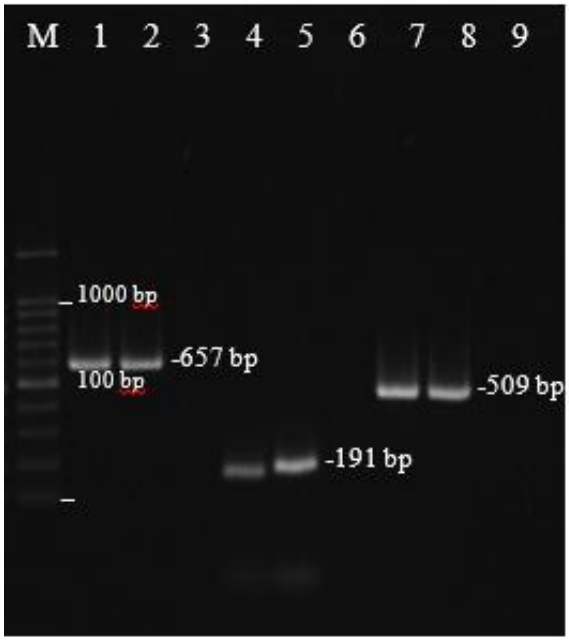

Fig. 3: Amplification of *icaA*, *fnbA*, *clfA* genes from *S. aureus* isolates.
Lane M, DNA marker (100 bp); Lane1 and 2, *clfA* (657 bp); Lane 4 and 5, *fnbA* (191 bp);
Lane 7 and 8, *icaA* (509 bp); Lane 3, 6 and 9, negative controls

Discussion

S. aureus is one of the important Gram-positive bacteria involved in urinary tract infections (UTIs). Although infection caused by this pathogen included low percentage of UTI, it should not be underestimated as untreated infection because can lead to severe health threatening conditions (6, 13). The ever-increasing emergence of antibiotic resistance in such organism has become a health concern (22). In recent years there has been an alarming increase in the prevalence of resistance to methicillin and reduced susceptibility to vancomycin in the *S. aureus* strains. Monitoring of antimicrobial susceptibility can lead physician for prescription of appropriate antibiotics and prevention of emergence of drug resistance (23, 24).

In this study, susceptibility pattern of S. *aureus* isolated from UTI was assessed. According to results of our study, linezolid, quinupristin/dalfopristin and chloramphenicol were the most effective agents against *S. aureus* isolates. Whereas, high resistance of *S. aureus* isolates to tetracycline, ciprofloxacin and erythromycin reported in the current study (66.7%, 64.1%, and 64.1%, respectively) that is consistent with other studies (1, 25). Antimicrobial resistance of MRSA strains to different antibiotics was significantly higher than MSSA strains. Although resistance to nitrofurantoin (59%) has been seen among our isolates, but it seems that it can be effective drugs for treatment of UTI associated with Gram-positive cocci.

Microbial cell adherence to surfaces and the development of multi-cellular communities is a key step in infection. Furthermore, bacterial biofilms can play an important role in recurrent urinary tract infections and resistance to antimicrobial agents (10). In the present study, modified Congo red agar and Microtiter plates methods used for the detection of biofilm production. The results showed that overall 30.8% and 69.2% of *S. aureus* isolates were non-biofilm and biofilm producers, respectively. In our study, resistance to nitrofurantoin, tetracycline, erythromycin and ciprofloxacin was significantly higher among biofilm producers than non-biofilm producers. In addition, resistance to trimethoprim-sulfamethoxazole and doxycycline was relatively less common among

biofilm than non-biofilm producing isolates. Therefore, these antibiotics may be used as an effective treatment for UTI caused by biofilm producers *S. aureus*. Progressive increase in antimicrobial resistance of *S. aureus* isolates to various antibiotics in the present study may be related to increased usage of different antibiotics for treatment of UTI, as well as biofilm-forming ability of strains and acquisition of resistance genes.

In this study, although 69.2% of the *S. aureus* isolates produced biofilms in Microtiter plate and Congo red agar method, the *icaA*, *fnbA*, *clfA* genes were detected in 100% of the isolates by PCR. Other studies have also shown that despite the presence of the *icaA*, *fnbA*, *clfA* genes, biofilm formation may not occur *in vitro* (26, 27). Although some studies have shown the role of biofilm-associated protein (Bap) in biofilm formation rarely (28), in current study the gene for the biofilm associated protein was not detected. These results correspond to previous reports on *S. aureus* isolates (29). Our results indicated biofilm formation regulated by several factors such as, environmental condition. Despite the presence of the *ica* gene, biofilm formation may not occur under *in vitro* conditions since *S. aureus* isolates are highly sensitive to environmental factors, such as the amount of glucose or glucosamine available for matrix formation (30). Furthermore, the difference between phenotypic and genotypic characterization of biofilm formation may result in heterogeneity in the genetic origins, and not because of the presence or absence of genes required for the biofilm.

Conclusion

The high level of antibiotic resistance among *S. aureus* causing UTI limits the use of antimicrobial agents for therapy and also the spread of MDR isolates is a threat for hospitalized patients. This study showed a significant relationship between biofilm formation of *S. aureus* isolates and some antibiotic resistance. So, bacterial biofilms can play an important role in resistance to antimicrobial agents. Trimethoprim-sulfamethoxazole and doxycycline may be used as an effective treatment for UTI caused by biofilm producers *S. aureus*. Our results suggest that biofilm formation is very complex and independent to only *icaA*, *fnbA*, *clfA* and *bap* genes harbor in *S. aureus* strains. Thus, molecular methods for detection of genes involved in biofilm formation are not appropriate methods for the actual biofilm phenotype under *in vitro* conditions.

Ethical considerations

Ethical issues (Including plagiarism, informed consent, misconduct, data fabrication and/or falsification, double publication and/or submission, redundancy, etc.) have been completely observed by the authors.

Acknowledgments

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