ISSN: 0067-2904

Gene Expression Evaluation of Intracellular Adhesins and Regulatory Genes among Biofilm Producing MRSA Isolates

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Received: 27/2/2022 Accepted: 16/5/2022 Published: 30/1/2023

Abstract

The gene expression of the most important structural genes *ica A* and *D* of biofilm, *sarA*, and *sigB* regulatory genes of some methicillin-resistant *Staphylococcus aureus* (MRSA) isolates were examined using the real-time polymerase chain reaction after 24 hours of growth. The results revealed that the isolates with strong biofilm production had the highest gene expression of the structural *icaA* and *D* genes. Whereas the isolates that showed moderate and weak biofilm production, recorded the lowest gene expression. The results of the regulatory genes *sarA*, and *sigB* fluctuated among all MRSA isolates. Isolate No. 64 recorded the highest gene expression, while isolate 50 recorded the lowest gene expression. The purpose of this study was to determine the gene expression of structural genes *ica A* and *D*, and regulator genes *sigB* and *sarA* in some MRSA isolates with different abilities of biofilm formation where the *sarA* and *sigB* play important role in positive regulation of biofilm formation.

Keywords: Gene expression, MRSA, Biofilm, *SarA* and *SigB*, Real-time PCR.

تقييم التعبير الجيني لعوامل الإلتصاق داخل الخلايا والجينات التنظيمية بين عزلات المكورات العنقودية الذهبية المقاومة لمضاد المثيسيلن المنتجة للغشاء الحيوي

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الخلاصة

تم فحص التعبير الجيني لأهم الجينات التركيبة *ica A* and *D* والتنظيمية *sarA* و *sig B* للأغشية الحيوية لبعض عزلات المكورات العنقودية الذهبية المقاومة لمضاد المثيسلين باستخدام تفاعل انزيم البلمرة المتسلسل اللحظي بعد 24 ساعة من النمو . أظهرت النتائج أن بعض العزلات المنتجة للأغشية الحيوية القوية كان لها أعلى تعبير جيني لجينات *ica A* and *D* في حين أقل تعبير جيني كان في العزلات المتوسطة والضعيفة ، بينما كانت نتائج الجينات المنظمة متذبذبة في جميع العزلات وسجلت العزلة رقم 64 أعلى تعبير جيني، في حين سجلت العزلة 50 أقل تعبير جيني.

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هدفت هذه الدراسة إلى تحديد التعبير الجيني للجينات البنيوية *icaA* and *D* والجينات المنظمة *sigB* و *sarA* في بعض عزلات المكورات العنقودية المقاومة لمضاد المثيسيلين المتباينة في قدراتها على تكوين الاغشية الحيوية.

1 . Introduction

Staphylococcus aureus, resistant to methicillin infections, are notoriously difficult to treat due to their resistance to the entire family of β-lactam antibiotics, including methicillin and penicillin [1, 2].

Methicillin-resistant *Staphylococcus aureus* (MRSA) strains associated with hospitals are frequently multidrug-resistant, leaving only low efficiency antibiotics such as vancomycin available for therapy [3]. The capacity of bacteria to build biofilms is connected with pathogenicity and a variety of chronic bacterial illnesses [4].

Antibiotic resistance is becoming more prevalent with an increasing variety of species. Even microbes that are not genetically resistant to antibiotics are extremely resistant when formed into biofilms [5].

Apart from antibiotic resistance, a crucial complicating factor is the capacity of bacteria to form biofilms, a dynamic architecturally complicated multilayered cellular matrix. Biofilms are three-dimensional mosaic microbial consortia that cling to and build on implant surfaces via extracellular polymers known as glycocalyx. Biofilm formation requires the expression of polysaccharide intracellular adhesion (PIA). PIA is transcribed on the chromosome by the *ica*-genes' operon products (intercellular adhesion), when activated, this operon produces the following proteins: Ica A, D, B and C. These four proteins are necessary for the synthesis of PIA [6].

The regulatory systems of *S. aureus* are very complicated. Environmental factors greatly influence *ica* expression. Inducers for biofilm development include subinhibitory antibiotic concentrations, osmolarity and anaerobic growth conditions [7].

Several global regulators, including Agr quorum sensing, sarA and sigma factor B have been connected to Staphylococcal biofilm production. Biofilm development is influenced by several genes controlled by *sarA*. With a high affinity for the *icaA* promoter, *sarA* significantly activates it, *sarA* mutations diminish but do not eliminate PIA synthesis in *S. aureus*. The transcription of *icaADBC* and its repressor *icaR* by *sarA* is intriguing [8]. *S. aureus* utilizes the sigma factor B(*sigB)* which regulates genes that are resistant to heat, oxidative damage and antibiotics. Bacterial persistence and adaptation within cells are dependent on *sigB*. A *sigB* mutant exhibits reduced biofilm formation and *ica* gene expression, which inhibits PIA production. There has been little research regarding the gene expression associated with biofilm development in *S. aureus*. as well as the processes involved in binding expression. The effects of proteins and EPS on staphylococci biofilms should be clarified. Gene expression linked with biofilm production in *S. aureus* has received minimal attention [9-11]. The present study aims to understand how proteins affect staphylococci biofilms. Therefore we focus our study on this part that is correlated with PIA expression and regulation.

2 . Materials and Methods

2.1 Isolation and Detection of MRSA Isolates

Forty-five clinical MRSA isolates were obtained from 217 different clinical samples in Baghdad Governorate hospitals, Iraq, during the period extending from December 2020 to October 2021. Methicillin resistance was evaluated by disc diffusion method utilizing cefoxitin disc (30 µg/mL) [12], finally identified by the colorimetric ID-GP VITEK 2 system (BioMérieux/French) and molecular detection of *mecA* gene and presence of *icaA* and *icaD* genes [13].

2.2 Quantification of MRSA Biofilms by Microtiter Method

Using the microtiter plate assay, according to Atshan *et al*.[14], the ability to form biofilm formation was investigated for all 45 MRSA isolates. , and were then cultured in tryptic soy broth with 1% glucose in 6-well polystyrene microtiter plates for 24 hours under aerobic conditions at 37°C. Following incubation, planktonic cells were washed three times with deionized water and the adherent bacterial cells were fixed for 20 minutes with absolute methanol. After emptying the plates, they were left to dry completely. 1ml of 0.1 % crystal violet was used to stain the adherent cells for 15 minutes.Any excess stain was later washed away. The plates were then rinsed with distilled water and allowed to dry overnight in the air. The adhering cells crystal violates dye was dissolved in 1ml of 95 % ethanol in each well, and the plates were scanned at 490nm (A490) using a spectrophotometer. The experiment was repeated three times, with the absorbance (A490) of wells containing sterile TSB serving as a negative control where the result was estimated according to Atshan *et al*. [14].

2.3 RNA Purification from MRSA Isolates Biofilm by Using TransZol Up Plus RNA Kit

Manufacturer's methodology was used to extract and purify RNA from MRSA clinical isolates, and then the manufacturer's protocol was used to determine the RNA concentration (Quantus Fluorometer).RNA was isolated from the ten MRSA biofilm according to the protocol of TransZol UpPlus RNA Kit (TransGen/china) as the following steps:

1. Homogenization
• The suspension of bacterial cells was transferred to a microcentrifuge tube along with the growth medium then centrifuged at 8,000×g for 2 minutes at a temperature of 2-8^{o}C, for removing the supernatant.
• 1ml TransZol Up reagent was pipetted up and down until no visible precipitate remained in the lysate.
2. Chloroform(0.2ml) was added to TransZol Up (1ml) and then vortexed vigorously for 30 seconds, later on incubated at room temperature for 3 minutes.
3. The tube was centrifuged at 10,000×g for 15 minutes at a temperature of 2-8^{o}C. The mixture separated into a pink organic phase at the bottom, and interphase and a colorless aqueous phase at the top that included RNA. The aqueous phase volume is approximately 50% - 60% of the volume of the TransZol Up reagent employed.
4. The colorless RNA-containing aqueous phase was then transferred to a second microcentrifuge tube. An equivalent volume of absolute ethanol was added and the tube was later inverted gently to mix.
5. The produced solution and precipitate were transferred to a spin column, at room temperature, centrifuged at 12,000×g for 30 seconds, before removing the flow-through..
6. Five hundred microliter of CB9 solution was added to the spin column and was then centrifuged at 12,000×g for 30 seconds. The flow-through was thrown away and this step was repeated again.

7. Five hundred microliter of WB9 was added to the spin column, at room temperature and was centrifuge at 12,000×g for 30 seconds. The flow-through was then removed and this step was repeated once.
8. RNase-free tube was prepared for the spin column (supplied with the kit).
9. To elute RNA, it was centrifuged at 12,000× g for 1 minute. Isolated RNA was stored at -80°C.

2.4 Synthesis of cDNA

The primers used for cDNA synthesis from mRNA are shown in Table 1.
We utilized the EasyScript® one-step gDNA removal and cDNA Synthesis SuperMix kit for cDNA synthesis as following:
1. Five microliters of the extracted RNA were transferred to a new separated PCR tube.
2. Each sample tube received 10 µl of protoscript reaction mix (including dNTPs, buffer and other reaction components).
3. Two microliters of provided MuLV reverse transcriptase enzyme were added into each sample tube.
4. Two microliters from random hexamine primers were added and the volume was completed up to 20 µl by adding 1µl of nuclease free water.
5. Thermo-cycler was used to incubate this mixture for 1 hour at 42 ^{o}C, followed by an increase to 80 ^{o}C to inactivate the enzyme. The cDNA product was kept in the freezer until the qPCR technique was performed.

2.5 Determining the Yield of RNA and cDNA

The concentration of extracted RNA was determined by using a Quantus Fluorometer to determine the quality of samples for downstream applications. 199µl of diluted QuantyFlour Dye was combined with 1µl of RNA. After 5 minutes of incubation at 35^{o}C in a dark environment, the RNA concentrations were finally determined.

2.6 RT-qPCR

Quantitative detection using SYBR-fluorescence Green power. The PCR tubes were immediately spinned to eliminate any bubbles before collecting the liquid (4000×g for one minute). The Quantitative Real-Time PCR methodology was used for four genes: *sarA*, *sigB*, *icaA* and *icaD*, while *gyrB* was used as a housekeeping gene, primer and their sequence of housekeeping genes used in RT-qPCR as shown in Table 1. Then, the program for Real-Time PCR was set up with thermo-cycling protocol according to Altshan *et al*. [11].

Table 1: The primers and their sequences that were used in RT-qPCR

NO.	Primer Name	Sequence 5' → 3'	Size (bp)	References
1	***sar A*-F**	**ACATGGCAATTACAAAAATCAATGAT**	720	[14]
	***sar A*-R**	**TCTTTCTCTTTGTTTTCGCTGATG**		
2	***sig B*-F**	**ATG TACGTTTATTGAAGGATTG**	786	
	***sig B*-R**	**TAATTTCTTAATTGCCGTTCTC**		
3	**gyr B-F**	**GGTGCTGGGCAAAATCAAGT**	107	[15]
	gyr-B-R	**TCCCACACTAAATGGTGCAA**		

2.7 Analysis of Gene Expression

We recorded the cycle number (Ct) at which signals crossed a logarithmic phase threshold. Variations in the cycle threshold (ΔCt) and fold changes were evaluated between the target and calibrators of each gene. These values were normalized to the housekeeping gene [16].

3 . Results and Discussion

3.1 Quantification of Biofilm Formation

Forty-five MRSA clinical isolates that confirmed the presence of *mecA* gene that is responsible for methicillin resistance and molecular detection of *ica A* and *icaD* genes, were utilized to identify biofilm formation by using the polystyrene microtiter plate method as described by Atshan *et al*. [14], using crystal violet dye. The O.D was measured at 490 nm by using the microtiter-plate reader device.

The results revealed that 100% MRSA isolates were biofilm producers, 19 (42%) isolates had a robust biofilm, 9 (20%) isolates had a moderate biofilm and 17 (38%) isolates had a weak biofilm.

Microtiter plate assays are critical for studying the earliest phases of biofilm development. This semi-quantitative assay is based on a static model of biofilm formation and is a highly reliable and reproducible approach for measuring biofilm formation *in vitro* [17]. Piechota *et al*. [19] and Mohammed & Al-Mathkhury [20] revealed that 99.2% of MRSA isolates produced biofilms. Around 37% of isolates were strong producers, whereas over 49% were moderate and over 13% were weak producers [19, 20]. Biofilm development is influenced by a variety of factors, including the environment, nutrition availability and most importantly, the presence and expression of biofilm-associated genes [17].

3.2 Gene expression

3.2.1 Gene Expression of Structural Genes (icaA, icaD) and Regulatory Gene (sarA, sigB).

Based on our phenotypic and molecular study results, ten isolates varieties with their ability to form biofilm and their genes were checked for their gene expression for the structural gene (*icaA* and *icaD*) and regulatory genes (*sarA* and *sigB*) by using quantitative Real-Time PCR as shown in Tables 2-4.

Table 2: The expression fold in mRNA level of adhesion genes *icaA* and *D* and regulatory gene *sarA*, *sigB* in cultures of strong biofilm-producing isolates.

No.of isolate	*ica A*	*ica D*	*sarA*	*Sig B*
64	794	12	4.11	**25**
51	663	4	2	**3.4**
19	1.8	4.5	0.3	**0.1**
C	1	1	1	**1**

Control :1 *Below 1, down-regulation occurs. *Above 1, up-regulation occurs.*icaA* and *D*: Intercellular adhesion biofilm gene, *sarA*: Staphylococcal accessory protein A, *sigB*: Sigma factor B, *gyrB*: DNA gyrase (subunit B) as housekeeping gene, C; Control

Table 3: The expression fold in mRNA level of adhesion genes *icaA* and *D* and regulatory gene *sarA, sigB* in cultures of moderate biofilm-producing MRSA isolates.

No.of Isolate	*ica A*	*ica D*	*sarA*	*Sig B*
39	1.3	3.4	1.2	**0.02**
25	1.8	2.20	0.003	**0.08**
27	4.0	2	0.039	**0.04**
C	1	1	1	**1**

Control :1 *Below 1, down-regulation occurs. *Above 1, up-regulation occurs. *ica A* and *D*: Intercellular adhesion biofilm gene, *sarA*: Staphylococcal accessory protein A, *sigB:* sigma factor B, *gyrB*: DNA gyrase (subunit B) as housekeeping gene, C: Control

Table 4: The expression fold in mRNA level of adhesion genes *ica A* and *D* and regulatory gene *sarA, sigB* in cultures of weak biofilm-producing MRSA isolates.

No.of isolate	*ica A*	*ica D*	*sarA*	*Sig B*
47	1	1.2	0.1	**1.2**
8	0.4	1	0.1	**0.007**
50	0.05	0.5	1	**0.001**
C	1	1	1	**1**

Control :1 *Below 1, down-regulation occurs. *Above 1, up-regulation occurs.*ica A* and *D*: Intercellular adhesion biofilm gene, *sarA*: Staphylococcal accessory protein A, *sigB*: Sigma factor B , *gyrB*: DNA gyrase (subunit B) as housekeeping gene, : C:control

The result showed up-regulation in both *ica A* and *D* genes in strong isolates, where the highest result was recorded by isolate no.46 which represents a stronger biofilm producer. While the results of the regulatory genes were fluctuating in all isolates, isolate No. 64 recorded the highest gene expression and isolate 50 recorded the lowest gene expression. *ica* genes have been demonstrated to be multifaceted and a complex process, involving a multitude of external environmental stimuli and internal regulators not only under the control of *sarA* and *sigB* genes. The research community is still struggling to comprehend the differential expression of genes associated with biofilm during its development. Additionally, the phenotypes of biofilm cells are not precisely characterized over time. There are presently few studies on the gene expression associated with the production of biofilms in *S. aureus* [11].

Additionally, regarding the ways via which binding has expressed the role of proteins in staphylococci biofilms, a clarification is required [21]. According to Marques *et al*. [22], the relative mRNA expression of the *ica A* and *D* genes in the strong biofilm-producer was more elevated in 24 hours and less expressed in moderate and weak isolates after 24 hours. Whereas *icaD* expression in moderate isolate recorded the lowest expression [22].

Yibao Ma *et al*. [23] observed an 88 % rise in *icaA* expression during the exponential phase of growth and a 40% decline in *icaA* expression during the stationary phase, implying that the *ica* genes are involved in initial colonization [23]. Mohammed and Radif [24]

revealed that strong biofilm-producing isolates had significantly higher gene expression levels when compared with the moderate and the weak isolates [24]. Atshan *et al*. [11] found that the primary adhesion of *S. aureus* was significantly higher at 24 h and lower at 48 h. The expression of *icaC* was 6 times higher at 24 h of growth, with *icaC* being the most up-regulated gene with a change in fold about 1855 [11].

sarA and *sigB* play important role in positive regulation for biofilm formation, Beenken *et al*. [25] approved this by inhibition of *sarA* leading to limit biofilm formation [25]. *sarA* and *sigB* was reported to be positive regulators of *ica A* and *D* expression, whereas *ica R* was revealed to be a negative regulator. Moreover, this study establishes that *sarA* and *sig B* are necessary for *S. aureus icaR* expression [26]. Due to its simplicity, specificity and sensitivity, reverse transcription-quantitative PCR has become the dominant approach for quantifying gene expression. The most often used method for gene expression analysis is relative quantification, which compares the expression of a target gene to that of a reference gene. It has gained widespread usage in basic research, functional genomics, pharmaceutical, forensic and water quality diagnostics [27, 28].

4 . Conclusion

Both *sigB* and *sarA* have an effect on the gene expression of *icaA* and *icaD* in biofilms, with *sarA* having a direct effect on *icaA* and *D* transcription and *sigB* having an indirect effect; since *sigB* affects *sarA* expression, which in turn has an effect on biofilm regulation. Although the current work focused on the expression of four genes associated with biofilms, further analysis of additional genes such as *agr* and the use of microarrays will provide additional information about the molecular regulation of biofilms.

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