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Assessment of *pelA*-carried *Pseudomonas aeruginosa* isolates in respect to biofilm formation

Mahmood Abd AL- Razzaq Hassan AL-Sheikhly*[1], Laith N. Musleh[2] and Harith. J. F. Al-Mathkhury[3]

[1]Laboratory Department, Directorate of technical matters, Ministry of Health, Baghdad, Iraq
[2]Department of Biology, College of Science, University of AL-Anbar, AL-Anbar, Iraq
[3]Department of Biology, College of Science, University of Baghdad, Baghdad, Iraq

Abstract

Owing to high antibacterial resistance of *Pseudomonas aeruginosa*, it could be considered as the main reason behind the nosocomial infections. *P. aeruginosa* has a well-known biofilm forming ability. The expression of polysaccharide encoding locus (*pelA* gene) by *P. aeruginosa* is essential for this ability. The purpose of the current research was to determine the biofilm formation in *P. aeruginosa* isolated from clinical samples and to evaluate the role of the selected *PelA* gene in biofilm formation using PCR method in Iraqi patients. Results revealed that 24 (96%) isolates were found to have the ability to form biofilm that was remarkably related to gentamicin resistance. Moreover, the *pelA* gene was found in all biofilm-producers. In conclusion, the results of the current study revealed that the *P. aeruginosa* biofilm-producer isolates were resistant to the antibiotics in question. Likewise, because of wide spreading, it appears that the *pelA* gene is related to biofilm formation.

Keywords: Biofilm, pelA gene, antibiotic resistance and PCR

تقييم عزلات الزوائف الزنجارية الحاملة لجين*pelA* فيما يتعلق بتكوينها للغشاء الحياتي

محمود عبد الرزاق حسن*[1]،ليث نجيب مصلح[2] ، حارث جبار فهد المذخوري[3]

[1]قسم المختبرات، دائرة الأمور الفنية، وزارة الصحة، بغداد، العراق
[2] قسم علوم الحياة، كلية العلوم، جامعة الانبار، الانبار، العراق
[3] قسم علوم الحياة، كلية العلوم، جامعة بغداد، بغداد، العراق

الخلاصة

بسبب المقاومة العالية للمضادات الحياتية، يمكن اعتبار بكتريا الزوائف الزنجارية المسبب الرئيسي للعدوى المكتسبة من المستشفيات. تشتهر الزوائف الزنجارية بإمكانية تكوين الاغشية الحياتية، أن التعبير عن الجين السكري *pelA* لبكتريا الزوائف الزنجارية ضروري لقدرة البكتريا لتكوين الاغشية الحياتية. هدف البحث الحالي لتحديد تكوين الاغشية الحياتية في الزوائف الزنجارية المعزولة من العينات السريرية وتقييم دور جين الـ *pelA* في تكوين الأغشية الحياتية باستخدام طريقة تفاعل البلمرة المتسلسل في المرضى العراقيين. أوضحت النتائج أن 24 (96٪) عزلة لها القابلية على تكون الغشاء الحياتي والذي ارتبط بشكل ملحوظ بمقاومة الجنتاميسين. علاوة على ذلك، تم العثور على جين الـ *pelA* في جميع العزلات المكونة للغشاء الحياتي. الاستنتاج، أظهرت نتائج الدراسة الحالية أن عزلات الزوائف الزنجارية المكونة للغشاء الحياتي كانت مقاومة للمضادات الحياتية

*Email: alimahsh70@gmail.com

قيد الدراسة. ايضا، وبسبب الانتشار الواسع للزوائف الزنجارية، يبدو أن جين *pelA* مرتبط بتكوين الأغشية الحياتية.

Introduction

Pseudomonas aeruginosa is a prototype microbe in the context of biofilms [1, 2]. Biofilm formation is considered as one of the main problems in the treatment of infection [3, 4]. There are robust indications that organism forms a multicellular aggregate in which cells adhere to each other as well as to a surface and elaborate a matrix of extracellular polymeric substance within the location of the infection [5, 6].

The efficacy of enduring infections on biofilm-forming *P. aeruginosa* strains has established serious problematic issues in burn hospitals, even in healthy individuals [1]. Genetically, the formation of the biofilm matrix in various *P.aeruginosa* strains still poorly clear [5, 6], Biofilm development is organized by the expression of polysaccharide intracellular adhesion molecule that facilitates cell to cell adhesion [7]. Biofilm formation shows a protective style of growth being permits the microorganism to stay alive in various environments. [8, 9]. Certain studies reveal that the *P. aeruginosa* strains produce biofilm, have the ability to withstand different antimicrobials at concentrations higher than needed to kill planktonic cells [10].

The matrix of bacterial biofilm is made of different polymers such as polysaccharide, proteins, and extracellular DNA (eDNA) [11]). Various kinds of polysaccharides have been detected in biofilm matrix: alginate, polysaccharide encoding locus (Pel), and polysaccharide synthesis locus (Psl) [12]. Pel is a cationic polymer that mediates cell to cell adhesion within the biofilm exopolymeric matrix via electrostatic interactions with eDNA, it is synthesized by *P. aeruginosa* as an important biofilm constituent highly needed for bacterial virulence and persistence [13]).

The aim of the current study was to evaluate the apparent correlation of *pelA* among biofilm producing *P. aeruginosa* clinical isolates alongside with antibiotic resistance.

Materials and methods

Clinical isolates and phenotypic identification

86 different specimens were collected from patients referring to several hospitals in Baghdad covered Baghdad teaching Hospital, Teaching laboratories department, Burn hospital / Medical City and Al-Yarmouk teaching hospital. All the 86 specimens were cultured on enrichment media such as Blood agar and transferred onto selective and differential media (MacConkey agar). The specimens demonstrating the growth on both media, appeared as pale colonies on MacConkey agar (lactose non-fermenter) were chosen for further experiments.

The discrete colonies were picked for the identification process based on some morphological and biochemical characterization. Gram stainability was conducted alongside the activities of oxidase, catalase [14]. API20E test was employed to confirm the results of identification.

Antimicrobial susceptibility testing

Antibiotic susceptibility was assayed by Kirby-Bauer disk diffusion method [15] on Mueller-Hinton agar (Salucea, Holland). The antibiotic disks were of Mast group, U.K, comprising Gentamicin (10 µg), Amikacin (30µg), Ciprofloxacin (5µg), Imipenem (10µg), and Ceftazidime (30 µg). An isolate was designated as sensitive, intermediate resistant, and resistant in according to criteria recommended by the Clinical and Laboratory Standards Institute (CLSI) [16]. A standard strain (*P. aeruginosa* ATCC 27853) was obtained from the Teaching laboratories department of the Medicine City, served as a quality control strain.

Biofilm formation assay

Biofilm formation was determined by microtiter plate assay [17]. Briefly, 24 hours old cultures 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 was 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 the negative control. The amount of crystal violet was

removed by the 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).

Molecular study

DNA of *P. aeruginosa* isolates was extracted and purified using Genomic DNA Extraction Kit (Promega, USA).

The primers pairs used are designed to amplify a 118 bp fragment *PelA* gene by Colvin *et al*.2011 [18]. The primers pairs are pelA F: 5′-CCTTCAGCCATCCGTTCTTCT-3′ and pelA R: 5′-TCGCGTACGAAGTCGACCTT-3′. PCR amplification was performed using Green master mix (Bioneer, Korea), 2 µl of 10 pmol/µl of each forward and reverse primers, 2 µl of template DNA (15-25 ng), volume was completed up to 20 µl with nuclease free water (Promega, USA). The PCR tubes containing the mixture were transferred to thermocycling (5 min at 94°C, 35 cycles of 30 sec at 94°C, 40 sec at 52°C, 50 sec at 72°C, and a final extension step 5 min at 72°C using Bio Rad, USA [19]. Thereafter, amplicons were resolved in 1.5% agarose gel.

Statistical Analysis

In order to determine the impact of parameters in this study using statistical package for social science (SPSS) 21.0 and Microsoft excel 2013. Categorical data formulated as count and percentage. Fisher exact test and chi square test was used to describe the association of these parameters. The lowest level of accepted statistical significant difference is bellow or equal to 0.01[20].

Results

Isolation and Identification

All the 60 isolates had a growth on nutrient, cetrimide agar and developed pale colonies on MacConkey agar and; nevertheless, they were oxidase and catalase positive. Hence, these isolates were primarily identified as *P. aeruginosa* (Table-1). Identification was confirmed using API 20E test.

Table 1-Morphological and biochemical tests for identification of *Pseudomonas aeruginosa*

Test	Result
Gram stain	Gram negative rods
MacConkey agar	Pale non-lactose fermenter colonies
Oxidase	Positive
Catalase	Positive
Growth on Cetrimide agar	Growth with fluorescent green colour, elevated colonies, and grape-like odour
Growth at 42°C on Nutrient agar	Positive

According to the specimen type, 60 isolates were categorized into seven groups, as shown in Table-2.

Table 2-The numbers and percentage of *Pseudomonas aeruginosa* isolates in accordance to specimen type

Source of isolates	No. of Isolates	(%)
Burn	**22**	**36.67**
Urine	**14**	**23.33**
Wound	**11**	**18.33**
Sputum	**4**	**6.67**
Broncho alveolar lavage	**4**	**6.67**
Ear infect	**4**	**6.67**
Nasal swab	**1**	**1,67**
Sum (%)	**60**	**100**

Antimicrobial susceptibility

The results showed highly significant (P≤0.01) variation in levels of resistance among the groups of antibiotics. The highest resistance results (68.3%) were recorded against each of Gentamicin and Ciprofloxacin. While the lowest resistance levels: 36.6% and 53.3% were against β-lactams represented by Ceftazidime and Imipenem, respectively (Figure-1).

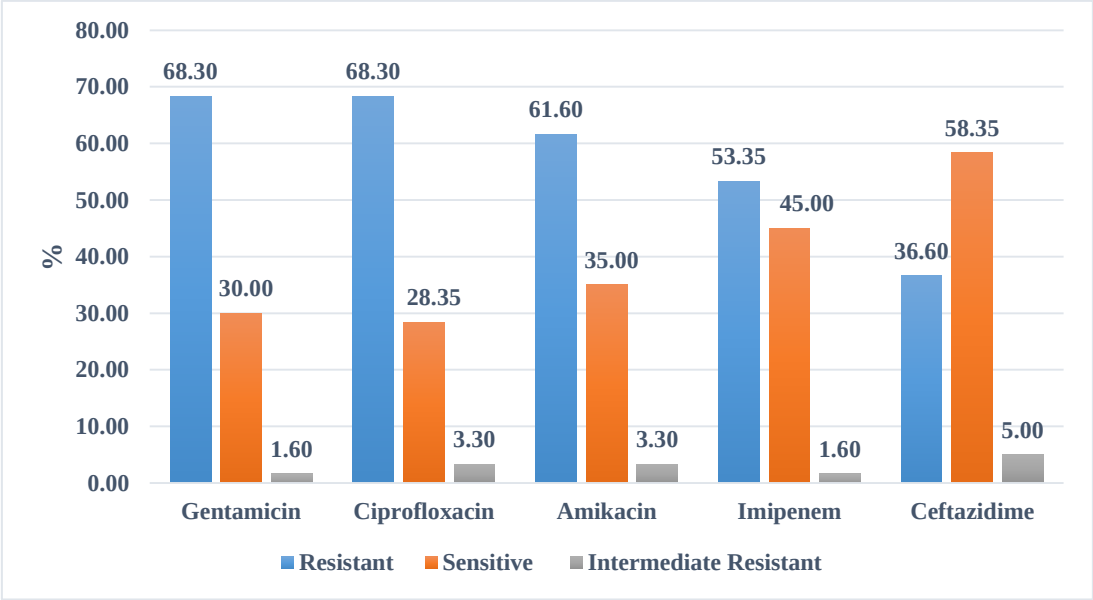

Figure 1-Antibiotic susceptibility test of *Pseudomonas aeruginosa* isolates

Biofilm formation assay

This study showed a trend among the clinical isolates of *P. aeruginosa* to form a biofilm 24/25 (96%), except one isolate (4%) represented non-biofilm producer. Upon the criteria listed in Figure-2, the present study declared that three isolates (12%) formed weak biofilm, fourteen (56%) isolates formed moderate biofilm, whereas seven (28%) isolates formed strong biofilm.

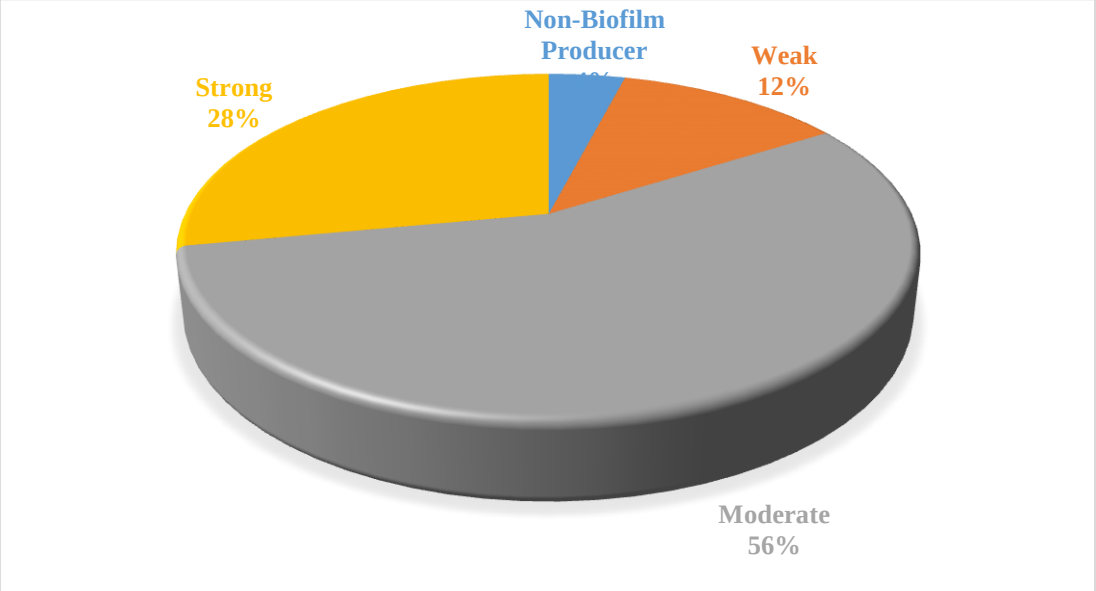

Figure 2-The percentage of biofilm formation by *Pseudomonas aeruginosa*

Molecular study

PCR was carried out for the detection of *PelA* gene, which was involved in the formation of biofilm among the clinical isolates; which was expressed in all 25 tested isolates that developed a resistance to Gentamicin (Figure-3).

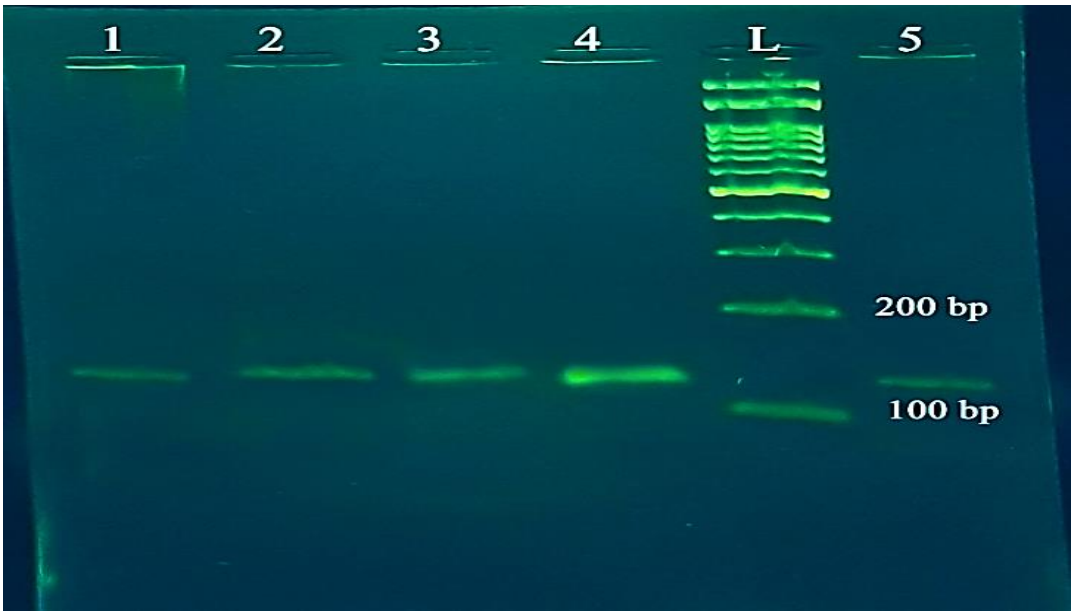

Figure 3-Agarose gel electrophoresis (1.5% agarose, 90 min) of *pelA* gene (118bp). Lane L represents 100bp DNA ladder, Lanes 1-5 represent bands of *P. aeruginosa* isolates.

Discussion

Pseudomonas aeruginosa is an opportunistic pathogen capable of causing a wide array of life threatening acute and chronic infections particularly in patients with compromised immune defense [21]. Earlier reports have shown 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 [22].

The variability in *P. aeruginosa* isolation percentage may be attributed to geographic, climatic, and hygienic factors among different areas. As well as, the high prevalence of *P. aeruginosa* in our community may be related to the rise of burn and wound patients than other samples in our population [23], which may be the result of different increased kitchen accidents, terrorist incidents, and electrical fires.

The differences in biofilm intensity among isolates of the present study might be due to several reasons; differences of isolates capacity to form biofilm or perhaps differences in primary number of cells that succeeded in adherence and differences of quality and quantity of quorum sensing signaling molecules (autoinducer) that produced from each isolate play important roles [24]. However, this high biofilm intensity may be related to susceptibility of microtiter plate assay in determining the few amounts formed, and is regarded as an essential method in studying the early stages of biofilm formation due to its use of constant conditions and it can be effective in studying different types of virulence factors to form biofilm such as pili, and flagella. In addition we can use this method with different types of bacteria [25].

On the other hand, Heydari and Eftekhar [26] indicated that the diversity of the ability of isolates to form biofilm is due to the association of production with its capability to produce different types of ß-Lactamase leading to forming a strong biofilm in comparison with the isolates that produced one type of enzyme. On the contrary, the isolates did not produce this enzyme were unable to form a biofilm. Besides, the variety in results might be due to the kind of media or the laboratory conditions that accompanied the detection of biofilm formation.

Numerous bacteria occur in turn between two forms of growth: planktonic cells and biofilms. These organized communities of bacteria are capable of growing on different surfaces and are the major factor of several diseases. Approximately, 80% of all microbial infections are related to biofilms [27]. Besides, several bacteria can form biofilms on abiotic surfaces. These microorganisms are usually considered as responsible for bio corrosion and biofouling in a variety of industry sectors. They may lead to the destruction of the equipment and accordingly to an increase in the maintenance costs [28].

The antibiotic susceptibility test was performed for aminoglycosides, fluoroquinolones, and beta lactam antibiotics with all the 60 isolates. Nearly 68.3% and 61.6% of isolates developed resistance toward gentamicin and amikacin, respectively. Starkey *et al*.2009 [29] reported that gentamicin in comparison to other aminoglycosides exhibited increased levels of resistance which may be due to the presence of *PelA* gene. Colvin *et al*. [18] assumed that pel is capable of providing preservation to planktonic cells when artificially overexpressed, thus suggesting that *Pel* plays an important protective role in biofilms of *Pseudomonas* strains. The aminoglycosides and fluoroquinolones antibiotics developed the highest resistance with respect to biofilm producing strains. Gentamicin and ciprofloxacin expressed 68.3% resistance. The carbapenems antibiotics revealed 53.3% resistant towards imipenem. The beta lactam antibiotics showed resistance lower level in biofilm producing strains; yet, ceftazidime expressed 36.6% resistance. Production of β-lactams enzymes in some isolates, which act to destroy β-lactams ring thereby leads to modification of antibiotics structure and spoilage their effects [30]. Moradali *et al*. [31] stated that *P. aeruginosa* is regarded as a main causative agent of nosocomial infections owing to its wide spectrum of antibiotic resistance. Saderi and Owlia [32] clarified that this ability either be normal or may be acquired through mutations in their genetic material or through the horizontal transference of genes.

In the present study, the isolates produced strong, intermediate and weak biofilms were screened for *PelA* gene, which is responsible for production of biofilm. Pel is essential for conserving cell-to-cell interactions in *P. aeruginosa* biofilm, service as a primary structural frame for the community. It also plays a secondary role by increasing resistance to aminoglycoside antibiotics and this defense happens only in biofilm populations [33].

The current study observed that *pelA* gene was expressed heavily 25/25(100%) amongst the biofilm producing isolates. The phenotypically positive isolates for production of biofilms in vitro were genotypically positive for expression of *PelA* gene, while those isolates phenotypically non-producing biofilm and harboring *PelA* gene were also found to be resistant towards aminoglycosides [19]. This gene was found in almost all clinical strains of *P. aeruginosa*. The presence of this gene could not predict which strains will produce a biofilm because the biofilm formation is affected by many factors. On the other hand, there was no gene expression appear in some isolates, despite the formation of the biofilm in these isolates, this means a contribution of other genes that are not studied in current research has a role in the formation of the biofilm.

Conclusion

The current study showed that most of the biofilm formed isolates were highly resistant to even higher generations of aminoglycosides, fluoroquinolones and beta lactam group of antibiotics. It was also observed that most of the isolates showing resistance to aminoglycosides antibiotic carried *PelA* gene. Henceforth, more work is needed to fully elucidate the antibiotic resistance mechanisms in biofilms and develop new therapeutic strategies.

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