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Prevalence and Expression of *PSM A* Gene in Biofilm-Producing *Staphylococcus aureus* Clinical Isolates

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Abstract

Background: *Staphylococcus aureus* is a frequent cause of hospital and community-associated infections on a global scale. This pathogen is responsible for causing an extensive range of diseases and sometimes create biofilms for their survival. Biofilm formation, leads to difficulty in treatment with antibiotics and antibiotic resistance which is another rising concern in health centers. Some *S. aureus* virulence factors encode on the core genome, such as alpha-toxin and phenol-soluble modulins (*PSMs*), which are produced by nearly all strains.
Objectives: There is no information about the expression of *PSM A* gene in clinical isolates of biofilm-producing *S. aureus* in Iran. This study was performed on clinical samples to determine the prevalence and expression of *PSM A* gene in biofilm-producing *S. aureus* clinical isolates.
Methods: The clinical samples were collected and examined for *S. aureus* by microbiological and biochemical tests. Then, the biofilm formation in *S. aureus* isolates was detected by microtiter plate. Finally, the expression of *PSM A* was determined using SYBR Green real-time PCR.
Results: From a total of 60 isolates of *S. aureus*, 47 strains (78.3%) had ability of biofilm formation and the others were negative for biofilm formation. Real-time PCR testing showed that 100% of the strains were positive for biofilms and *PSM A* genes. The results of phenotypic and genotypic tests of biofilm were closely related to each other and the expression of *PSM A* gene was 80%. It was found that 100% of strains were biofilm producing and *PSM A* gene was present in 78.3% (47 strains) of them.
Conclusions: The prevalence of biofilm production in *S. aureus* strains isolated from clinical samples was high, so it is highly important to monitor the prevalence of these organisms in hospitals and community as well as their antimicrobial resistance.

Keywords: *PSM A* Gene, *Staphylococcus aureus*, Clinical Isolates, Biofilm

1. Background

Hospital infections are a global health problem associated with a variety of factors. The causes of these infections are various among different countries. Since the 1980s, Gram-positive bacteria, especially *Staphylococcus aureus*, has been considered as the main cause of hospital infections (1). The biofilm formation by *S. aureus* and its resistance to antimicrobial agents have made this organism a major problem for hospitals and medical staff (2). The *S. aureus* causes a wide range of infections, including bacteremia, septicemia, and pneumonia, as well as skin, soft tissue and bone infections (3). *Staphylococcus aureus* is a frequent cause of hospital- and community-associated infections on a global scale (4). However, *S. aureus* can also cause a series of other diseases, including endocarditis or osteomyelitis. Often, *S. aureus* infection can proceed to septicemia and become life-threatening. Furthermore, *S. aureus* is a frequent cause of biofilm-associated infections, in particular those developing on indwelling medical devices (5). Finally, *S. aureus* can cause food poisoning (3).

Some *S. aureus* virulence factors such as alpha-toxin and phenol-soluble modulins (PSMs) encode on the core genome, which is virtually produced by all strains. Alpha-PSM peptides of *S. aureus* are toxins that play a role in infection and neutrophil lysis after phagocytosis. It is an important mechanism in the pathogenesis of highly invasive strains of *S. aureus* (6). The alpha 1 *PSM* and alpha 2 PSM peptides have antibacterial activity by lysing competitive bacteria (7). The *PSM* activates the host immune system by regulating the production level of lymphokine (8). Phenol-soluble modulins, also have a wide range of func-

tional activities, including supporting biofilm formation during *S. aureus* infections (9). During *S. aureus* growth in biofilms, bacteria become tolerant to concentrations of antimicrobials that could eliminate single-cell bacteria, making biofilm infections particularly difficult to eradicate (10).

Studies show the multi-faceted role of PSMs in biofilm development: it helps in making bacterial biofilms' structure by its surfactant activity but on the other hand, its expression can also lead to biofilm dispersal, i.e. the detachment of cells or cellular clusters from biofilms, which is a key strategy leading to the systemic dissemination of biofilm infection (11). In regard to the importance of biofilm as a key factor in survival and antimicrobial resistance of *S. aureus* strains, the prevalence of biofilm-producing *S. aureus* and identification of genes involved in biofilm formation is highly significant, especially in clinical samples. The discovery that PSMs have been recognized as key players in staphylococcal pathogenesis, prompted us to determine expression of the *PSM A* as a first gene of PSM class of peptides (9).

2. Objectives

There is no information about the expression of *PSM A* gene in clinical isolates of biofilm-producing *S. aureus* in Iran. In the present study, we report, for the first time, the prevalence and expression of *PSM A* gene in biofilm-producing *S. aureus* clinical isolates in Iran.

3. Methods

3.1. Samples and Identification

Samples were collected from different wards of 5th Azar Hospital, Gorgan, Golestan province, Iran from different sources (wound, CSF fluid, urine culture, abscess, pleural fluid and ascites fluid) from September 2018 to March 2019. The samples were examined for *S. aureus* using conventional bacteriological methods such as culture on manitol salt agar, Gram staining, other biochemical tests (catalase, oxidase and coagulase) and manitol fermentation or DNase test if necessary (12). For all biochemical tests, the standard strain of *S. aureus* ATCC 25923 was used as positive control.

3.2. Biofilm Formation

Biofilm study on *S. aureus* (Biofilm Power): The ability of biofilm formation in the isolates was examined by microtiter plate (crystal violet) (13, 14) as following: A 24-hour culture of each bacterial isolate was prepared and inoculated in Mueller Hinton agar medium, TSB medium containing 1% glucose and incubated at 37°C. When the opacity of the tubes were equal to 0.5 McFarland standard (0.08% - 0.13% absorbance at a wavelength 625 nm) (12), 200 μL of suspension was inoculated into a 96-well polystyrene microplate. As a negative control, 200 μL of TSB medium with 1% glucose without bacteria was added to other wells and *S. aureus* ATCC 35556 (the strain of strong biofilm producer) was used as a positive control (15). To view biofilms, 200 μL of crystalline violet 2% was added to each well for 5 minutes.

The excess stain was discarded and then washed three times with PBS. To remove the crystal from the bacteria and biofilm, 200 μL of ethanol-acetone (20% to 80%) was added to each well. After 30 minutes, the optical absorption of each well was measured by an ELISA reader at a wavelength of 570 nm. A semi-quantitative study of biofilm formation using cut-off calculations was carried out in accordance with the following formula (16, 17). The mean OD of negative controls + three times the standard deviation was considered as a cut- off point. To ensure the correctness of the work for the isolates studied, the absorbance of each isolate was examined three times. The method of calculating the quotient amount for each group is presented in Table 1 and Figure 1.

Table 1. Classification of Isolates Ability to Form a Biofilm Using Microtiter Plate Method (18)

Biofilm Formation Ability	Calculate the Cut-Off[a]	Results of Mean (Maximum OD Absorption)
Strong	$OD > 4 \times ODc$	$OD > 1.2$
Moderate	$2 \times ODc < OD \leq 4 \times ODc$	$0.7 < OD \leq 1.2$
Weak	$ODc < OD \leq 2 \times ODc$	$0.3 < OD \leq 0.6$
None	$OD <$ cut off	$OD \leq 0.3$

[a] Cut-off: average of OD negative control + (3 × SD of negative control).

3.3. Evaluation of PSM A Expression in Biofilm-Producing Staphylococcus aureus Isolates

3.3.1. Extraction and Purification of RNA

RNA extracted from *S. aureus* biofilm for studying on *S. aureus* PSM A gene expression. RNA extraction from bacterial biofilm was performed using the RNX-Plus kit (CinnaGene Co.), these steps were as follows: For total RNA isolation RNX-Plus, a guanidine/phenol solution which contains guanidine thiocyanate and phenol, was used to extract the entire RNA from homogeneous samples. The solution was prepared in a volume of 25 mL from the CinnaGene company and maintained at 2 - 8°C. The bacterial

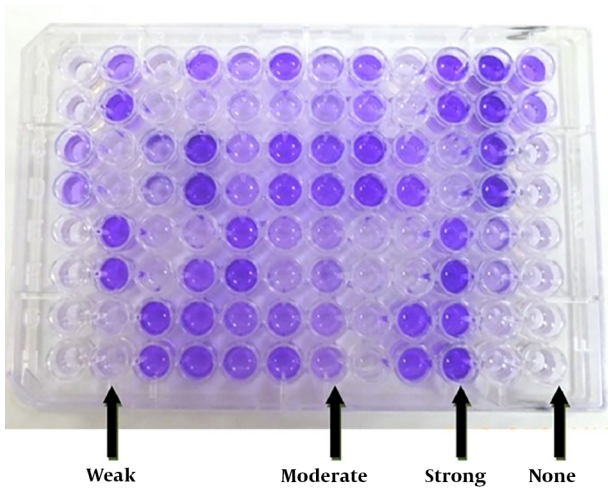

Figure 1. Biofilm formation based on microtiter plate method in ELISA plates

isolates were each grown for 24 hours at 37°C in Mueller Hinton agar. The medium was poured and washed three times with distilled water to remove non-adherence free cells and replaced by fresh preheated medium every day .For extraction, in addition to the solution, chloroform, isopropanol, 75% ethanol and water treated with DEPC were required. After the disruption, 1 mL of RNX-Plus solution was added to the lysed product and mixed well for 5 to 10 seconds and incubated at room temperature for 5 minutes. After that, 200 μL of chloroform was added and washed with vigorous shaking for 15 seconds (not vortex). The micro tube was incubated for 4 minutes at 5°C. The sample was centrifuged for 15 minutes at 12000°C and at 4°C.

3.3.2. Evaluation of Extracted RNA Quality

The quality and quantity of extracted RNA was evaluated using electrophoresis and agarose gel 1.2% for qualitative evaluation and nanotubes or biophotometers for quantitative ones.

3.3.3. Expression of PSM A

To determine the amount of gene expression in extracted RNA, the real-time PCR of the SYBR Green bark was used. To perform this RealQ Plus 2x Master Mix Green Kit was applied. Real-time PCR was achieved by an ABI Prism 7300 Applied Biosystems thermocycler. The primers were confirmed at the NCBI site, using the Blastas shown in Table 2. Genomic amplification was performed by using QuantiFast SYBR Green Master Mix (Qiagen, Hilden, Germany) (as described in Table 3). The reactions were mixed appropriately into the PCR stacks and cDNA sample (1 - 10 ng) added to each well containing a reaction mixture. The amplifications were performed on a ABI Prism 7300 Applied Biosystems device (Applied Biosystems, Foster, CA, USA) and thermal cycling protocol (as shown in Table 4).

Table 2. Features of *PSM A* Primers Used in the Study

Name	Sequence (5 to 3)	Product Size (bp)	Reference
***PSM A* (F)**	CTTTCACATGGGTATCATTGCAGG	266	(8)
***PSM A* (R)**	CAATAGCCATCGTTTTGTCCTCCT		

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	1 x
Primer F	1 μL	1 μM
Primer R	1 μL	1 μM
Template of cDNA	4 μL	5 ng (1 - 10 ng)
RNase-free water	6.5 μL	-
Total reaction volume	25 μL	-

Table 4. Real-Time PCR Cyclic Conditions

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

3.4. Statistical Analysis

Descriptive quantitative variables were computed by calculating central indexes and dispersion and plotting, and qualitative variables were computed by calculating the percentage of frequency. Also, information obtained from the samples and the results of the evaluations was entered into SPSS V. 16 software and analyzed by ANOVA and chi-square. In all cases a significant level of $P < 0.05$ was considered.

4. Results

4.1. Isolation and Identification of Staphylococcus aureus

A total of 60 strains of *S. aureus* were isolated from 1800 clinical isolates. Of 60 *S. aureus* 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 of *S. aureus* carriers was in the age group of 15 - 25 years (40%). There was no statistically significant correlation (P = 0.03) between the mean age of patients with *S. aureus* infection.

4.2. Quantitative Biofilm Production Test

Of the 60 *S. aureus* clinical isolates included in this study, 47 strains (78.3%) were identified as biofilm producer and the others 13 (21.7%) were negative 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. The microtiter plates assay results showed that 30 (50%) isolates were strong biofilm producers and 17 (28.3%) isolates were weak biofilm producers and 13 isolates (21.7%) were found as non-adhesive. No obvious correlation was observed (P < 0.05) between source of bacteria and sex of patients, and potential of biofilm formation in strains.

4.3. Determination of PSM A Gene

All the primers used in the study showed specificity with a single band. After real-time PCR testing to detect PSM gene it was found that 100% of the strains were positive for biofilms and *PSM A* genes. The results of phenotypic and genotypic tests of biofilm were closely related to each other and the expression of *PSM A* gene was 80%. In addition, the results showed that 100% of strains were biofilm producers and the *PSM A* gene was present in 47 (78.3%) strains.

5. Discussion

Staphylococcus aureus is one of the important bacteria with the capability to stick and produce biofilms on external surfaces which cause community-acquired infections. Increasing antibiotic resistance among biofilm producers in hospital settings makes the therapy of staphylococcal infection an international challenge (13). Several studies showed the biofilm formation and genetics characteristics of different isolates of *S. aureus* (19-22). Consistent with the result of our study, Nourbakhsh and Momtaz reported the 86% of *S. aureus* isolates isolated from hospital infections in Shahrekord (the capital city of Chaharmahal and Bakhtiari province, Iran) were able to form biofilms (23). Arya et al. also reported the same results. They isolated 52 strains of *S. aureus* from blood culture of volunteers. In this study, 87% of strains isolated from blood culture were positive for biofilms (24). Christensen et al. showed that 48.5% of the clinical strains of *S. aureus* were able to produce biofilms (25). Similar to the current research, in a study published in 2007, Cafiso et al. reported that 57.5% of *S. aureus* strains isolated from in-patient medical equipment were biofilm producers (26).

In this study, according to quantitative evaluation of biofilm production, 30 isolates (50%) as sticky strains, 17 strains (28.3%) as weak adhesive strains and 13 strains (21.7%) as non-adhesive strains were considered. Mirzaee et al. in 2014 reported that 46% of clinical isolates of methicillin resistant *S. aureus* (MRSA) were strong producer of biofilm on microtiter tissue culture plates (21). Croes et al. in 2009 proposed that strong biofilm formation in *S. aureus* could not be attributed to a specific accessory gene regulator (agr) genotype, as suggested previously (27). Eftekhar and Dadaei in 2011 showed that 53.3% of MRSA isolates had the potential to form biofilm by colony morphology of which, 75% carried the ica operon. Weak biofilm production was observed in the microtitre plate assay by 57.8%, of which 53.8% harbored the ica operon. However, about 70% of biofilm non-producers also carried the ica operon (28).

Namvar et al. detected the intercellular adhesion gene cluster (ica) in clinical *S. aureus* isolates. Their results showed that in the quantitative biofilm assay, 58% produced biofilm while 42% isolates did not exhibit this property (29). In present study, 47 (78.3%) strains were identified as biofilm- producing strains and 13 (21.7%) strains were negative for biofilm. According to the results of studies from all different parts of the world, it seems that the ability to produce biofilms among strains isolated from patients is very close to the present study, which indicates the cloning of patients with these strains has a high prevalence in Iran.

Based on our knowledge, expression of some genes is responsible for adhesion and biofilm formation in bacteria. Quantification of the gene expression involved in biofilm formation by *S. aureus* strains has been investigated in several studies (21, 22). In *S. aureus*, all PSMs have biofilm-structuring activities through their physicochemical properties (11). In this study, the prevalence and expression of *PSM A* gene in clinical isolates of biofilm producing *S. aureus* was evaluated. The *PSM A* genes were present in 78.3% of all biofilm-producing isolates. Consistent with our result, in the Fursova et al. study, the *PSM A* gene was detected in 81.6% of the isolates (30).

Given that biofilm production and pathophysiology of *S. aureus* infection are multifactorial and controlled by various genetic factors, a complete review of the genes involved in the process of biofilm formation is needed. The high prevalence of biofilm strains of *S. aureus* in patients is a warning and a serious health risk to the community. The introduction of these bacteria into the genitourinary system will become a major problem that will make it really difficult to eliminate and eradicate. Such a large-scale study in Iran is absolutely essential for screening and ac-

cess to patient information.

5.1. Conclusions

The high prevalence of biofilm producing *S. aureus* isolates could pose a major health challenge with serious consequences for hospitalized patients. Therefore, it is crucial to disinfect and sterilize hospital surfaces and equipment effectively to minimize the risk of contamination and spread of bacteria in the hospital settings.

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Footnotes

Authors' Contribution: Hami Kaboosi and Ailar Jamalli conceived and designed the experiment. Abolfazl Khandan Del and Fatemeh Peyravii Ghadikolaii conducted the study and collected the samples. Abolfazl Khandan Del, Hami Kaboosi and Fatemeh Peyravii Ghadikolaii performed the experiments and analyzed the data. All authors contributed to paper writing. The authors have read and approved the final manuscript.

Conflict of Interests: The authors declare that there is no conflict of interest regarding the publication of the current paper.

Ethical Approval: This study was approved by the Research Ethics Committee of Golestan University of Medical Sciences, Gorgan, Iran (project approval code: 110152 and code of ethics: IR.GOUMS.REC.1397.116).

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