Correlation between Biofilm Formation, Multi-Drug Resistance and AlgD Gene among *Pseudomonas aeruginosa* Clinical Isolates

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Abstract

Background: *Pseudomonas aeruginosa* is an opportunistic pathogenic bacterium that associated with persistent infections that are difficult to eradicate. **Objective:** This study aims to determine biofilm forming potential of *P. aeruginosa* and its correlation with antibiotics resistance and presence of AlgD gene. **Materilals and Methods:** The present study carried out in Azady teaching general hospital in Kirkuk city during the period from August 2017 to May 2018, one hundred isolates of *Pseudomonas aeruginosa* had been obtained from (1260) different clinical specimens. Antibiotic susceptibility was determined by Kirby-Bauer method, biofilm formation analyzed by Microtiter plate quantitative method, and AlgD gene was determined by polymerase chain reaction (PCR).**Results:** A lower percentage of antibiotic resistance was against piperacillin/tazobactam (5%), cefepime (24%), and ceftazidime (26%) while, higher resistance percentage was seen against amoxicillin/clavulanic acid (98%) and trimethoprim/sulfamethoxazole (97%), multi-drug resistance (MDR) formed (60%) of total isolates. Biofilm formation found in (98%) of total isolates. AlgD gene detected by PCR technique found in (98%) of total isolates, AlgD gene was found only in biofilm former isolates. **Conclusions:** Significant correlation found between biofilm formation and antibiotic resistance. Strong correlation observed between presence of AlgD gene and biofilm formation.
Key Words: Multi-drug resistance (MDR), biofilm formation, AlgD gene.

Introduction

Biofilm is one of the most important virulence factors that form on the surface of bacteria; like *Pseudomonas aeruginosa*, biofilms are organized communities of bacterial cells embedded to an inactive or living surface and enclosed in an extracellular polysaccharide matrix, that protect the bacterial community from harsh environment conditions such as host immune system and antibiotics [(1)] .

Biofilms have been implicated in numerous sub-acute and chronic infections [2]. *Pseudomonas aeruginosa* synthesizes an exopolysaccharide called alginate, protect the bacteria from changed environmental conditions and encourages adhesion to solid surfaces [3]. Transcription of the alginate biosynthetic genes (AlgD gene) is induced by attachment to the substratum and which leads to increased alginate production that leads to biofilms formation [4, 5]. Currently *P. aeruginosa* shows resistance to various antibiotics, because of excessive antibiotic administration lead to accumulation of antibiotic resistance and cross-resistance between antibiotics to develop multi-drug resistant (MDR) forms of *P. aeruginosa* [6]. Multi-drug resistant *Pseudomonas aeruginosa* is defined as the isolate that resistant to three or more than three groups of antibiotics as mentioned: penicillins, cephalosporins, monopactam, carbapenems, fluoroquinolones, and aminoglycosides [7].

Materials and Methods

In the present study 100 isolates of *P.aeruginosa* were collected from 1260 different clinical specimens that include: sputum, urine, bronchial wash, swabs (burn, wound, and ear swabs). All specimens cultured on Blood agar, MacConkey agar and brain heart infusion broth (BHI) incubated over-night at 37 °C, then the colonies tested for oxidase production and for biochemical, which had been performed by VITEK®2 GN ID card.

Antibiotic susceptibility tests:

Antibiotic susceptibility test had been performed for all isolates by depending on Kirby-Bauer method, after cultured on Mueller-Hinton agar. Interpretation of results based on clinical and laboratory standard institute (CLSI) guidelines [8]. Antibiotic discs that had been used included: ticarcillin (75µg), ticarcillin(75µg)/clavulanic acid (10µg),piperacillin(100µg)/tazobactam(10µg),aztereonam(30µg),levofloxacin(5µg),cipro floxacin(5µg), ceftazidime(30µg), cefepime(30µg), imipenem(10µg), meropenem(10µg), amikacin(30µg), Trimethoprim(1.25µg)/sulfamethoxazole(23.7µg), tobramycin(10µg), amoxicillin/clavulanic acid(20/10meg).

Biofilm formation:

Quantitative microtiter plate method was used to analyze the ability of *P.aeruginosa* to biofilm formation. In this method *P. aeruginosa* isolates inoculated in 3–5 ml Tryptone Soy Broth(TSB) and incubated for 24 hours at 37°C, After incubation cultures were diluted 1:100 in the TSB, and 100 µl of each diluted culture broth was pipetted in each well of 96-well flat-bottom microtiter plate, then incubated at 37°C for 48 hours. Contents of the well were aspirated out after incubation and each well was washed thoroughly with Phosphate Buffer Saline (PBS; pH 7). Then after, the wells were stained for 10 min by adding 125 µl of 0.1% Crystal Violet (w/v) solution to each well, then stain was removed and the plate was washed with clean tap water and left to air dry. Subsequently, 200 µl of 95% Ethanol was added to each stained well and plate incubated for 10 to 15 min. at room temperature. Contents of each well were mixed and then 125 µl of the Crystal Violet/Ethanol solution was transported from each well to another well of an optically clear flat-bottom 96-well plate. Optical densities (OD) of each of these 125-µl samples were measured at 630 nm using ELISA reader. Experiments were performed

in duplicate. The value of the optical densities for each isolate (OD_i) was obtained by averaging the double wells, and this value was compared to the optical density of the negative control (ODc)*. The isolates were classified into four categories, according to the mean optical densities (OD) in relation to the ODc results. The categories were based on the following criteria: non-adherent if $OD_i \leq ODc$; weakly adherent (+) if $ODc < OD_i \leq 2 \times ODc$; moderately adherent (++) if $2 \times ODc < OD_i \leq 4 \times ODc$; or strongly adherent (+++) if $4x\ ODc < OD_i$. TSB broth without bacterial inoculums was used as the (negative control) [9].

DNA extraction:

Half of our isolates (50 isolates) had been chosen for molecular study, DNA extracted by using Wizard® Genomic DNA extraction Kit (PROMEGA, Germany) by depending on manufacture procedure, extracted DNA stored at -20°C for PCR.

Primers: In the present study primers of AlgD gene designed by depending on NCBI gene sequence information base. The primer prepared by Integrated DNA Technologies Company, Canada; as shown in (Table1).

Table 1 Primers used in PCR amplification for detection of virulence genes [10]

Gene name		primers	Product size
AlgD	F	5'-CGT CTG CCG CGA GAT CGG CT - 3'	313bp
	R	5'-GAC CTC GAC GGT CTT GCG GA - 3’	

PCR method:

INtRON's Maxime PCR PreMix Kit used, amplification of DNA was carried out at final volume of (25µl) containing the following mixture: Taq PCR PreMix 5µl, Forward primer 1 µl, Reverse primer 1 µl, DNA 1.5 µl, Distill water 16.5 µl, PCR amplification carried out by MultiGeneOptiMax Gradient Thermal Cycler under this conditions for AlgD gene; 35 cycle for 3minute at 95°C, 45seconds at 95°C, 45seconds at 68°C, 1minute at 72°C, 7minute at 72°C. The amplified genes were detected by electrophoresis in a (2%) agarose gel, the gel has been tested by a source of the UV (336 nm) after put the gel in pool contain on 30µl Red safe Nucleic acid staining solution.

Statistical analysis: Chi-square statistical analysis test used to determine if AlgD gene significantly distributed in biofilm former *P. aeruginosa* isolates or not, and the relation between biofilm formation and MDR is significant or not. P value was set at 0.05.

Results: Antibiotic susceptibility test for *P. aeruginosa* isolates revealed that, lower percentage of resistant found against piperacillin/tazobactam (5%), cefepime (24%), and ceftazidime (26%) while, higher resistant percentage was against amoxicillin/clavulanic acid (98%) and (97%) trimethoprim/ sulfamethoxazole, (60%) of total isolates were MDR, statistical analysis revealed the presence of resistant isolates was very significant ($P < 0.05$) as shown in Table 2

Table 2 Antibiotic susceptibility for *P. aeruginosa* isolates

ANT.	TC	TIM	PTZ	ATM	LEV	CIP	CAZ	CPM	IMI	MER	AK	TS	TN	AMC
R	45 %	44 %	5%	45 %	48 %	40 %	26 %	24 %	40 %	43 %	36 %	97 %	42 %	98 %
I	36 %	36 %	31 %	16 %	1%	2%	0%	0%	2%	4%	6%	0%	1%	0%
S	19 %	20 %	64 %	39 %	51 %	58 %	74 %	76 %	58 %	53 %	61 %	3%	57 %	2%

X_2= 629.402 ** p-value = 0.00009 highly significant

*abbreviations: ticarcillin(TC), ticarcillin/ clavulanic acid(TIM), piperacillin/tazobactam(PTZ), aztereonam(ATM), levofloxacin(LEV), ciprofloxacin(CIP), ceftazidime(CAZ), cefepime(CPM), imipenem(IMI), meropenem(MER), amikacin(AK), trimethoprim/ sulfamethoxazole(TS), tobramycin(TN), amoxicillin/ clavulanic acid(AMC).R (resistant), S(sensitive), I (intermediate).

Quantitative microtiter plate method revealed that (98%) of total isolates were biofilm former. Regarding to the divisions of biofilm formation, Multi-drug resistant (MDR) form 90.9% of strongly adherent isolates, followed by moderately adherent isolates (76%) and weakly adherent isolates (7.7%). Statistically the relation between biofilm formation and MDR is highly significant ($P<0.05$) as shown in Table 3.

Table 3 Correlation between biofilm formation and antibiotic resistance

Biofilm / Antibiotic susceptibility	strong adherent (22%)	Moderate adherent (50%)	Weak adherent (26%)	Total (98%)
MDR	20/22 (90.9%)	38/50 (76%)	2/26 (7.7%)	(58.2%)
Sensitive	2/22 (9.1%)	12/50 (24%)	24/26 (92.3)	(41.8%)

**Chi-Square = 46.732, P-Value = 0.0008(Highly significant)

AlgD gene was found in (98%) of *P. aeruginosa* isolates, the gel electrophoresis showed that molecular weight of AlgD gene (313bp), as show Figure1.
AlgD gene was found (100%) in all biofilm former while absent in non biofilm former, statistically the distribution of AlgD gene regarding to biofilm formation not significant ($P > 0.05$), as shown in Table 4.

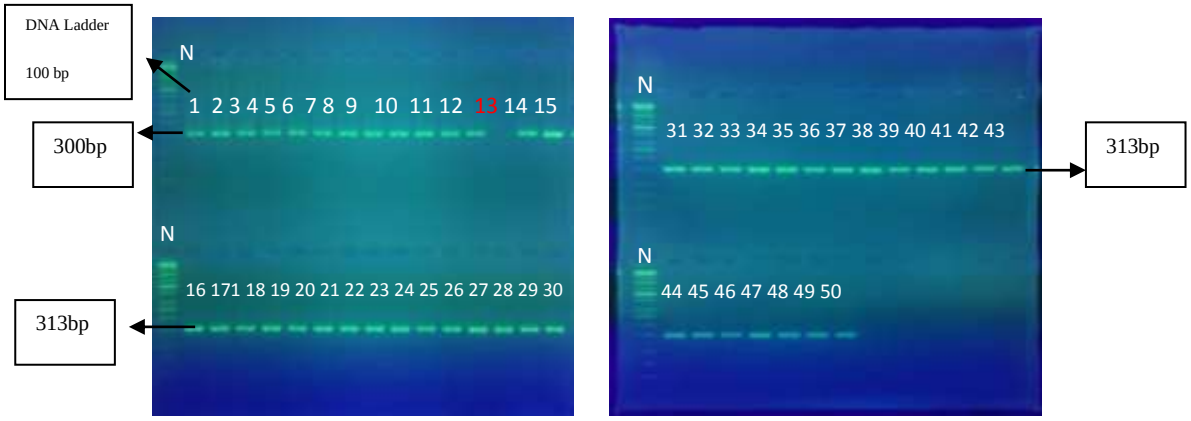

Figure 1 Agarose gel electrophoresis for PCR product AlgD gene (the band 313 bp). Electrophoresis had run on (2% agarose at 5 volt/cm^2, 1x TBE buffer for 1:30 hours). N: DNA ladder (100) Bp, lanes (1-50) PCR product positive AlgD gene, except lane (13) was negative for AlgD gene (red colored).

Table 4 Correlation between biofilm formation and presence of AlgD gene

Biofilm (n=50) / AlgD(n=50)	strong adherent 11/50 (22%)	Moderate adherent 25/50 (50%)	Weak adherent 13/50 (26%)	Non adherent (2%) 1/50
Present	11/11 (100%)	25/25 (100%)	13/13 (100%)	0/1 (0%)
Absent	0/11 (0%)	0/25 (0%)	0/13 (0%)	1/1 (100%)

Chi-Square = 3.618, P-Value = 0.328

Discussion

Antibiotic resistant pathogenic bacteria started to be concern huge problem that lead to nosocomial infections and increase morbidity and mortality rate of hospitalized patients [(11)]. The present study results showed that, (60%) of isolates were MDR that is agrees with Unan *et al* [(12)], who found same percentage of MDR (60%) in his isolates, and the present study disagree with Corehtash *et al* [(13)] that found (93.1%) of isolates were MDR.

In the current study lower percentage of antibiotic resistance was seen against piperacillin/tazobactam (5%) this result agrees with Hoque *et al* [(14)] who reported piperacillin/ tazobactam resistance (3.37%) and Direkel *et al* [(15)] who reported (7%). Higher resistance percentage was seen against amoxicillin/clavulanic acid (98%) and trimethoprim/ sulfamethoxazole (97%), the results agree with Hussain Qadr [(16)] who reported that no isolation were susceptible to amoxicillin/ clavulanic, and the resistance to trimethoprim/ sulfamethoxazole (1%). In the current study regarding to the correlation between biofilm formation and antibiotic resistance, higher percentage of MDR (90.9%) observed in strong adherent biofilm former, statistically the relation between biofilm formation and MDR is highly significant. The results statically agree with Abidi *et al* [(17)] that found MDR isolates displayed significant biofilm production as compared to susceptible isolates ($p < 0.0001$), and with Yekani *et al* [(18)] who reported the same results, also agrees with Corehtash *et al* [(13)] who documented biofilm producer in MDR

isolates was significantly higher than non MDR isolates (P< 0.001). The results disagree with Costa Lima *et al* [19] who reported (75%) of *P.aeruginosa* isolates were biofilm producers (48.4%) were MDR and (51.6%) were MDS (multi drug sensitive). The high prevalence of MDR in biofilm former isolates due to the elevation of minimum inhibitory concentration of various antibiotics from 10-1000 fold, because of failure of antibiotics in penetration of the dense matrix, inability of antimicrobial agent to reach deeper layers of biofilm and disposal of antibiotics from the biofilm by aid of microbial communities efflux action[17]. In the study of the correlation between AlgD gene and biofilm formation in *P.aeruginosa* found that AlgD gene present in high percentage in all biofilm former and absent in non biofilm former, the result agrees with Pournajaf *et al* [20] who documented that the ability of biofilm formation was (54.5%) of isolates and prevalence of the AlgD genes was (92.3%), also correlated with Heidari *et al* [21] who reported that all biofilm producers *P. aeruginosa* isolates carried the AlgD gene (100%).

Conclusions

Significant correlation between biofilm formation and antibiotic resistance, also between strength of biofilm (strength of adherence) and MDR, as well as strong correlation was found between presence of AlgD gene and biofilm formation.

CONFLICT OF INTERESTS.
There are non-conflicts of interest .

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الخلاصة

الخلفية: الزوائف الزنجارية هي عبارة عن بكتريا انتهازية مسببة للامراض التي ترتبط بالإصابة بالعدوى المستمرة التي يصعب استئصالها.**الهدف:** تهدف هذه الدراسة إلى تحديد قدرة تكوين الغشاء الحيوي في الزوائف الزنجارية وارتباطه بمقاومة المضادات الحيوية وعلاقتها بوجود جين ال AlgD .**المرضى وطريقة العمل:** أجريت الدراسة الحالية في مستشفى أزادي التعليمي العام في مدينة كركوك خلال الفترة من أغسطس 2017 حتى مايو 2018 ، تم الحصول على 100 عزلة من الزائفة الزنجارية من 1260 عينة سريرية مختلفة. تم تحديد الحساسية للمضادات الحيوية بواسطة طريقة كيربي باور، تم تحليل تكوين الغشاء الحيوي بطريقة الميكروتايتر بليت ، جين AlgD تم الكشف عنه بواسطة تفاعل البلمرة المتسلسل.**النتائج:** اقل مقاومة للمضادات الحيوية وجد ضد: ببراسلين/ تازوباكتام (5%) , سيفيبيم (24%), سيفتازيديم (26%), بينما كانت النسبة الاكبر مقاومة ضد: الاموكسيسيلين/ حامض الكلافيولانك (98%) و ترايميثوبريم/ سلفامثوكزازول (97%).وكانت (60%) من هذه العزلات مقاومة للعديد من الأدوية. وجد أن (98%) من العزلات كانت لها قابلية تكوين الغشاء الحيوي. تم الكشف عن وجود جين AlgDبواسطة تفاعل البلمرة المتسلسل حيث وجد أن (98%) من العزلات حاملة للجين, مع جود هذا الجين AlgD في العزلات المكونة للغشاء الحيوي فقط. **الاستنتاجات:** كان الإرتباط معنويا بين قابلية تكوين الغشاء الحيوي ومقاومة المضادات الحيوية, ووجد ان هنالك علاقة قوية بين وجود AlgD جين و تكوين الغشاء الحيوي.