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Investigation of the *ndvB* gene related to antibiotic resistance in a biofilm of *Pseudomonas aeruginosa* isolates in Iraq

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Abstract

The high morbidity and mortality are caused by *Pseudomonas aeruginosa*, which behaves as an opportunistic organism that resists many disinfectants through biofilm. The current study aims to detect variation in the *ndvB* gene and its relation with biofilm construction through the correlation of the change in the DNA sequence of the *ndvB* gene in different isolates and their phenotypic change in biofilm formation (weak, moderate, and strong biofilm producers). The biofilm was constructed, and the Minimum Inhibitory Concentration (MIC) for planktonic cells and Minimum Bactericidal Concentrations (MBC) for biofilm-forming cells were determined for the 20 isolates under the study. The prevalence of *ndvB* gene was detected, and its sequence was detected through Sanger sequencing and compared to the analysis of the genic variation for the *ndvB* gene and their correlation with phenotypic change for biofilm construction. Most of the isolates were biofilm producers and were resistant to different antibiotics. In addition, MBC for biofilm-producing isolates was high, while MIC for the planktonic cell was low. This result may indicate the isolates can resist ciprofloxacin, gentamicin, and tobramycin antibiotics in the presence of biofilm. Furthermore, the SNP detection in the coding sequence of the *ndvB* gene in isolate with ID 14 compared with the reference genome revealed that SNP located at position 792 transferring G to A. This variation may contribute to the resistance of the *ndvB* gene to antibiotics in the presence of biofilm in *P. aeruginosa* but needs to be further proven experimentally.

Keywords: antibiotic resistance, biofilm construction, genic variation, *Pseudomonas aeruginosa*, *ndvB* gene

التحري عن جين ndvB المرتبط بمقاومة المضادات الحيوية في الأغشية الحيوية لعزلات *Pseudomonas aeruginosa* في العراق

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الخلاصة

معدلات الامراضية والوفيات الناتجة عن *Pseudomonas aeruginosa* تكون جدا مرتفعة وهذه البكتريا هي جدا انتهازية وتقاوم العديد من المضادات الحيوية بوجود الأغشية الحيوية. هدف الدراسة الحالية هو الكشف عن التغاير بجين *ndvB* وعلاقته بتكوين الاغشية الحيوية من خلال الربط بين التغاير بالمادة

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الوراثية لجين *ndvB* في العزلات المختلفة والتغاير المظهري لتكوين الاغشية الحيوية. قمنا بتكوين غشاء حيوي وفحص MIC و MBC للخلايا العوالق والخلايا المكونة للغشاء الحيوي للعزلات قيد الدراسة على التوالي. قمنا بتحديد النسبة المئوية للعزلات التي حملت جين *ndvB* وتم تحديد التسلسل الجيني *لجين ndvB ومقارنة التغاير بالتسلسل الجيني بالتغاير المظهري لتكوين الاغشية الحيوية*. كانت معظم العزلات مكونة للغشاء الحيوي ومقاومة للعديد من المضادات الحيوية وقيم MBC ضد الغشاء الحيوي عالية ، على النقيض من ذلك ، كان MIC لخلية العوالق منخفضًا. قد تشير هذه النتيجة إلى قدرة العزلات على مقاومة المضادات الحيوية ciprofloxacin, gentamicin, and tobramycin بوجود الأغشية الحيوية. بالإضافة إلى ذلك ، ان التباين في المنطقة الجينية لجين *ndvB* المعزول من السلالة ID 14 مقارنة مع الجينوم المرجعي كان عند الموقع 792 وذلك بتحويل نيوكلوتيدة G الى نيوكلوتيدة A . هذا التباين قد يعطي دليلاً على أهمية الجين *ndvB* في مقاومة المضادات الحيوية للعزلات التي شكلت الأغشية الحيوية لكن يحتاج الى ان يثبت باجراء التجارب المختبرية.

Introduction

Pseudomonas aeruginosa is considered one of the main causative agents for nosocomial infection, mainly enrolled in patients with cystic fibrosis (CF)[1-4]. *P. aeruginosa* in CF patients can cause infection due to their capacity to engage with biofilm and disinfectant resistance [5-9]. This bacterium is an opportunistic pathogen and is characterized by its ability to resist different antibiotics [10-13]. *P. aeruginosa* possesses many factors that help in pathogenesis; one of these is the ability to form biofilm that helps in resisting antibiotics and disinfectants [10, 14-17]. It is impossible to eliminate *P. aeruginosa* infection, which possesses antibiotic resistance and forms biofilm and protects the bacterium from immune clearance[18-20] . In the case of antibiotic resistance, *P. aeruginosa* forming biofilm can be more tolerant than planktonic cells in more than 1000 fold [16, 21-24]. Biofilm consists of 15% microcolonies and 85% polymeric extracellular substances, and it contains polysaccharides, nucleic acid, and protein[25, 26]. Eradication of the biofilm of *P. aeruginosa* in tissue infection in humans is quite challenging [10, 27]. Genetic destabilization can lead to the development of biofilm through observing phenotypic variation and lead to the determination of biofilm architecture [28-30]. Genomic changes in clinical *P. aeruginosa* isolates are more than that of environmental isolates, especially in the transcriptional profile, and they also showed higher divergent transcriptional profiles in biofilm-forming isolates compared with planktonic cells. [5, 31-35]. The phenotypic and genomic diversity of *P. aeruginosa* participate in survival of this organism in many habitat [36-39]. Expression of the genes related with antibiotic resistance and virulence factors in biofilm model may differ from the expression in planktonic cell as the level of antibiotic resistance increase in biofilm [29, 40, 41]. In addition, multidrug resistance mediating biofilm arises due to an increase in mutation and a decrease in metabolic activity, especially mutations occurring in the *ndvB* gene [25, 42, 43]. *ndvB* gene participates in antibiotic resistance through biofilm construction [40, 44]. Many studies aimed to detect the presence and absence of the *ndvB* gene in biofilm-forming *P. aeruginosa* isolates; detection of the variation in the coding sequence of the *ndvB* gene in biofilm-forming and non-forming isolates is rarely studied. Therefore, this study focused on the detection of the variation in the genic region of the *ndvB* gene in different isolates and its correlation with phenotypic change for the biofilm formation as the isolates formed weak, moderate, and strong biofilm.

Materials and methods

Collection of isolates

Isolates of *P. aeruginosa* were collected from different samples taken from hospitals in Baghdad city from patients of different ages for the period between 2018 and 2019 using

sterile cotton swabs; around 20 isolates were detected belonging to *P. aeruginosa,* relying on morphological and biochemical tests. Different media, including blood, cetrimide, and MacConkey agars, were used to culture the swabs, then relying on the phenotypic characteristics of bacteria on culture media (such as texture, size, edge, shape, and odor), *P. aeruginosa* isolates were initially detected. For microscopic examination, a part of the colonies was transferred by sterile loop into a glass slide to detect the morphological characteristics and gram stain. The isolates were further identified using biochemical tests such as oxidase, catalase, motility, sugar fermentation, and indol tests. Finally, to clarify the diagnosis of bacterial strains, the Vitek 2 system was used according to the manufacturer's protocol.

Biofilm construction assay

Microtiter plate assay was used to detect the possibility of *P. aeruginosa* isolates to construct strong, moderate, or weak biofilm [45]. The culture media of tryptic soy broth was used to cultivate the *P. aeruginosa* isolates with the addition of glucose (0.25%) and incubated at a temperature of 37 ^{0}C for 24 h. A suspension of bacteria with 125 µl was poured in each well of 96 well of microtitre plates and kept at a temperature of 37 ^{0}C for 24 h. Then, the wells were washed with Distilled water (D.W) and left to dray by inverting the microtitre plates at room temperature. After that, the wells were stained by the addition of 125 µl of crystal violet (0.1%) for 10–15 min. Then, wells were destained using acetic acid. Finally, the optical density (OD) at 570nm for the biofilm that was constructed in each well was measured using the ELISA apparatus. Each test was carried out in triplicate, while the control was used as media without inoculation. Different intensities of biofilm with weak, moderate, and strong biofilm constructors were detected relying on the optical density of each sample with triplicate that was averaged and subtracted from OD of control (ODi) while ODc was represented by O.D of negative control plus three standard deviations. These intensities were detected as follows: ODi less than ODc (non-biofilm constructor), ODc less than ODi and ODi less than 2*ODc (biofilm constructor with a weak intensity), 2*ODc less than ODi and ODi less than 4*ODc (biofilm constructor with moderate intensity), 4*ODc less than ODi (biofilm constructor with strong intensity)[46] .

Detection of Minimum Bactericidal Concentrations (MBC-B) for biofilm

MBC-B for biofilm was detected as reported by [47, 48] as follows: biofilms were constructed for 24 h as previously described, then planktonic cells were removed. After that, antibiotics with serial dilutions were prepared (ciprofloxacin ranged between 2.5 to 160 mg/ml, gentamicin ranged between 12.5 to 800 mg/ml, and tobramycin ranged between 6.25 to 400 mg/ml) and poured into biofilms. Antibiotic treatment for biofilm was kept for 24 hours, and then the survival of bacteria was detected by cultivating a biofilm culture using plates containing nutrient agar.

Detection of Minimum Inhibitory Concentration (MIC) for planktonic cells

Minimum inhibitory concentrations for planktonic cells (MICs-P) were detected using the broth dilution approach with two-fold as reported by[49] as follows: antibiotics with serial dilutions were prepared (ciprofloxacin ranged between 2.5 to 160 mg/ml, gentamicin ranged between 12.5 to 800 mg/ml, and tobramycin ranged between 6.25 to 400 mg/ml) and added to planktonic cells cultured in 96 wells of microtitre plate. The MICs were detected by adding a tetrathiozune stain for each well, and the change in the color from blue to pink was an indicator of a MIC point.

ndvB gene detection and sequencing

DNA extraction was carried out using the ABIOpureTM Total DNA kit according to the ABIOpure protocol. The bacterial cell was lysis, and then purification and extraction were done using a mini-column for *P. aeruginosa* isolates with six isolates (two non-biofilm forming, two weak-biofilm forming and two moderate-biofilm forming). Amplification of the *ndvB* gene was achieved by PCR with an amplicon size of 990 bp using the following primers: ACAGTTGATCGCCTACCT for forward primer and TGTCGATCACCAGGACTT for reverse primer; these primers were designed using primer quest tools. The reaction mixture of the amplification was 25 µl that contained 1 µl reverse primer, 1 µl forward primer, 2 µl bacterial DNA template, 12.5 µl of Master mix and 8.5 µl distilled water. The PCR reaction was performed as illustrated in Table 1. Gel electrophoresis with 2% agarose was used to visualize DNA bands that were stained with ethidium bromide. The DNA samples for 6 *P. aeruginosa* isolates were sent for sequencing using ABI 3730 DNA Sequencer (ABI, Applied Biosystems). The raw data of sequencing results was filtered using the SNPviewer program, and the criteria of filtration of spurious data relied on phred quality score for each nucleotide and peak characterization. The SNPs were detected through the alignment of DNA sequencing for the *ndvB* gene in different isolates using the BioEdit program. Finally, the BLAST tool was used to align reference genomes with isolates under study and to search the database.

Table 1 : Condition of PCR reaction for *ndvB* gene

Cycles of PCR	Temperature	Time
Initial denaturation	95 ^{0}C	2 minute
Denaturation	95 ^{0}C	1 minute
Annealing	56 ^{0}C	1 minute
Extension	72 ^{0}C	90 second
Final extension	72 ^{0}C	10 minute

Statistical test

A comparison of proportions was carried out using the Chi-squared test [50]. The following link was used to detect P-value
https://www.medcalc.org/calc/comparison_of_proportions.php
One way ANOVA test was enrolled to calculate the statistical significance of differences in MIC and MBC values between different biofilm producers. The following link was used to detect P-value
https://www.statskingdom.com/two-way-anova-calculator.html

Results

The biofilm formation in 20 *P. aeruginosa* isolates revealed that non-biofilm producers and weak biofilm constructors were 20% and 45%, respectively. However, the moderate biofilm producer constructs around 35% (Figure-1). The isolates forming biofilm were significantly higher, with a P value equal to 0.0002, than the isolates that were unable to form biofilm.

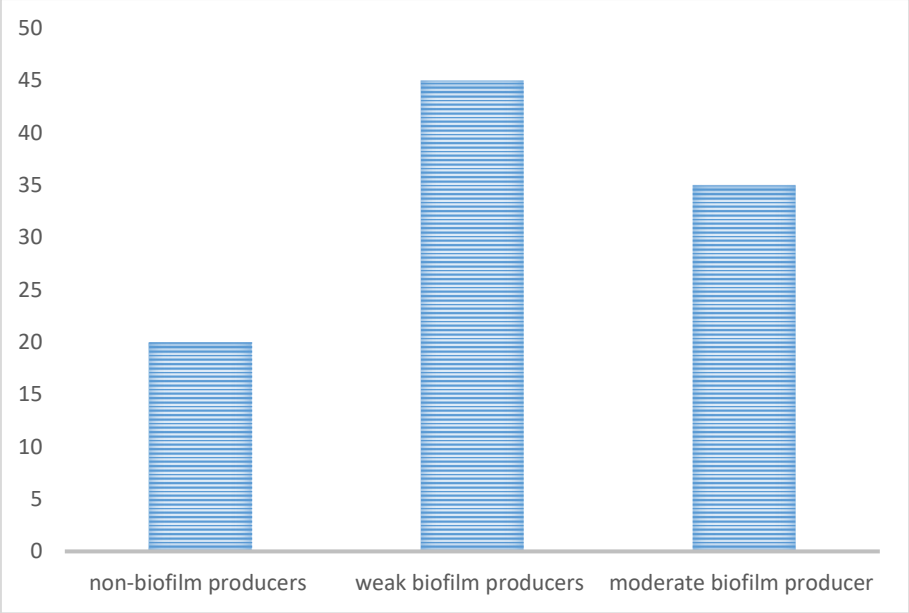

Figure1: Biofilm formation by *P. aeruginosa*

MIC for planktonic cells of *P. aeruginosa* isolates was determined relying on color indicator (change the color from blue to pink) using tetrathiozune pigment.

MIC of planktonic cell to Tobramycin antibiotic for non-biofilm producer, weak and moderate biofilm producer was 6.25, however MBC for biofilm maturation to Tobramycin antibiotic was higher with values ranged from (25-400) and there was no a big difference between MBC values among non- biofilm, weak and moderate intensity of biofilm constructors. For the Gentamycin antibiotic, MIC of the planktonic cell for non-biofilm producers was 12.5, while it was 25 for weak and moderate biofilm constructors. Conversely, MBC for biofilm maturation was also higher and ranged between (12.5-400), and it was higher in weak and moderate, comparable with non-biofilm producers. MIC of planktonic was very low using Ciprofloxacin with 2.5, while MBC for biofilm maturation to Ciprofloxacin was higher and ranged between (10-80). There was no difference between MBC values among non-biofilm, weak and moderate biofilm producers, as shown in Table 2. Using Tobramycin and Ciprofloxacin antibiotics, the proportion of isolates with a high concentration of MBC-biofilm compared with MIC-planktonic cells was highly significant with P-value = 0.0009 while for Gentamycin, the P-value was 0.02.

The significance of differences in MIC and MBC values between different biofilm producers was calculated for different antibiotics. For Tobramycin, different biofilm producers were not statistically different, with a P-value equal to 0.4 for MIC values and 0.6 for MBC values. For Gentamicin, different biofilm producers were statistically different, with a P-value equal to 0 for MIC values and 0.3 for MBC values. Finally, different biofilm producers using Ciprofloxacin antibiotic were not statistically different, with a P-value equal to 0.6 for the MBC value.

Table2: MIC for planktonic and MBC for biofilm (µg/mL) of Tobramycin, Gentamicin, and Ciprofloxacin

strains	Tobramycin MIC of planktonic cell (µg/mL)	Tobramycin MBC of biofilm (µg/mL)	Gentamicin MIC of planktonic cell (µg/mL)	Gentamicin MBC of biofilm (µg/mL)	Ciprofloxacin MIC of planktonic cell (µg/mL)	Ciprofloxacin MBCof biofilm (µg/mL)
strain-1: non-biofilm former	6.25	25	12.5	12.5	2.5	40
strain-6:non-biofilm former	6.25	400	12.5	200	2.5	10
strain-12:weak-biofilm former	25	100	25	50	2.5	80
strain-19: weak-biofilm former	6.25	25	25	200	2.5	40
strain-15:moderate-biofilm former	6.25	100	25	400	2.5	80
strain-16:moderate-biofilm former	6.25	200	25	200	2.5	10

After subjecting biofilm formation of 6 *P. aeruginosa* (2-non-biofilm, 2 weak-biofilm, and 2 strong biofilm constructors) isolates to different concentrations of antibiotics, the DNA was extracted, and PCR analysis was carried out. The PCR result revealed that the *ndvB* gene was only found in the isolate number 14 (moderate biofilm producers), while it was missing from the rest of the isolates (figure -2).

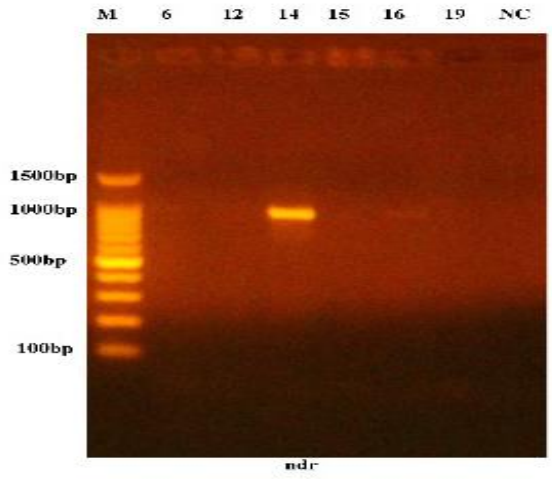

Figure 2: Visualization bands of *ndvB* gene (999bp) on an agarose gel (2%). M represents the DNA ladder, and numbers 6 to 19, represent the bacterial isolates. NC: represents negative control.

To clarify the deletion in the *ndvB* gene in 5 *P. aeruginosa*, the sequencing of the *ndvB* gene was carried out. The sequencing result was compatible with the PCR result, and we got sequencing for the *ndvB* gene in isolate 14 only. Comparing the sequence of the *ndvB* gene between isolate number 14 in the current study with isolate taken from the database as reference genome showed SNP that was determined at position 792 converting A to G, as shown in Figure 3.

Figure 3: Schematic representation for the alignment for isolate number 14 with reference genome. The variation was detected at position 792, converting A to G.

Discussion

Biofilm formation participates in the pathogenicity of *P. aeruginosa*[51] , a high percentage of isolates under the study formed biofilm, and this was compatible with Saffari *et a.l* [52] and Kamali *et a.l* [53] results, thereby they revealed that all the isolates constructed biofilm. For antimicrobial treatment, MIC was very low for all antibiotics that were investigated in the current study with all isolates. This could be explained as low doses of antibiotics inhibiting the growth of *P. aeruginosa*; therefore, all antibiotics are effective against *P. aeruginosa* isolates under study. This result agreed with Cigana *et al*. [54], who showed low MIC and excellent antibiotic activity against *P. aeruginosa* for the new generation of antibiotics compared with the old one. This result is also agreed with Sousa et *al.* [53], who showed that most antibiotics are effective against *P. aeruginosa.* However, MBC was very high for all antibiotics that were investigated in the current study with all isolates. This could be explained as high doses of antibiotics required to penetrate the biofilm and eliminate the bacteria, and this reflects the important role of biofilm formation in resistance to different antibiotics; this result was agreed with Silva *et al.* [55], who declared important of biofilm construction in prevalent of *P. aeruginosa* isolates. Akel *et al*. [56]

showed that Ciprofloxacin antibiotic was able to eliminate biofilm and kill the bacterial cells with 67%. Banar *et al*. [57] also showed that most of the isolates were resistant to antibacterial agents used in their study, and they almost formed biofilm; therefore, he stated that the resistance to antimicrobial agents raised from the fact that these isolates were able to form biofilm. According to the PCR result, only one isolate, which is a moderate biofilm producer, carried the *ndvB* gene. The prevalence of *ndvB* gene for the selected 6 isolates was very low at 16.6%, and the reason for missing the *ndvB* gene in most of the isolates may be that the isolates were subjected to various concentration of antibiotics before detection the presence of this gene. On contrast, Saffari *et al*., [52] found that the prevalence of *ndvB* gene was in most of the isolates with 97.6%. While Ganjo *et a.l* [58] showed, only 17% of isolates carried the *ndvB* gene. However, in our study, the isolates producing biofilm were highly resistant to antibiotics, which may be due to many reasons, but it was not only concerned with the presence of the *ndvB* gene, especially since the gene is missing and was confirmed by PCR and sequencing technique. Comparing the genic sequence of the *ndvB* gene between our isolate ID: 14 and reference genome showed SNP located at position 792 transferring G to A, this variation may participate in the resistance of the *ndvB* gene to the antibiotic in the presence of biofilm in *P. aeruginosa*, this variation could leads to change affinity of glucosyltransferase enzyme coded by *ndvB* gene for production of cyclic-b 1, 3 glucans hence increase the amount of glycans and the amount of antibiotics sequestered by the glucans. This interpretation needs to be confirmed experimentally, showing the importance of this SNP in changing the affinity of glucosyltransferase enzyme. In addition, the limitation of the current study regarding sample size and methodology gives evidence for the fact that this SNP may be less crucial in antibiotic resistance of *P. aeruginosa* in the presence of biofilm. Moreover, Ismail and Altaai [58] showed that most of the variation mediating antibiotic resistance in the presence of biofilm occurred in promoters; therefore, it changed the level of expression.

Conclusion

Most isolates that formed biofilm can resist the antibiotics under study. Moreover, Studying the genic variation pattern for the *ndvB* gene may indicate this gene contributes to antibiotic resistance mediated by the formation of biofilm.

Conflict of Interest:

The authors declare that they have no conflicts of interest

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