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Prevalence, Antibiotic Resistance Pattern, Screening of *psm* A and *psm* B Gene Expression, and in Vitro Analysis of Biofilm Formation of *Staphylococcus aureus* Isolated from Clinical Samples

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ABSTRACT

Backgrounds: *Staphylococcus aureus* is one of the major causes of nosocomial infections. Biofilm formation is an important virulence factor of *S. aureus*, leading to its high resistance to antibiotics and evasion from host defenses. This study aimed to assess the prevalence and antimicrobial resistance profile of biofilm-producing *S. aureus* strains and characterize genes involved in biofilm formation.

Materials & Methods: A total of 79 *S. aureus* strains were isolated from 1000 clinical samples and characterized using phenotypic, biochemical, and molecular tests. The biofilm production ability of isolates was examined using the microtiter assay. Moreover, the expression of genes involved in biofilm production (*psm* A and *psm* B) was screened using real-time PCR. Finally, antibiotic susceptibility testing was done using the Kirby-Bauer method and interpreted according to the CLSI M100 standard.

Findings: Out of 79 *S. aureus* isolates, 43 (54.4%) isolates were strong biofilm producers, 21 (26.6%) isolates were weak biofilm producers, and 15 (19%) isolates were non-adhesive. The results of real-time PCR showed that 55 (86%), 60 (93.7%), and 46 (58.2%) isolates were positive for *psm* A, *psm* B, and both genes, respectively. The results of antibiotic susceptibility testing showed that all the isolates were resistant to two or more antibiotics.

Conclusion: The high prevalence of biofilm-forming *S. aureus* strains in hospital environments could be a major health challenge with serious outcomes for hospitalized patients. Thus, it is necessary to disinfect hospital environments to reduce the risk of infection and spread of these microorganisms.

Keywords: *Staphylococcus aureus*, Biofilm, Real-time PCR, Antibiotic susceptibility.

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Introduction

Staphylococcus aureus strains are Gram-positive cocci that typically colonize the skin surface as well as the nasopharynx [1]. This bacterium is found in 30–50% of healthy people worldwide, and 1 in 1000 of this population is colonized with methicillin-resistant *S. aureus* (MRSA) strains [2]. This resistant pathogen is easily transmitted through direct contact, predisposing a huge population of individuals to infection [1]. *S. aureus* is one of the major causes of nosocomial infections and transmitted through direct contact with contaminated healthcare facilities or during invasive medical procedures, such as applying catheters and medical implants or performing surgeries [3]. Nosocomial infections are considered as a global public health concern. They are associated with a variety of factors including geographical location, type of disinfectant used in hospitals, circulating bacterial strains, and their inherent resistance to environmental conditions [3, 4]. The most important bacterial species causing nosocomial infections include *S. aureus, Escherichia coli, Pseudomonas aeruginosa, Acinetobacter baumannii,* and *Enterococcus faecalis* [4]. Among these species, *S. aureus* is considered as the main cause of nosocomial infections, especially in developing countries, due to its high intrinsic resistance to harsh environmental conditions such as drought and high temperatures as well as its ability to form biofilm and acquire multiple antibiotic resistance genes [5].

S. aureus causes a wide range of infections, such as pneumonia, septicemia, bacteremia, and soft tissue, bone, and skin infections [6]. *S. aureus* has many virulence factors; however, most infections caused by this species are related to biofilms formed on medical facilities and devices [7]. Biofilms are structured aggregates of bacterial communities enclosed in an extracellular polymeric matrix. Bacteria in biofilms exhibit high resistance to disinfectants and antibiotics and could also evade host defenses, making biofilm infections particularly difficult to eradicate [7]. Recent studies have shown that *S. aureus* in biofilms exhibits increased mutability, thereby accelerating the emergence of heritable antibiotic resistance phenotype through spontaneous mutation [7, 8]. Therefore, evaluating the antibiotic resistance pattern of biofilm-producing *S. aureus* strains could be helpful in the treatment and control of these infections.

Alpha-toxin and phenol-soluble modulins (*psm*) including *psm* A and *psm* B, which are encoded on the core genome, are produced by all staphylococcal strains [9]. These proteins have a wide range of functions, most importantly they support biofilm formation during *S. aureus* infections [9]. By reviewing the literature, it was found that *psm* (s) plays a multifaceted role in *S. aureus* biofilm formation; for example, it helps make the structure of bacterial biofilms by its surfactant activity; in addition, its expression could also lead to biofilm dispersal. Detachment of cells or cellular clusters from biofilms is a key strategy leading to the systemic dissemination of biofilm infection [10].

Objectives: Due to the fact that biofilm formation is a key factor for the survival and resistance of *S. aureus*, this study aimed to assess the prevalence and antimicrobial resistance profile of biofilm-producing *S. aureus* isolates and characterize genes involved in biofilm formation, especially in clinical isolates.

Materials and Methods

Bacterial isolates: In this cross-sectional study, 1000 clinical samples of different sources, including urine culture, abscess, CSF fluid, wound, ascites, and pleural fluid, were collected from 5th Azar hospital in Gorgan

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city from October 2018 to July 2019. A total of 79 *S. aureus* isolates were collected and characterized using phenotypic tests, such as growth on mannitol salt agar (Merck, Germany), Gram staining, catalase, oxidase, coagulase, and DNase tests, as well as PCR (polymerase chain reaction) amplification of a specific region of the 16S rRNA gene for *S. aureus* detection [11]. In addition, the antibiotic resistance profile of all 79 isolates was evaluated based on the Kirby-Bauer disk diffusion and broth microdilution methods according to the CLSI M100 standard [12]. This study was approved by the Ethics Committee of Golestan University of Medical Sciences. All participating patients provided informed consent on their own behalf.

Identification of biofilm formation: The biofilm formation ability of *S. aureus* isolates was analyzed using the microtiter plate method [13]. In brief, a fresh culture of each isolate was transferred into tryptic soy broth (TSB) (Merck, Germany) culture medium containing 1% glucose and then incubated overnight at 37 °C. When the turbidity of the suspensions reached the 0.5 McFarland standard, 200 μL of each bacterial isolate suspension was transferred into a 96-well microplate and incubated at 37°C for 48 h. Un-inoculated TSB medium with 1% glucose and the standard strain *S. aureus* ATCC 35556 (as a strong biofilm producer) were used as negative and positive controls, respectively. To evaluate the isolates biofilm formation ability, each well was treated with 200 μL of 2% crystal-violet for five minutes and then washed three times with phosphate buffered saline (PBS). Then 200 μL of ethanol-acetone mixed solution (20: 80) was added to each well to remove the crystal-violet from the isolates and biofilm. After 30 minutes, the optical absorption of each well was measured at a wavelength of 570 nm using an ELISA reader. A semi-quantitative analysis of biofilm formation was carried out using cut-off calculations based on the following formula [14]. The mean OD (optical density) of the negative controls plus three times the standard deviation was considered as a cut-off point. Accordingly, the isolates were classified into four groups in terms of their ability to form biofilm, including strong (OD > 1.2), moderate (0.7 < OD<1.2), weak (0.3 < OD<0.6), and none (OD<0.3) biofilm producers.

Molecular methods: The isolates with the ability to form biofilm were selected and subjected to PCR test to detect biofilm-related *psm* A and B genes.

Extraction and purification of RNA: Bacterial RNA was extracted from all biofilm-producing *S. aureus* isolates using the RNA-Plus kit (Cina-Gene Co., Iran) based on the standard procedure described previously [15]. The quantity and quality of the extracted RNA samples were evaluated by spectrophotometry and electrophoresis.

cDNA synthesis: Reverse transcription was performed using 5 μL of hot-denatured DNA-free RNA, 100 pmol of random hexamer primers (*psm* A and *psm* B) (Bioneer, Korea), and 100 U of Superscript II reverse transcriptase (Roche Diagnostics, Mannheim, Germany). The reaction mixture was incubated at 20 ^{0}C for 10 min, 42 ^{0}C for 30 min, and 37 ^{0}C for 5 min. The resulting products were precipitated by NaOAc (0.3 M, pH 6) and 2.5 volumes of ethanol (95%). They were then resuspended in 30 μL of DEPC–water and stored at -20 ^{0}C.

Expression of biofilm-related *psm* A and *psm* B genes: The real-time PCR method (ABI Prism 7300 Applied Biosystems) was performed using the Real-Q Plus 2x Master Mix Green Kit (Qiagen, Hilden, Germany) and specific primers to detect *psm A* and *psm B* genes in order to determine the level of biofilm-related gene expression in the extracted RNA [16].

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Antimicrobial susceptibility testing: Biofilm-producing *S. aureus* strains were selected for antimicrobial susceptibility testing by applying a panel of 16 antibiotic discs on Mueller-Hinton agar medium (Mast, UK) using the Kirby-Bauer disk diffusion method, and the results were recorded after incubation at 37 °C for 24 hrs. The interpretive criteria used in antibiotic susceptibility testing were based on CLSI guidelines (ver M100, 2019) [12]. The antibiotic discs used in this study included penicillin (PG, 10 µg), clindamycin (CD, 2 µg), teicoplanin (TEC, 30 µg), ampicillin (AP, 10 µg), gatifloxacin (GAT, 5 µg), vancomycin (VA, 30 µg), levofloxacin (LEV, 5 µg), gentamicin (GM, 10 µg), amikacin (AK, 30 µg), ceftriaxone (CRO, 30 µg), tobramycin (TN, 10 µg), erythromycin (E, 15 µg), kanamycin (K, 30 µg), linezolid (LZD, 30 µg), ciprofloxacin (CIP, 5 µg), and trimethoprim-sulfamethoxazole (TS, 25 µg). Intermediate susceptibility was scored as resistance. Multidrug resistance was defined as resistance to three or more antibiotic classes in addition to beta-lactams. *S. aureus* ATCC25923 was used as a control strain in the experiments.

Statistical analysis: Descriptive quantitative variables were calculated by measuring the central indices, dispersion, and plotting, then qualitative variables were computed by measuring the percentage of frequency. Also, the information obtained from the samples and the evaluation results were entered into SPSS software V. 20 and analyzed using Chi-square and ANOVA tests. In all cases, a *p* value of < .05 was considered as significant.

Findings

A total of 79 *S. aureus* isolates were recovered from 1000 various clinical specimens. Out of 79 *S. aureus* isolates, 47 (59.5%) isolates were collected from women, and32 (40.5%) isolates were collected from men. The age distribution of patients infected with *S. aureus* was as follows: four (5%) patients belonged to the age group of 10-20 years, 18 (22.78%) patients to the age group of 20-30 years, 16 (20.25%) patients to the age group of 30-40 years, 14 (17.72%) patients to the age group of 40-50 years, 10 (12.65%) patients to the age group of 50-60 years, five (6.32%) patients to the age group of 60-70 years, and 12 (15.2%) patients to the age group of over 70 years. There was no statistically significant correlation (p = .03) between the mean age of patients and *S. aureus* infection.

Quantitative biofilm production test: Out of the 79 *S. aureus* isolates analyzed in this study, 64 (81%) isolates showed the ability to produce biofilm based on the microtiter plate method results. Among them 30 (37.9%) isolates were strong biofilm producers, 13 (16.5%) isolates were moderate biofilm producers, 21 (26.6%) isolates were weak biofilm producers, and 15 (19%) isolates were found to be non-adhesive. The biofilm-producing isolates were recovered from blood (n=8), urine (n=14), wound (n=20), abscess (n=17), and cerebrospinal fluid (n=5) samples (Figure 1). No significant correlation was found (p= .03) between the source of isolation, gender of patients, and biofilm formation potential of *S. aureus* isolates.

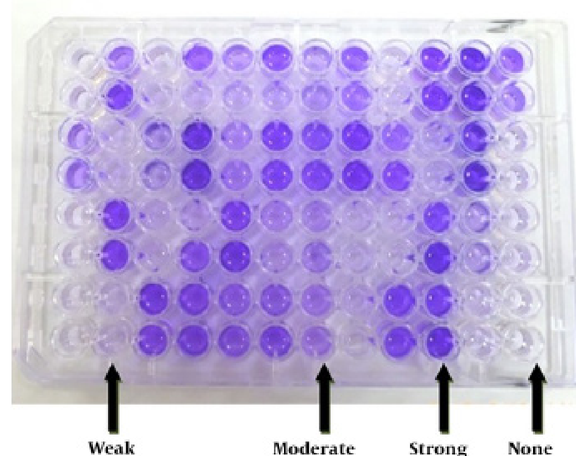

Figure 1) Analysis of biofilm formation of *S. aureus* isolates based on the microtiter plate method

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Table 1) Antimicrobial resistance pattern and distribution of *psm* A and *psm* B genes of biofilm-producing *S. aureus* isolates

	Biofilm Related Gene		Resistance Pattern		
Isolates code	*psm A*	*psm B*	Resistance	Intermediate	Susceptible
AS1/2/5/9/24/36/37	+	+	PG, AP, E, AK,	CD, TN, GM	CIP
AS3/7/11/18/27	+	+	PG, AP, AK, CD, TN,		GAT, LEV
AS12/22/28/29/40/41/46	+	+	PG, TN, AK,	AP, CD,	GAT, LEV
AS4/13/26/33	-	+	E, PG, AP, GM, K,	CIP, CD,	TN
AS14/20/31/47/52	+	+	PG, , CD, GM	AP	CIP, E, AK
AS3/19/21/34/43/50	+	+	, AK, K, TN, TS,	PG, AP	GM,GAT, E, CIP
AS6/15/17/23/30/	+	+	PG, AP, GM, CD		E, CIP, K, TN
AS8/16/25/51/55/60/62	+	+	PG, AP, , CD, TN, GAT,		AK LEV
AS10/68/71/73/75/	-	+	PG, AP, E, CD, GAT, TS, TN,		LEV, CRO
AS35/54/78	-	+	PG, K, GAT, TS,	AP, CD,	CRO
AS66/79/72/74	+	-	CD, K,	PG, AP,	GAT, TS, CRO
AS42/49/70	+	+	PG, AP	E, CIP,	CD, GM, K, TN
AS53/63/69	-	+	PG, AP, AK, CD, TN,		GAT, LEV
AS38/46/48/	-	-	PG, AP, E,		AK, CD, CIP, GM
AS39/44/45/56/57/	-	-	PG, GAT, LEV	AP,	AK, CD, TN,
AS58/59/61/64//	-	-	PG, AP, E, AK, CD, GM		, CIP
AS 65/67/76/	-	-	PG, AP, E, CIP, AK, GM,		K, TN, TS, GAT

PG: penicillin, VA: vancomycin, TEC: teicoplanin, TN: tobramycin, LZD: linezolid, TS: trimethoprim-sulfamethoxazole, AK: amikacin, AP: ampicillin, CD: clindamycin, CIP: ciprofloxacin, CRO: ceftriaxone, E: erythromycin, GAT: gatifloxacin, GM: gentamicin, K: kanamycin, LEV: levofloxacin

Determination of *psm* A and *psm* B genes: After the phenotypic characterization of biofilm-producing *S. aureus* isolates, the presence of biofilm-related genes in these isolates was analyzed using real-time PCR assay by applying specific primers to detect *psm* A and *psm* B genes. The results showed that all tested isolates harbored one or two biofilm-related genes: 55 isolates (86%) were positive for *psm* A gene, 60 isolates (93.7%) were positive for *psm* B gene, and 46 isolates were positive for both genes (Figure 2).

Antimicrobial susceptibility pattern and distribution of biofilm-related genes: The antimicrobial susceptibility pattern of biofilm-producing S. *aureus* isolates and the distribution of *psm* A and *psm* B genes in these isolates are given in Table 1.

Discussion

S. aureus is one of the most important bacterial species with the ability to produce biofilm on external surfaces, causing infections in individuals in hospitals [16]. Biofilms are compressed communities of bacteria encased in a matrix composed of polysaccharide, protein, and DNA. During growth in biofilms, bacteria showed tolerance to higher concentrations of antimicrobials agents commonly used to kill single-cell bacteria. Therefore, treatment and eradication of infections caused by biofilm-producing *S. aureus* strains are

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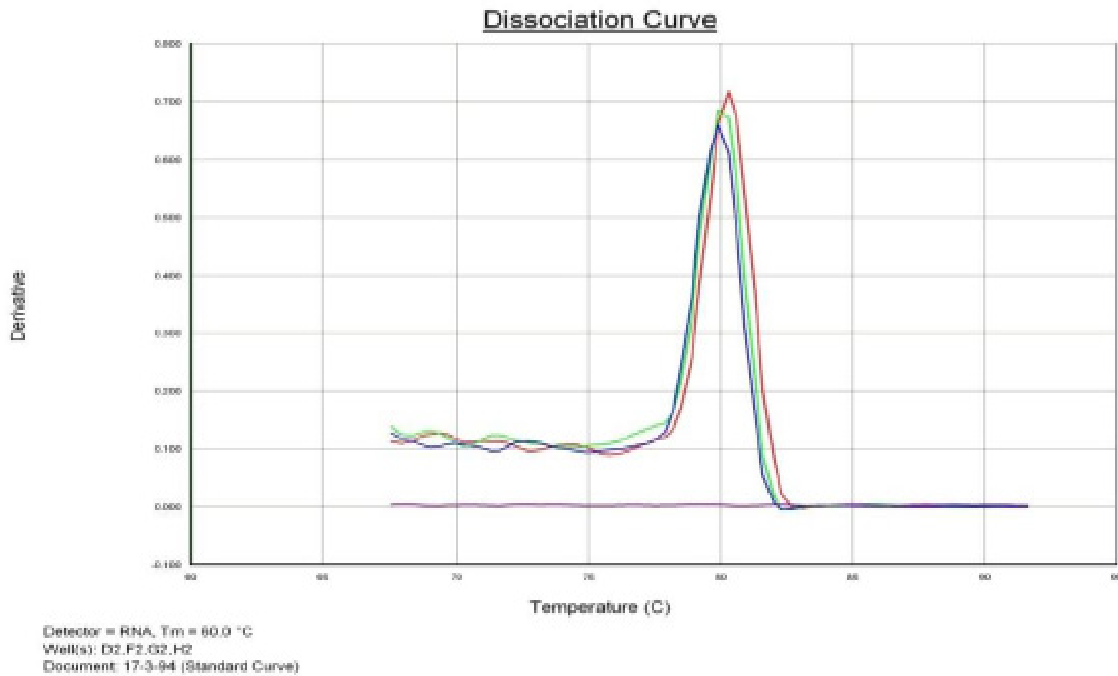

Figure 2) Standard diagram of real-time PCR of *psm* A and *psm* B genes on the axes of temperature and time

difficult [17, 8]. Additionally, the lack of specific biofilm markers makes the detection and diagnosis of biofilm infections challenging. As a result, the use of biofilm-specific diagnostic markers and the development of suitable diagnostic methods could lead to a better understanding of the pathogenesis of *S. aureus* and significant progress in the development of therapies against staphylococcal biofilm infections [18].

The current study results showed that the prevalence of *S. aureus* strains in various clinical samples was 7.9% (79 of 1000), of which 64 (81%) isolates showed the ability to produce biofilm. These results are consistent with the results of various studies conducted by Nourbakhsh and Momtaz (2016), Arya et al. (2005), and Namvar et al. (2013), showing that 86, 82, and 78% of *S. aureus* clinical isolates had the ability to form biofilm, respectively [19-21]. The present study results are higher than the results of other studies carried out by Christensen et al. (2002) and Cafiso et al. (2007). In these studies, 48.5 and 57.5% of *S. aureus* strains were able to produce biofilm [22, 23].

In this study, the microtiter plate assay results revealed that 43 (54.4%) isolates were strong biofilm producers, and 21 (26.6%) isolates were weak biofilm producers. These results suggest that different gene expression patterns may be involved in biofilm production. Following this idea, the results of real-time PCR performed to detect *psm* A and *psm* B genes in biofilm-producing isolates showed that all 64 isolates expressed at least one or two biofilm-related genes. According to the results, 43 isolates producing strong biofilms expressed both *psm* A and *psm* B genes, while 21 isolates producing weak biofilms expressed only *psm* A or *psm* B genes. These results are in agreement with the results of other studies reporting the effects of different gene

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expression patterns on biofilm formation by *S. aureus* strains [24, 25].

One of the most important problems related to *S. aureus* infections is the development of antibiotic resistance. In infections caused by biofilm-producing *S. aureus* strains, the produced biofilm may lead to an increase in minimum inhibitory concentrations of antibiotics compared to the single planktonic form of bacteria, indicating that biofilm production increases bacterial antibiotic tolerance [26]. In the present study, the antimicrobial susceptibility pattern of all biofilm-producing *S. aureus* isolates showed that these strains were highly resistant to antimicrobial agents.

Multidrug resistance was considered as resistance to three or more antibiotic drugs, and accordingly, 55 (69.6%) isolates in this study were multidrug-resistant. Also, 30 isolates (38%) were resistant to four antibiotics with different resistance levels. The current study results are consistent with the results of other studies that have reported a similar high prevalence of multidrug resistance in *S. aureus* isolates worldwide [27-29]. This suggests the possibility of developing *S. aureus* infections with limited treatment options. Hence, there is a need to adopt measures to eradicate and prevent the transmission of this organism to vulnerable populations.

Conclusion

The high prevalence of biofilm-forming *S. aureus* strains in hospital environments could be considered as a major health challenge with serious outcomes for hospitalized patients. Thus, it is necessary to disinfect hospital surfaces and equipment to reduce the risk of infection and spread of these microorganisms.

List of abbreviations: MRSA: methicillin-resistant *S. aureus*, *psm* s: phenol-soluble modulins, PCR: polymerase chain reaction, TSB: tryptic soy broth, CLSL: clinical and laboratory standards institute

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Ethical Permissions: The Ethics Committee of Golestan University of Medical Sciences approved this study, and the study was performed in accordance with the approved guidelines.

Conflicts of Interests: The authors declare that they have no conflict of interest.

Authors' contributions: All authors contributed to the study conception and design. Material preparation, data collection, and data analysis were performed by D.A, MSH, AKD, and PKD. The first draft of the manuscript was written by DA, and AAA commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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Consent to participate: Before participating in the study, all patients signed an informed consent form regarding the goals of the study and the willingness to participate.

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