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Gene Expression of *pelA* and *pslA* in *Pseudomonas Aeruginosa* under Gentamicin Stress

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Abstract

Pseudomonas aeruginosa produces an extracellular biofilm matrix that consists of nucleic acids, exopolysaccharides, lipid vesicles, and proteins. Alginate, Psl and Pel are three exopolysaccharides that constitute the main components in biofilm matrix, with many biological functions attributed to them, especially concerning the protection of the bacterial cell from antimicrobial agents and immune responses. A total of 25 gentamicin-resistant *P. aeruginosa* selected isolates were enrolled in this study. Biofilm development was observed in 96% of the isolates. In addition, the present results clarified the presence of *pelA* and *pslA* in all the studied isolates. The expression of these genes was very low. Even though all biofilms were affected by gentamicin, the results of fold change showed a wide variation. In conclusion, all *P. aeruginosa* isolates carried *psl* and *pel* regardless of the intensity of the biofilm. A strongly positive correlation with gentamicin minimum inhibitory concentration was noticed.

Keywords: PelA, PslA, biofilm, RT-qPCR.

التعبير الجيني ل *pelA* و *pslA* في الزوائف الزنجارية عند اجهاد الجنتامايسين

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الخلاصه

تنتج الزوائف الزنجارية قالب غشاء حياتي خارج خلوي يتألف من احماض نووية و عديد السكريد الخارجي وحويصلات دهنية و بروتينات. تعد الالجينات و psl و pel ثلاثة من عديد السكريد الأهم في تركيب الغشاء الحياتي والذي يضطلع بوظائف حياتية عديدة وخصوصا حماية الخلية البكتيرية من المضادات الحياتية و الجهاز المناعي. اختير لهذه الدراسة ما مجموعه 25 عزلة من الزوائف الزنجارية المقاومة للجنتامايسين. لوحظ تكون الغشاء الحياتي في 96% من العزلات. كما أوضحت النتائج الحالية وجود كل من *pslA* و *pelA* في جميع عزلات الدراسة. علاوة على ذلك كان التعبير الجيني لهذين الجينين منخفضا جدا. على الرغم من الجنتامايسين قد اثر في الغشاء الحياتي لكل العزلات، الا ان تاثيره في التعبير الجيني كان متغاير بدرجة واسعة. من الممكن ان نستنتج من هذه الدراسة ان جميع عزلات الزوائف الزنجارية تمتلك كل

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من *pslA* و *pelA* بغض النظر عن شدة و قوة الغشاء الحياتي. كما لوحظ وجود ارتباط ضعيف بين التركيز المثبط الأدنى للجنتامايسين و التعبير الجيني لكل من *pslA* و *pelA* .

Introduction

Pseudomonas aeruginosa is a Gram-negative bacterium that is found widely in the environment and engages in various forms of interactions with eukaryotic host organisms. It is an opportunistic pathogen widely spread in humans, giving rise to a broad spectrum of infections in community and healthcare facilities [1, 2]. Due to the extended spread of *P. aeruginosa* habitats, the control of the organism in a hospital setting is very difficult and it is practically impossible to prevent contamination [3]. The major threat is the infection of immunocompromised persons or those in burns, neonatals, and cancer compartments [4]. Infection of *P. aeruginosa* is still one of the main causes of death among the critically ill and patients with impaired immune systems, in spite of the development of newer and stronger antibiotics [5].

Biofilm production is an important mechanism for the survival of *P. aeruginosa* and its relationship with antimicrobial resistance represents a challenge for patient therapeutics [6]. Biofilms are associated with over 80% of all microbial infections [7]. Exopolysaccharides and extracellular DNA are important structural components that contribute to the self-assembly of large aggregates or microcolonies that are characteristic of biofilms. *P. aeruginosa* is capable of producing multiple exopolysaccharides, including alginate, Psl, and Pel [8]. A key component of biofilm formation in *P. aeruginosa* is the biosynthesis of exopolysaccharides known as polysaccharide encoding locus (Pel) and polysaccharide synthesis locus (Psl), which are involved in the formation and maintenance of the structural biofilm scaffold and protection against antimicrobials and host defenses [9]. Although the preference of Pel or Psl is often strain-specific, many isolates are capable of switching between the synthesis of Pel and that of Psl in response to stress to maintain the infection in the host [10]. This adaptive mechanism underscores the importance of developing therapies that target both exopolysaccharides [9].

Aminoglycosides are used to treat many Gram-negative and some Gram-positive infections and, importantly, multidrug-resistant tuberculosis. Among various bacterial species, resistance to aminoglycosides arises through a variety of intrinsic and acquired mechanisms [11]. *P. aeruginosa* has developed several mechanisms to resist antimicrobial agents that can be associated with high mortality [12, 2].

Materials and Methods

Pseudomonas aeruginosa isolates

A total of 25 *P. aeruginosa* isolates were obtained from the Department of Biology, College of Science, University of Anbar. They were originally isolated from different clinical specimens as previously mentioned by AL-Sheikhly *et al*. [13]. *P. aeruginosa* ATCC (27853) was used as a quality control strain.

Microplate Biofilm Development Test

Biofilm formation was determined by microtiter plate assay [14]. Briefly, 24 hours old cultures were kept in tryptic soy broth (TSB) containing 1% glucose. Thereafter, broth cultures were checked to McFarland standard No. 0.5 using the same medium as diluent. About 200 µl of an isolate suspension were transferred into each of three wells of a 96-well flat-bottomed polystyrene plate and incubated for 24 hr. at 37°C. Thereafter, each well was washed twice using sterile deionized water with rough shaking and later dried thoroughly. The adhering bacterial cells were fixed with 200 µl of absolute methanol. Afterward, each well was stained with 200 µl of 0.1% crystal violet for 15 minutes. Repetitive washing was performed to remove the excess stain. Later on, the crystal violet bound to the adherent cells was retained with 200 µl of ethanol per well. The test was made in triplicates, and the absorbance of wells filled with bacteria-free TSB served as a negative control. The amount of crystal violet removed by ethanol in each well was directly quantified spectrophotometrically by measuring the OD_{630} using microplate reader. The cut-off value (ODc) was calculated as three standard deviations plus the mean OD of the negative control. Given that, absorbance values represented the intensity of the biofilm formed by the studied isolates on the surface of the microtiter well. The obtained results were categorized into four groups (viz., non-biofilm producer, weak, moderate, and strong).

The effects of gentamicin at sub-MIC on biofilm formation

The MIC was assessed for gentamicin by the broth dilution method [15] and was carried out in triplicate. The antibacterial activity was examined after incubation at 37°C for 18 - 24 h. MIC was resolved as the lowest concentration of test samples that resulted in a whole inhibition of the observable growth in the broth.

Detection of *pelA* and *pslA*

A- Extraction of DNA and Polymerase Chain Reaction Amplification

DNA was extracted from 25 *P. aeruginosa* selected isolates using genomic DNA extraction kit (Promega, USA), following the manufacturer's instructions. The purity and concentration of the DNA were estimated by Nanodrop.

B- Primer Selection

Specific primers that were needed for amplifying a fragment of *16SrRNA, pelA* and *pslA* are listed in Table- 1. Primers were provided in a lyophilized form and dissolved in sterile nuclease-free water to give a final concentration of 100 pmol /µl. Afterward, they were stored in a deep freezer until use.

Table 1-Primers utilized in this study

Primer name		Sequence (5´-3´)	Product size	Reference
pelA	F	CCTTCAGCCATCCGTTCTTCT	118 bp	[10]
	R	TCGCGTACGAAGTCGACCTT		
pslA	F	ATAAGATCAAGAAACGCGTGGA	70 bp	[10]
	R	TGTAGAGGTCGAACCACACCG		
16SrRNA	F	ACCTGGACTGATACTGACACTGA		[16]
	R	GTGGACTACCAGGGTATCTAATCCT		

C- Preparation of PCR mixture

The extracted DNA and primers were added to the PCR premix (Acuu Power PCR pre mix) tubes and vortexed to have homogenous contents. A PCR mixture was made in a total volume of 20 µl as described in Table-2.

Table 2-Components of the conventional PCR reaction

Component	Volume (µl)
Forward primer (10 pmol)	**2**
Reverse primer (10 pmol)	**2**
Nuclease free water	**14**
Template DNA	**2**
Final volume	**20**

C- PCR program

The PCR tubes containing the mixture were transferred to thermo-cycler and the program in Table-3 was started [17].

Table 3-Conditions of PCR for *pelA* and *pslA* genes

Step	Temperature (˚C)	Time	No. of cycle
Initial Denaturation	**94**	**5 min.**	**1**
Denaturation	**94**	**30 sec.**	
Annealing	**52**	**40 sec.**	**35**
Extension	**72**	**50 sec.**	
Final Extension	**72**	**5 min.**	**1**

Gene expression

Seven isolates (PA1, PA6, PA50, PA58, PA59, PA60 and PA17) were chosen for this study. In regard to planktonic cells, aliquots of bacterial cells were harvested from media and suspended in

sterile Muller Hinton Broth. All tubes were incubated overnight at 37°C. In order to test the effect of gentamicin on gene expression of *pelA* and *pslA*, a similar protocol was followed with the use of gentamicin-containing Muller Hinton Broth at sub-MIC.

A- RNA Extraction from *P. aeruginosa* isolates

RNA was isolated from *P. aeruginosa* planktonic cells using Trizol reagent (Promega, USA) according to the protocol described by the manufacturer.

B- Quantitative reverse transcription-PCR

In order to assess the gene expression of *pelA* and *pslA*, the results were normalized using *16SrRNA*. The reaction mixture is summarized in Table- 4. Moreover, after several trials, the thermo-cycler protocol was optimized and the resultant protocol is listed in Table-5.

Table 4-Components of qRT-PCR used in *pelA* and *pslA* genes expression

Master mix components	Volume (µl)
qPCR Master Mix	**5**
RT mix	**0.25**
Forward primer	**0.5**
Reverse primer	**0.5**
Nuclease Free Water	**1.75**
RNA	**2**
Total volume	**10**

Table 5-qRT-PCR protocol

Step	Temperature (˚C)	Time	No. of cycle
Reverse transcription (RT). Enzyme activation	**37**	**15 min**	**1**
Initial Denaturation	**95**	**10 min.**	**1**
Denaturation	**95**	**15 sec.**	
Annealing	**52**	**30 sec.**	**40**
Extension	**72**	**30 sec.**	

Expression levels were quantified using relative quantitation. The difference in cycle thresholds (ΔCt) and fold changes were evaluated between the treated groups and the calibrators of each gene [18]. Fold change of less than 2-fold was considered insignificant [19]. A melting curve was obtained with temperatures ranging from 60˚C to 95˚C with a 1˚C increase in temperature every one second.

Statistical analysis

In order to determine the impact of parameters in this study, the statistical package for social science (SPSS) 21.0 and Microsoft excel 2013 were used. Categorical data were formulated as count and percentage. T-test was used in evaluating the effect of gentamicin on biofilm. Regarding other experiments, Fisher exact test and chi-square test were used to describe the association of these parameters. Furthermore, Pearson correlation coefficient was used to check the correlation between fold change and gentamicin sub-MIC. The lowest level of accepted statistical significant difference is bellow or equal to 0.05 [20, 21].

Results and Discussion

Biofilm forming capacity

In the current study, the ability of *P. aeruginosa* biofilm production was evaluated using pre-sterilized 96-well polystyrene microtiter plates, which is considered as a standard test for the detection of biofilm biomass [14, 22]. According to the results listed in Table- 8, the present study declared that out of 25 gentamicin-resistant isolates, three (12%) isolates formed a weak biofilm, fourteen (56%) isolates developed moderate biofilm, whereas seven (28%) isolates constituted strong biofilm. Nevertheless, only one (4%) isolate was unable to form a biofilm.

Beenken *et al*. [23] concluded that the differences in biofilm thickness among isolates might be owing to several reasons; differences of isolates capacity to form biofilm or perhaps differences in primary number of cells that succeeded in adherence, along with differences in the quality and

quantity of quorum sensing signaling molecules (autoinducers) that are produced from each isolate. A part form moderate biofilm, there is no specific pattern that governs the distribution of biofilm intensity among specimens, i.e. each biofilm intensity is a specimen-specific. Perhaps the reason behind such findings is the variation in the genetic makeup of each strain.

Current results corroborate the findings of other local studies [24, 25, 26]. In addition, there was an agreement with other previous studies [6, 17].

This high productivity of biofilm formation may be attributed to the sensitivity of MTP method to measure the few quantities formed. It was considered an important method in studying the early stages of biofilm formation because it uses constant conditions and it can be effective in studying many virulence factors to form biofilms such as pili and flagella. Furthermore, this method was adopted to explore biofilm forming capacity by different types of bacteria [27].

Heydari and Eftekhar [28] indicated that the variation in the ability of isolates to form biofilm is due to the association of the production with its ability to produce ß-Lactamase. The isolates produced multiple types of enzymes that produced a strong biofilm compared with isolates that produced one type of enzymes. While, the isolates that do not produce this enzyme are unable to form biofilm.

The Effects of Gentamicin on Biofilm Formation

The results of the present study, summarized in Table- 6, revealed that gentamicin has significantly ($P < 0.05$) decreased the density of biofilm formation in four isolates (PA1, PA58, PA59, and PA60). While, no change in biofilm intensity was detected in two isolates (PA6 and PA50). Furthermore, gentamicin induced biofilm formation significantly ($P < 0.05$) in only one isolate PA17. Yet, the effect differs insignificantly from one isolate to another.

Table 6-The formation of biofilm by *P. aeruginosa* treated with gentamicin at sub-MIC.

Code of Isolates	Before Treatment			After Treatment				P-Value
	OD_{630}	**SD**	**Biofilm intensity**	**Sub-MIC(µg/ml)**	**OD_{630}**	**SD**	**Biofilm intensity**	
PA1	**0.420**	**0.099**	**Moderate**	**8**	**0.0663**	**0.0092**	**Weak**	**0.024**
PA6	**0.302**	**0.136**	**Weak**	**256**	**0.0606**	**0.0025**	**Weak**	**0.091**
PA50	**0.322**	**0.011**	**Weak**	**16**	**0.0753**	**0.0045**	**weak**	**0.0001**
PA58	**0.769**	**0.126**	**Strong**	**16**	**0.0743**	**0.0046**	**Weak**	**0.010**
PA59	**1.015**	**0.103**	**Strong**	**8**	**0.0916**	**0.0065**	**Weak**	**0.004**
PA60	**0.493**	**0.136**	**Moderate**	**16**	**0.0813**	**0.0025**	**Weak**	**0.034**
PA17	**0.154**	**0.022**	**Non biofilm producer**	**512**	**0.0583**	**0.0035**	**Weak**	**0.016**

A similar variation was noticed by other studies [29, 30]. Such variation may be considered normal due to the types of studied isolates and their source as well as the genetic makeup of isolates, or the laboratory conditions that accompanied the detection of the sub-MIC.

It has been reported that when antibiotics are present at concentrations below the MIC, it can significantly induce biofilm formation in a variety of bacterial species *in vitro*. Kaplan [31] reported that the first study that demonstrated that the sub-MIC of antibiotics can induce bacterial biofilm formation *in vitro* was reported in 1988 by Gordon Christensen.

Marr *et al*. [32] also investigated the mechanism of aminoglycoside-induced biofilm formation in *P. aeruginosa*, whereas Hoffman *et al*. [33] found that the sub-MIC concentrations of tobramycin

readily induced *P. aeruginosa* biofilm formation. Otani *et al*. [34] noticed that the ceftazidime at sub-MIC significantly inhibited *P. aeruginosa* biofilm formation.
Generally, the antibiotics reduced the biofilm formation; however, several studies showed that the antibiotics could significantly induce biofilm formation depending on antibiotics class and the bacterial strain [30].

Molecular Identification

A- Genomic DNA Extraction and Purity

The bacterial genomic DNA was extracted from overnight cultures of isolates. It was found that the purity ranged from 1.88 to 2.01 ng/µl, while the concentration fluctuated between 59 and 539 ng/µl. Furthermore, Figure- 1 illustrates the presence of a single band of extracted DNA, which indicates the efficiency of the method used in the extraction of DNA.

Figure 1-Agarose gel electrophoresis of DNA bands from *P. aeruginosa* isolates in (1% agarose, 5 V/cm) stained with Ethidium Bromide. Lane N represents negative control.

Detection of *pelA* and *pslA*

PCR was conducted over 25 isolates, using the *pelA* and *pslA* specific primers to amplify the constitutional genes *pelA* and *pslA*. The present results clarified the presence of these genes in all *P. aeruginosa* isolates Figures-(2 and 3).

The results of the current study are in agreement with those of Maita and Boonbumrung [17] who stated that the percentage of pelA was 97.8% while that of *pslA* was 94.9%, which were found in almost all clinical isolates of *P. aeruginosa*. Al-Wrafy *et al*. [35] suggested that biofilm represents an important virulence factor for these bacteria and plays a role in *P. aeruginosa* infections and avoidance of immune defense mechanisms; it can protect the bacteria from antibiotics. Alginate, Psl and Pel are three exopolysaccharides which constitute the main components in biofilm matrix, with many biological functions attributed to them, especially with respect to the protection of the bacterial cell from antibiotics and the immune responses.

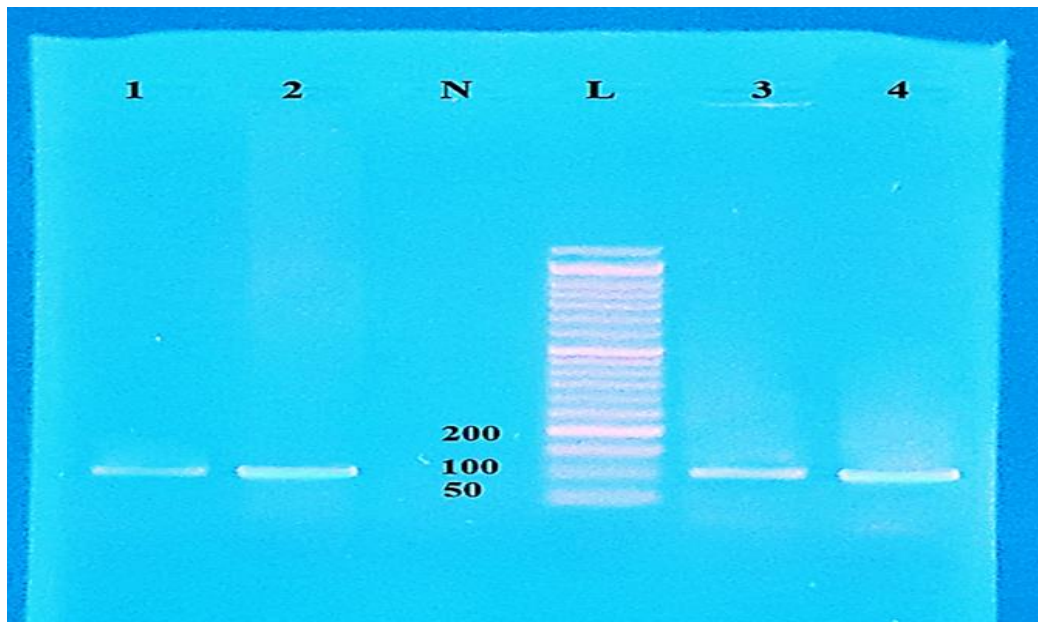

Figure 2-Agarose gel electrophoresis (1.5% agarose, 5 V/cm) of *pelA* (118bp). Lane L represents 50 bp DNA ladder, Lanes 1-4 represent bands of *pelA* (118bp).

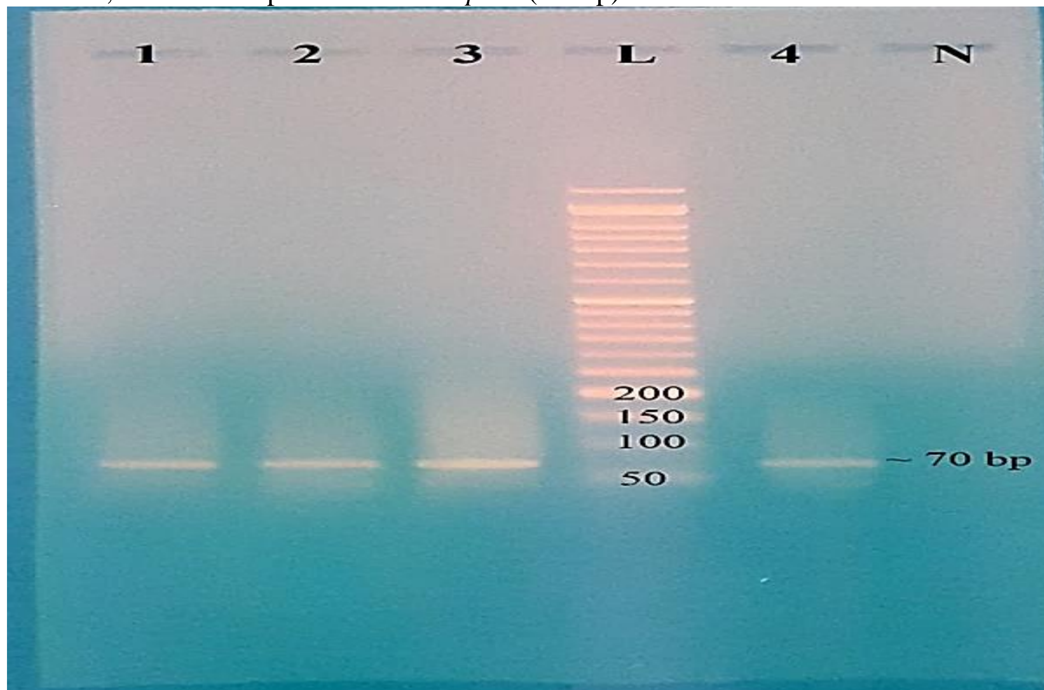

Figure 3-Agarose gel electrophoresis (1.5%agarose, 5 V/cm) of *pslA* (70bp). Lane L represents 50 bp DNA ladder, Lane N denotes negative control. Lanes 1, 2, 3, and 4 represent bands of *pslA* (70bp).

Many local studies investigated other types of biofilm genes. Musafer [24] revealed that the *OprD* plays a major role in the acquired resistance to imipenem, while it also participates in biofilm formation; AL-Dulami [26] observed that lasI has an important role in the production of biofilm. AL-Sabawi [25] found that the pelF is responsible for biofilm formation.

Recalling the results of biofilm formation, one isolate was unable to form biofilm despite carrying both of *pelA* and *pslA*. Obviously, it can be realized that there are other genes responsible for biofilm formation. Accordingly, the presence of these genes could not predict which isolate will produce biofilm.

Although the preference of Pel or Psl is often strain-specific, many isolates are capable of switching between the synthesis of Pel and that of Psl in response to stress to maintain infection in the host [10]. This adaptive mechanism underscores the importance of developing therapies that target both exopolysaccharides [9].

Gene expression

The expression of *pelA* and *pslA* of *P. aeruginosa* biofilm was studied by RT-qPCR. The isolates were characterized by different sub-MIC levels of gentamicin (8, 16, 256, and 512 µg /ml). Seven isolates (PA1, PA6, PA17, PA50, PA58, PA59, and PA60) enrolled in this experiment were chosen for the following reasons:

a)Different biofilm intensity.

b)Six of them are of the same clinical source (PA1, PA6, PA50, PA58, PA59, and PA60).

c)Isolate PA17 was a biofilm non-producer.

RNA Extraction from *P. aeruginosa* isolates and RT-qPCR

The isolates PA1 and PA60 are moderate biofilm formers, PA58 and PA59 are strong biofilm producers, and PA6 and PA50 are weak biofilm producers. While, isolate PA17 is non-biofilm former. RNA was extracted from the aforementioned isolates. Total RNA was extracted by using TRIzol™ Reagent and its concentration was measured by using quantusflorometer. It ranged from 59.9 to 245 ng /µl.

Gene expression of *pelA* and *pslA*

Obviously, gene expression levels presented in Table-7 were very low in all isolates; however, such results were explained by Huse *et al*. [36]; he revealed that biofilm polysaccharides production is increased throughout the infection. Furthermore, an increase in gene expression of one gene corresponded with a decrease in the other gene. Colvin *et al*. [10] stated that such overlapping is owing to compensating the lack of gene expression of one gene with overexpression in the other one.

Albeit all biofilms were affected by gentamicin at sub-MIC (Table-6), the results of fold change presented a wide variation. For instance, both genes have suffered an increase in the isolate PA6. Nevertheless, the isolate PA50 showed an increase in fold change of *pelA*, whereas the isolates PA60 and PA17 showed an increase in fold change of *pslA*. A part of these three isolates (PA1, PA58 and PA59), none of the rest developed an increase; on the contrary, both genes were under-expressed. Such findings, perhaps, emphasize the contribution of other genes alongside with *pel* and *psl*. Remarkably, it was noted that the isolate PA17 was unable to form biofilm before the treatment with gentamicin; hitherto, it formed the biofilm when treated with gentamicin at 512 µg /ml (sub-MIC). The expression of both genes (*pelA* and *pslA*) before treatment with gentamicin was low, but the gene expression of the *pslA* increased after treatment. This indicates that this gene plays a role in the formation of biofilm in this particular isolate (PA17) at least. Yet, it might play a role in the formation of biofilm in the rest of the selected isolates (PA6and PA60). Moreover, a strong correlation was found (r = 0.999) between the fold change of *pelA* and *pslA*.

Table 7-Results of gene expression and fold change for *pelA* in *P. aeruginosa* biofilm

Isolate Code	Source of isolated	Gentamicin at sub-MIC (µg/ml)	*pelA*		*pslA*	
			Gene expression	**Fold change**	**Gene expression**	**Fold change**
PA1	**Burn**	**8**	**2.15792×10^{-5}**	**0.56**	**0.0019667**	**0.76**
PA6	**Burn**	**256**	**8.39564×10^{-5}**	**106.44**	**0.0332615**	**1218.61**
PA50	**Burn**	**16**	**3.58399×10^{-6}**	**1.01**	**8.17698×10^{-6}**	**0.47**
PA58	**Burn**	**16**	**1.69533×10^{-6}**	**0.28**	**0.0003599**	**0.91**
PA59	**Burn**	**8**	**4.14556×10^{-6}**	**0.04**	**0.0001876**	**0.06**
PA60	**Burn**	**16**	**4.82848×10^{-6}**	**0.58**	**0.0002125**	**52.95**
PA17	**Wound**	**512**	**2.03284×10^{-7}**	**0.19**	**2.98895×10^{-5}**	**1.71**

The results of the current study were somewhat in agreement with a previous study of Maita and Boonbumrung [17] who revealed that the biofilm formation is accompanied by drastic changes in gene regulation. The formation of microcolonies in *P. aeruginosa* has been attributed to many factors. These include type IV pili, flagella, free DNA, alginate and Pel and Psl polysaccharides. Even if one of the factors is not functioning, the biofilm is still able to perform well.

The findings of the present study clarified a weak correlation (r = 0.306) between antibiotic concentration at sub-MIC and folding change for pelA. In addition, a weak correlation was observed (r = 0.302) between antibiotic concentration at sub-MIC and folding change for *pslA* (Table- 8). On the contrary, the results of the present study disagreed with Coulon *et al* [37] who linked the Pel production with aminoglycoside tolerance in biofilms formation.

Table 8-Correlation between fold change (*pelA, pslA*) and antibiotic concentration at sub-MIC

Groups	Mean ± SE	Correlation coefficient
Gentamicin (Sub-MIC)	**118.857±73.951**	**0.306 (Weak)**
Folding Change (*pelA*)	**15.586 ±15.143**	
Gentamicin (Sub-MIC)	**118.857±73.951**	**0.302 (Weak)**
Folding Change (*pslA*)	**182.210±172.890**	

SE =Standard Error

Colvin *et al*. [10] and Thellin *et al*. [38] suggested that there was significant strain-to-strain variability in the contribution of Pel and Psl to mature biofilm structure. A similar interpretation suggested that Pel and Psl can serve a redundant function as a structural scaffold in mature biofilms. Depending on the strain studied, the role of Pel and Psl in biofilm formation can vary drastically.

Maita and Boonbumrung [17] stated that the antibiotic resistance of bacteria due to biofilm formation contributes to the persistence of bacterial cells and causes problems in the complete eradication of infection. The structure of biofilms is increasingly recognized as a crucial factor in the persistence of several infections. Chronic infections have been remarkably demonstrated to involve biofilm production, especially those infections associated with indwelling devices such as catheters and prostheses. The ability of the biofilm to contribute to bacterial protection is widely different among microbes. Biofilms not only contribute to the resistance mechanisms against a broad-spectrum of antibiotics but also against host immune systems. The antibiotic susceptibility of biofilm-producing bacteria was reduced because of a restricted antibiotic penetration, adaptive response and the presence of persisting cells.

In conclusion, all *P. aeruginosa* isolates carried *psl* and *pel* despite the intensity of biofilm. However, a weak correlation was noticed between the gene expression and gentamicin MIC.

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