International Journal of Advanced Biological and Biomedical Research
Available online at http:www.ijabbr.com
Volume 8, Issue 1 (2020) pp. 58-66 DOI: 10.33945/SAMI/IJABBR.2020.1.6

Original Article

Biofilm Formation with Microtiter Plate 96 and *pslA* Detection of *Pseudomonas Aeruginosa* Isolates from Clinical Samples in Iran

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Received: 6 June 2019, Revised: 24 August 2019, Accepted: 31 August 2019

ABSTRACT

Background: Microorganisms attach to various surfaces and they have manufactured biofilms by production polysaccharides like PSL in *P. aeruginosa*. Synthesis of this kind of polysaccharide has done by *PSL* gene cluster. The aim of this study is consideration of biofilm formation which is one of the major cause of antibiotic resistance.
Methods: In this study, 100 *P. aeruginosa* were isolated with bacteriological and biochemical methods and *pslA* gene detection with PCR in all of the *P. aeruginosa* isolated from patients. Then biofilm formation checked with microtiter plate method and it showed with SEM. Finally, expression of main attachment gene *pslA* in 6 strains could make moderate and strong biofilm were investigated by real-time PCR assay.
Results: In this study, 100 *P. aeruginosa* were isolated that these strains showed high rates of MDR. The presence *pslA* gene in all of the pseudomonas isolated from patients was proven. Microtiter plate method showed 24 (24%) strains could make biofilm among 100 strains that showed with SEM. The *pslA* expression in strains which making moderate and strong biofilms are more than other strains.
Conclusions: Hence, for bacterial biofilm treatment is recommended: before antibiotics are prescribed, biofilm formation by bacteria should be investigated.

Key words: *P. aeruginosa*, *Microtiter plate*, *Biofilm*, *Real Time PCR*, *Scanning electron microscopy (SEM)*.

Introduction

Pseudomonas aeruginosa is a gram-negative bacterium and part of the family Pseudomonadaceae that is readily isolated from soil and water environments. It is an opportunistic human pathogen special in burned patients. The main problem in treating *P. aeruginosa* infection is its high resistance to various antibiotics (Kolter *et al.*, 2010;

Shahandashti *et al*., 2012; Khan *et al*., 2010). The unique trait of *P. aeruginosa* is its resistance to a wide range of antibiotics that is adjective to allow permeability of the cell wall, the output of inductive cephalosporinases, active efflux and a poor affiliation for the target. Multidrug resistance *P. aeruginosa* is defined as an isolated intermediate or resistant to at least three groups of antibiotics *β*_lactamase, carbapenem, and fluoroquinolones (Zaidenstein *et al*., 2018; Verma *et al*., 2018; Buhl *et al*., 2015). *P. aeruginosa* is also notorious for its extensive intrinsic, adaptive and acquired resistance profiles to various antimicrobial agent classes (Porras-Gómez *et al*., 2012). Timely appropriate antibiotic treatment is therefore vital as *P. aeruginosa* infections are associated with devastating outcomes and high mortality rates (Seshadri *et al*., 2009).

Junction phenomenon is the most important stage in the development of the disease by pathogenic microorganisms. Most pathogenic bacteria for pathogenicity must connect to the target cell surface that this junction in the various environments causes formation biofilm. Biofilm cause to priority strain that it has more effect on the growth and stable state on the surfaces. Also, the biofilm is one of the reasons for antibiotic resistance and failure to treat bacterial infections. For this reason, study about biofilms and their environmental effects are particular importance (Seshadri *et al*., 2009; Peleg *et al*., 2010).

Bacteria could be able to make biofilm with attachment to various surfaces. They produce several extracellular polysaccharides as a scaffold and keeping the cells of the biofilm community together. One of the potential polysaccharide biosynthetic loci in *P. aeruginosa* is *psl* that has been identified and play a serious duty in biofilm initiation and formation in non-mucoid *P. aeruginosa* strains. The *psl* gene cluster contains 15 co-transcribed genes (*pslA* to *pslO*) that *pslA* has an important role in biofilm formation and PSL polysaccharide formation (Perez *et al*., 2011; Overhage *et al*., 2005).

The aim of this study was consideration biofilm formation which is one of the major cause for antibiotic resistance.

Materials and methods

Bacterial isolation

Within a year, 100 *p. aeruginosa* isolates of different clinical samples such as: burn, blood, wound and other liquids body from Firoozgar and Motahari hospitals in Tehran and Velayat hospital in Rasht hospitalized Patients. Initial identification based on tests of colony morphology, oxidase test, catalase test, Indol test, MRVP test, Motility test, citrate test and Fermentation of Sugar Types (TSI) and color production was done. Then *Pseudomonas* was separated with conventional bacteriological biochemical methods. Determination of antibiotic susceptibility test was done by Kirby-Bauer method as per CLSI guidelines 2016 for various antibiotics namely: Ciprofloxacin (5 µgm), Imipenem (10 µgm), Amikacin (30 µgm), Piperacillin (30 µgm), Gentamicin (30 µgm), colistin (10 µgm), ceftazidime (30 µgm) (Rosco, Germany).

Molecular Identification of pslA by PCR

DNA purification was performed by extraction kit (Iranian Biological Resource Center) and the primers used for the amplification of the *pslA* genes (Macrogene, Korea). Table 1 shows the used primers and PCR conditions.

Table 1. Primer sequence and PCR conditions

Gene	Primer sequences 5'_3'	Size of products(bp)	PCR Conditions	Volume Re-actions
pslA	F: CCC AGC ACA AGA TCA AGA R: ACA GTA CGG AAC AGG ATG TA	125	1cycle 94ċ.....13min 30cycle: 94ċ.....30s 58ċ.....30s 72ċ.....30s 1cycle: 72ċ.....8min	10X PCR Buffer:2.5 µl 10 mM dNTPs: 0.5 µl 10 mM $MgCl_2$:0.75 µl 10 pmol F+R Primer: 1.25 µl Taq DNA polymerase (5u $µl^{-}$): 0.2 µl Template DNA: 1 µl H_2O up to 25µl

Biofilm formation

Three to five colonies of each strain were suspended in 5 mL BHI medium broth and incubated for 16 h at 37 ºC. After incubated, 200 µl of these solutions were incubated in 96 during well plates 16 h at 37 ºC. Media with suspended bacteria was then removed. Then three times washed with 250 µl of sterile phosphate-buffered saline (PBS) solution removed unattached cells. The adherent cells were solubilized with 95% ethanol and the adherent biofilm layer formed in each microtiter plate well is stained with 2% crystal violet solution for 15 min. After staining and washing with water, the clunged dye was dissolved with 33% acetic acid and the optical density of the adherent biofilm was determined twice with a filter 492 nm in the microtiter plate reader. After that, the extent of biofilm formation was determined by applying this formula: BF=AB – CW (10, 11) (Table 2) (Laverty *et al.*, 2014; Naves *et al.*, 2008).

Table 2. AB is the OD 492nm of stained attached bacteria and CW is the OD 492nm of stained control wells containing bacteria-free medium only

Formula	Strong (S)	Moderate (M)	Weak (W)	Negative (N)
BF= AB - CW	≥0.300	0.200_0.299	0.100_0.199	<0.100

Scanning electron microscopy (SEM)

At first, the samples were fixed by glutaraldehyde in cacodylate buffer, then, exposed to different ethanol dehydration series, then subjected by a dehydration concentration of 100% ethanol+hexamethyldisilazane 2×100% (HMDS, Ted Pella, USA). All the coupons were covered with a thin palladium-gold. Finally, the co-aggregation was observed with an SEM system (FEI Quanta 400FEG ESEM/EDAX Genesis X4M, FEI Company, USA) in high-vacuum mode at 15 Kv (Fratesi *et al.*, 2004).

Gene expression

Isolation of total RNA

After inoculating the strains from glycerol stocks in nutrient agar (2 mL), they were grown at 37 ºC overnight. Next, the strains were sub-cultured in the nutrient medium (5 mL) and grown to the mid-exponential phase (OD600= 1.5–2.0). Afterward, an aliquot of the culture (0.25 mL) was added to 2 mL of RNX-plus (Sinaclon, Iran). In addition, isolation of total RNA was performed based on the manufacturer's guidelines. DNase treatment was applied to remove the residual DNA with 20 U of RQ1 DNase I (Sinaclon, Iran).

cDNA Synthesis

A reaction mixture, consisting of 5 µg of RNA, was incubated in DEPC-treated water and 1 µL of random hexamers (Pars Tous, Iran). Next, it was incubated for five minutes at 65 ºC and cooled down on the ice. After 10 µL of RT premixture (2X) was added, it was mixed by pipetting up and down gently in a total volume of 24 µL. Incubation was then performed at 25 ºC for 10 minutes and at 47 ºC for 60 minutes. Following that, the reaction was terminated by heating at 70 ºC for 10 minutes and chilling on ice. The collected cDNA was stored at 20 ºC until further analysis (Table 3).

Real-Time PCR assay

Changes in *pslA* gene expression were measured using RT-PCR in the strain with the capacity to produce moderate and strong biofilm. Quantitative PCR (qPCR) was performed with according to manufacturer's instructions SYBR Green and 2X Real Time PCR Master Mix Green-No Rox kit then detected by the real-time PCR detection system (Corbett, Australia). Based on the melt curve analysis after 40 cycles, the presence of RT-PCR product was examined. The reference housekeeping gene was the *Pro C* gene (Table 3). Then, the comparative Ct (ΔΔCt) method was applied to determine Ct values; fold differences were measured as $2^{-\Delta\Delta Ct}$.

Table 3. Primer sequences of studied biofilm genes in *P. aeruginosa* isolates and reaction setup for 2-step real-time PCR

Primer Sequences	RT-PCR(step1)		Real-Time PCR(step2)	
	Conditions	Volume Re-actions	Conditions	Volume Re-actions
pslA F: CCCAGCACAAGATCAAGA R: ACAGTACGGAACAGGATGTA *Pro C* F: ATCAGTTGCTGCGGCTTCAG R: CCATCGACGTGGTCGAGTC	1cycle 25ċ.....10min 47ċ.....60min 70ċ.....10min	Total RNA 5µl Random hexamer 2µl H_2O up to 10µl	1cycle 95ċ.....5min 40cycle: 95ċ.....30s 58ċ.....30s 72ċ.....45s 1cycle: 95ċ.....10min	*Real Q Plus 2x Master Mix, green*: 12.5 µl Primer F+R Primer: 2 µl Template DNA: 5 µl Nuclease-free water: 5.5µl Total reaction volume:25 µl

Statistical analysis

To analyze the obtained data, SPSS version 23.0 (SPSS, Chicago, IL) using t-test was employed; data analysis were performed of descriptive statistics (frequency, percentage, mean); the level of significance was 0.05.

Results

One hundred *P. aeruginosa* strains were collected from clinical samples. The antibiotic susceptibility pattern was exhibited seventeen isolates resistance to all antibiotics and all the samples were confirmed as multidrug-resistant (MDR) strains (Table 4).

Table 4. The pattern of Antibiotic Resistance in *Pseudomonas Aeruginosa* Strains Isolated from the wounds, burns, blood

Antibiotics / Samples	PIP	CO	AMI	CAZ	CP	GEN	IPM
Blood And Liquids	R=16 S=17	R=17 S=14	R=12 S=21	R=15 S=17	R=18 S=12	R=15 S=17	R= 18 S= 13
Wound And Burn	R=20 S=47	R=8 S=61	R=18 S=48 I=1	R=24 S=44	R=31 S=39	R=30 S=38	R=27 S=41 I=1
Total Strains	R=36% S= 64%	R=25% S=75%	R=30% S=69% I=1%	R=39% S=61%	R=49% S=51%	R=45% S=55%	R=45% S=54% I=1%

After that, *PslA* gene was identified in all of the strains using specific primers designed in all of the strains and its accuracy was determined with electrophoresis (Figure1). The result showed all of the strains had *PslA* gene.

Figure 1. Gel electrophoresis shows the PCR product of *pslA*: Lane 1. Gene ruler 25 bp DNA Ladder, Lane 2, 3: PCR product of *pslA*

Among 100 *P. aeruginosa* resistant to antibiotics, 24 (24%) strains could make biofilm. Among 24 *P. aeruginosa* subjected to biofilm production, 1 (1%) strain demonstrated strong biofilm strength, 5 (5%) strains demonstrated moderate biofilm and 18 (18%) strains demonstrated weak biofilm (Figure 2). *P. aeruginosa* biofilm showed with scanning electron microscopy (SEM) (Figure 3).

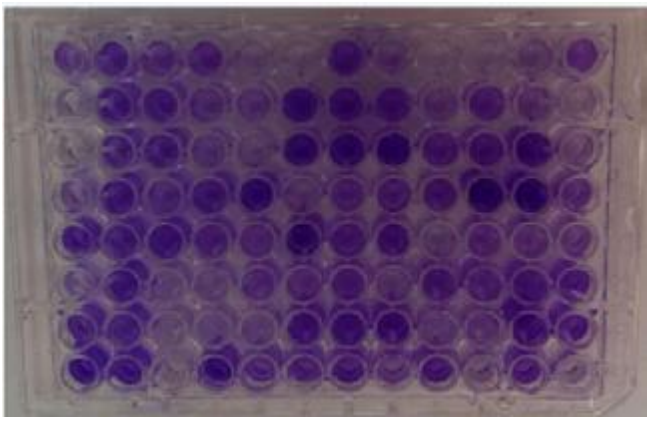

Figure 2. Biofilm formation in a microtiter plate method

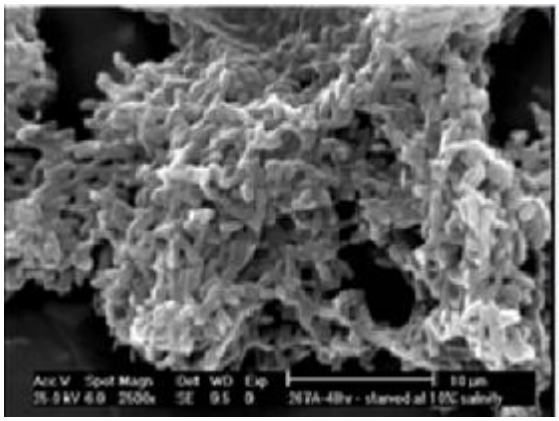

Figure 3. Scanning electron microscopy (SEM) of *P. aeruginosa* biofilm cells

The gene expression of the biofilm components *pslA* in strains with capacity produce biofilm showed that their fold change is 4.12 more than other strain respectively. The *pslA* expression was studied for the first time in Iran.

Discussion

Our study has shown high rates of MDR *P. aeruginosa* and the resistance of this bacterium to a variety of antibiotics is one of the issues for treating in hospitalized patients infected with *Pseudomonas* that is a challenge for microbiologists. Previously, closest to our findings, (Verma *et al*., 2018) were isolated *P. aeruginosa* from burn and ICU patients to evaluate multidrug resistance. They found, resistance rates to various antibiotics in the burn unit were Piperacillin/Tazobactam (33.3%), Piperacillin (75%), Aztreonam (75%), Imipenem (33.3%), Amikacin (83.3%), Ciprofloxacin (58.3%), Colistin (16.7%), Ceftazidime (75%), Cefepime (66.7%). In ICU unit resistance rates were Piperacillin/Tazobactam (33.3%), Piperacillin (77.8%), Aztreonam (66.7%), Imipenem (22.2%), Amikacin (55.6%),

Ciprofloxacin (44.4%), Colistin (11.11%), Ceftazidime (55.6%), Cefepime (44.4%) (Verma *et al*., 2018). All 21% of isolates were MDR patients. In another study, (Abidi *et al*., 2013) were isolated *P. aeruginosa* from 36 contact lenses and 14 contact lens protective fluid samples. These isolates were tested against eight commonly used antibiotics using the Kirby-Bauer disk diffusion method. The biofilm-forming potential of these isolates was also evaluated using various qualitative, quantitative techniques and a relationship between biofilm formation and antibiotic resistance were examined. They found the isolates of *Pseudomonas aeruginosa* tested were found resistant to most of the antibiotics tested. Also, qualitative and quantitative biofilm analysis revealed that most of the isolates exhibited strong biofilm production (Abidi *et al*., 2013).

In this study, the presence of a *pslA* gene as one of the main genes at biofilm formation was considered in *P. aeruginosa* strains isolated from patients. (Overhage *et al*., 2005) proved the mutation in *pslA* cause impaired in attachment and biofilm formation (Overhage *et al*., 2005).

Nowadays multi-drug resistance *P. aeruginosa* is the main problem around the world. One of the multidrug resistance reasons is the biofilm formation by these bacteria.

The findings of (Hassan *et al*., 2011) differed from our results, where they reported strong biofilm producer 64.7%, weak, and non-biofilm producer to be 36.3% but based on our findings 24 (24%) strains could make biofilm (Abidi *et al*., 2013). In another study, biofilm production among *P. aeruginosa* isolates recovered from cystic fibrosis(CF) patients were evaluated (Perez *et al*., 2011). The findings of their inquiry isolates from CF^{+}(25/35) were biofilm-producing while isolates from CF (25/39) were biofilm-producing (Perez *et al*., 2011). These findings are in line with the results of the current study.

In the current study, *P. aeruginosa* biofilm formation has shown with SEM, which is in agreement with the results of (Brandenburg *et al*., 2015). They used SEM for evaluating *P. aeruginosa* biofilm formation. Hence, they proved *P. aeruginosa* readily formed biofilms on the biological wound dressing within 48 hours of incubation in vitro.

Conclusion

Biofilms antibiotic resistance is one of the most important human problems for treating the bacterial infection, which one of the reasons can be the improper use of antibiotics and it is showing the motive to studying bacteria biofilms compared to the planktonic state. It is suggested, bacteria should investigate biofilm formation before antibiotics are prescribed.

Acknowledgments

The authors would like to thank various people for contribution, useful and constructive recommendations on this project, Especially thanks to Dr. Yadolah Mashayekhi. The authors declare that they have no conflict of interest regarding the publication of this article and, they received no specific funding for this work.

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How to cite this article: Mohammad Abootaleb, Mohammad Reza Zolfaghari, Nazila Arbab Soleimani, Nassim Ghorbanmehr, Mohammad Reza Yazdian, Biofilm Formation with Microtiter Plate 96 and pslA Detection of *Pseudomonas Aeruginosa* Isolates from Clinical Samples in Iran. *International Journal of Advanced Biological and Biomedical Research*, 2020, 8(1), 58-66. Link: http://www.ijabbr.com/article_36268.html