Prevalence of Biofilm Formation and Detection of *PSM B* Gene in Clinical Isolates of *Staphylococcus aureus*

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ABSTRACT

Background and Objectives: *Staphylococcus aureus* is a common cause of hospital- and community-acquired infections in the world. This microorganism causes a wide range of diseases, and biofilm formation is as an important mechanism for its virulence. Alpha-toxin and phenol-soluble modulins (PSMs) are among the main factors involved in the biofilm formation by *S. aureus*. The aim of this study was to detect *PSM B* gene among biofilm-forming *S. aureus* clinical isolates from hospitalized patients at the 5th Azar Hospital in Gorgan, Iran.

Methods: Clinical specimens were collected and examined for presence of *S. aureus* using conventional microbiological and biochemical tests. Then, biofilm formation ability of *S. aureus* isolates was evaluated using the microtiter plate assay. In addition, presence of the *PSM B* gene was assessed using real-time PCR.

Results: Of 1,800 clinical isolates, 60 (3.3%) were identified as *S. aureus*. Of these isolates, 47 (78.3%) were positive for biofilm formation. The *PSM B* gene was present in all biofilm-forming isolates. Results of the phenotypic and genotypic biofilm tests were closely linked and the rate of *PSM B* expression was 80%.

Conclusion: The prevalence of biofilm-producing *S. aureus* clinical isolates from patients hospitalized in the 5th Azar hospital of Gorgan (Iran) is relatively high, which could pose a serious challenge. Therefore, regular surveillance of biofilm formation in *S. aureus* isolates and their antimicrobial resistance profiles is highly recommended.

Keywords: *PSM B* gene, clinical isolates, *Staphylococcus aureus*, biofilms.

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INTRODUCTION

Staphylococcus aureus is a common human pathogen that has been considered as the main cause of hospital-acquired infections in the world (1). It causes a wide range of infections, including bacteremia, septicemia and pneumonia, as well as skin, soft tissue and bone infections (2). Respiratory tract infections accounts for majority of the hospital-associated *S. aureus* infections, while skin and soft tissue infections are dominant among community-associated *S. aureus* infections (3). This microorganism has many virulence factors among which biofilm formation is of upmost importance for development of resistance to antimicrobial agents, making it a major problem for hospitalized patients and medical staff (4). Infections caused by biofilm-producing *S. aureus* increase the length of hospital stay and are associated with more clinically important infections such as pneumonia, polyarthritis, necrotizing fasciitis, endocarditis and septicemia (5). Alpha-toxin and phenol-soluble modulins (PSMs) including PSM A and PSM B that are encoded on the core genome of *S. aureus*, have an extensive range of functional activities, such as supporting biofilm formation during *S. aureus* infections (6). Since biofilm formation is a key factor for survival and resistance of *S. aureus* strains, monitoring the prevalence of biofilm-producing *S. aureus* and identification of genes involved in the process are essential, especially in clinical isolates. Therefore, the aim of this study was to detect the *PSM B* gene among biofilm-forming *S. aureus* clinical isolates.

MATERIALS AND METHODS

The study received approval from the Golestan University of Medical Sciences, Iran (ethical approval code: IR.GOUMS.REC.1397.116). Samples were collected from different wards of the 5th Azar Hospital in Gorgan, Iran. The samples were examined for *S. aureus* using conventional biochemical and bacteriological tests such as culture on manitol salt agar, gram staining, catalase, oxidase, coagulase and DNase tests (7).

The biofilm formation ability was evaluated by microtiter plate assay. For this purpose, a 24-hour culture of each bacterial isolate was inoculated in Mueller Hinton agar and TSB medium containing 1% glucose and incubated at 37 °C. When the opacity of the tubes reached 0.5 McFarland standard (0.08%-0.13% absorbance at 625 nm), 200 µl of the bacterial suspension were inoculated into wells of a 96-well microplate. In addition, TSB medium with 1% glucose and *S. aureus* 35556 were used as the negative and positive control, respectively (8). To visualize biofilms, each well was treated with 200 µl of 2% crystalline violet for 5 minutes. The excess stain was discarded and the wells were washed with phosphate buffer saline three times. Next, 200 µl of ethanol-acetone (20-80%) were added to each well to remove the crystals from the bacteria and biofilms. After 30 minutes, absorbance of each well was measured at 570 nm using an ELISA reader. A semi-quantitative analysis of biofilm formation using cut-off calculations was carried out in accordance with the following formula: mean optical density of negative controls + three times the standard deviation was considered as a cut-off point (9, 10). The absorbance of each isolate was measured three times. The method for calculating the quotient amount for each group and interpretation of results is presented in Table 1 and Figure 1.

Table 1- Classification of *S. aureus* clinical isolates based on their biofilm-forming ability using the microtiter plate assay

Mean OD	Cut-off value	Biofilm-forming ability
OD > 1.2	OD > 4×ODc 2	Strong
0.7 < OD ≤1.2	2×ODc<OD ≤4×OD	Moderate
0.3 < OD ≤ 0.6	ODc< OD ≤ 2×ODc	Weak
OD ≤ 0.3	OD > cut off	None

Cut off = average OD of negative control + (3×standard deviation of negative control)

Figure 1- Biofilm formation based on the microtiter plate assay

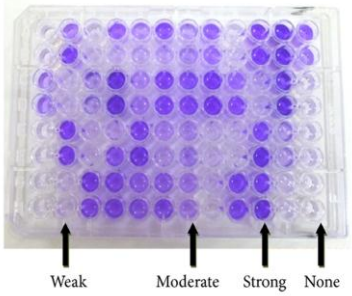

Table 2- Sequence of the primers used for the amplification of *PSM B*

Name	Sequence (5 3)	Product size (bp)	Reference
PSM B (F)	**TAATAATGACGGCGCAAAATTAGG**	**238**	**(20)**
PSM B (R)	**GCAACGATGTCTACGATACTTGTGC**		

Table 3- Preparation of the reaction mixture for real-time PCR

Component	Volume/reaction	Final Concentration
2x QuantiFast SYBR Green Master Mix	**12.5 µl**	**1x**
Primer F	**1µl**	**1µM**
Primer R	**1µl**	**1µM**
cDNA template	**4µl**	**5 ng (1 – 10 ng)**
RNase-free water	**6.5µl**	**-**
Total reaction volume	**25 µl**	**-**

Table 4-Cycling conditions for the real-time PCR

Step	Time	Temperature
PCR initial activations	**15 min**	**95°C**
Denaturation	**15s**	**95°C**
Combined annealing/extention	**60s**	**60°C**
Number of cycles	**35**	

RNA was extracted from the biofilm-producing *S. aureus* isolates using the RNX-Plus kit (CinaGene Co., Iran) as described previously (11). Quality and quantity of the extracted RNA was evaluated by electrophoresis on 1.2% agarose gel and spectrophotometry, respectively.

The amount of gene expression in the extracted RNA was determined by real-time PCR (ABI Prism 7300 Applied Biosystems). Table 2 shows the sequence of the primers used for gene expression analysis. Genomic amplification was performed using QuantiFast SYBR Green Master Mix (Qiagen, Hilden, Germany) as described in Table 3. The cycling conditions are displayed in table 4.Descriptive quantitative variables were computed by calculating central indexes, dispersion and plotting. Qualitative variables were computed by calculating the frequency percentage. All statistical analyses were performed in SPSS 16 using ANOVA and chi-square tests. A p-value of less than 0.05 was considered as statistically significant.

RESULTS

Of 1,800 clinical isolates, 60 isolates (3.3%) were identified as *S. aureus*. Of these isolates, 27 (47%) were isolated from men and 33 (53%) were isolated from women. The mean age of patients with *S. aureus* bacteremia was 37.8 years and the highest rate (40%) of *S. aureus* carriers was observed in patients aged 15-25 years. There was no statistically significant correlation between the mean age of patients and *S. aureus* infection (P=0.03).

Of the 60 *S. aureus* clinical isolates, 47 (78.3%) were positive for biofilm formation. The biofilm-forming strains were isolated from urine (n=12), blood (n=5), cerebrospinal fluid (n=8), wound (n=17) and abscess (n=5) specimens. Based on the results of the microtiter plate assay, 30 (50%) isolates were strong biofilm producer, 17 (28.3%) isolates were weak biofilm producer and 13 (21.7%) isolates were non-biofilm producer. No obvious correlation was found between specimen source, gender of patients and the biofilm formation ability (P>0.05).

Based on the results of the real-time PCR experiment, all 47 biofilm-producing isolates were positive for the *PSM B* gene. Moreover, results of the phenotypic and genotypic characterization of biofilm formation were closely related to each other and the rate of *PSM B* expression was 80%.

DISCUSSION

As a common cause of hospital- and community-acquired infections, biofilm-producing and resistant *S. aureus* strains are of great clinical importance. Biofilm formation helps bacteria adhere to tissues or medical indwelling devices, which could potentially cause life-threatening infections. Alpha-toxin and PSMs are involved in biofilm formation by *S. aureus* strains (12). Recently, concerns have been raised about the survival and resistance of biofilm-forming strains. As a result, monitoring the prevalence of biofilm-producing *S. aureus* and identification of genes involved in the process are essential, particularly in clinical isolates (13).

In two previous studies, more than 85% of *S. aureus* clinical isolates were able to form biofilm (14, 15). In a study conducted by Christensen et al., 48.5% of clinical isolates were able to produce biofilm (16). Cafiso et al. reported that 57.5% of isolates from in-patient medical equipment were biofilm producer (17). In the present study, the prevalence of strong, weak and non-biofilm producing isolates was 50%, 28.3% and 21.7%, respectively. In another study in Iran, 68% of clinical isolates from a hospital were strong biofilm producer (18). Consistent with the result of our study, the prevalence of biofilm-producing *S. aureus* isolates from hospital infections in Shahrekord (the capital city of Chaharmahal and Bakhtiari Province, Iran) was 86% (19). Namvar et al. detected the intercellular adhesion gene cluster (ica) in *S. aureus* clinical isolates and reported the prevalence of biofilm-producing isolates to be 58% in the quantitative biofilm assay (20). In our study, 47 (78.3%) isolates were identified as biofilm-producing strains. Moreover, all 47 biofilm-forming isolates contained the *PSM B* gene. In a study by Fursova et al., the *PSM B* gene was present in 95% of the isolates (21).

Given that biofilm formation is associated with development of antimicrobial resistance, detection of the genes involved in the biofilm formation process and regular monitoring of biofilm formation by clinically important bacteria such as *S. aureus* may contribute to early treatment of infections.

CONCLUSIONS

The prevalence of biofilm-producing *S. aureus* clinical isolates from hospitalized patients in the 5th Azar hospital of Gorgan (Iran) is relatively high, which could pose a serious challenge. Therefore, regular surveillance of biofilm formation in *S. aureus* isolates and their antimicrobial resistance profiles is highly recommended. It is also crucial to disinfect and sterilize surfaces and medical equipment in the hospital setting to minimize the risk of contamination and spread of bacteria.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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