Synergistic Effect of Amikacin and Ciprofloxacin on *pelA* and *algD* Genes in *Pseudomonas aeruginosa*

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Abstract

Pseudomonas aeruginosa is one of the most important pathogen in both hospital and community-acquired infections, it is a classical opportunistic pathogen, and this organism does not normally attack healthy tissue, and rarely causes infection in the general population. It is possess various virulence mechanisms to adhere, colonize and biofilm formation. Samples were collected from different hospitals in Baghdad, samples were collected and categorized in to two main groups: eighty clinical samples and forty as an environmental samples. Forty one isolates were identified as *P. aeruginosa* by using conventional methods and confirmed by VITEK-2 compact system, biofilm were detected by three methods: congo-red agar, tube method and microliter plate. Synergistic Effect of Amikacin and Ciprofloxacin on biofilm formation were detected phenotypically by using microliter plate and genetically by Real-time PCR for *pelA* and *algD* Genes which are responsible for biofilm formation. The results shows an obvious differences in biofilm formation phenotypically and genotypically for the treated isolates.

Keywords: *Pseudomonas aeruginosa; PelA; algD; biofilm; RT-qPCR.*

Introduction

Pseudomonas aeruginosa is a gram-negative opportunistic pathogen capable of causing several infections, mostly in immunocompromised or critically ill patients, including pneumonia, sepsis, bacteremia, and wound and skin infections [(1)]. *P. aeruginosa* infections can be very serious and life-threatening, mostly with the emergence of drug-resistant strains, one of the most worries about human health today is antimicrobial resistance. *P. aeruginosa* is one of the most prevalent nosocomial pathogens, it's responsible for 57% of total hospital-acquired infections[(2)].

One of the most primary characteristic of *P. aeruginosa* chronic infections is biofilm formation [(3)]. A biofilm is a bacterial community that attached to a surface or substratum, typically made of densely packed, multi-species populations of cells. The main feature of biofilms is the presence of highly hydrated extracellular polymeric substance (EPS), including proteins, polysaccharides, and extracellular DNA (eDNA) [(4)]. *Pseudomonas aeruginosa* biofilms are generated through a process whereby the bacterial cells are surrounded to form an aggregated structure, which exhibits increased resistance to antibiotics and other anti-infection agents [(5)]. Therefore, infections caused by biofilm-forming *P. aeruginosa*, such as in cystic fibrosis of the lung, are almost impossible to eradicate, and additional challenges are encountered when treating infections caused by multidrug-resistant strains. These complications lead to increased patient morbidity and mortality, higher costs of treatment, and greater rates and time of hospitalization, diverse strategies are being pursued to develop novel agents that can kill (new antibiotics) or disarm (antivirulence) the pathogen [(6)]. Exopolysaccharide is a critical biofilm matrix component, so the process of biofilm maturation is namely the production of the exopolysaccharides via the polysaccharide synthesis locus (Psl), pellicle formation

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(Pel) and alginic acid synthesis in *P. aeruginosa* [7].

Materials and Methods

Specimens' Collection:

Eighty different clinical specimens were collected from patients referring different hospitals in Baghdad and forty environmental samples were collected from the same hospitals, the collected specimens were ear, burn, urine, sputum, wound and Bronchoalveolar lavage (BAL).

Bacterial Isolation and Identification:

All the collected samples were cultured by streaking on cetrimide agar, macConkey agar and on blood agar, all samples incubated for 24 hours at 37ºC. *P. aeruginosa* colony characters appears on cetrimide agar plates was mucoid, smooth in shape with flat edges and elevated center, have fruity odor and creamy color. Moreover, it appeared as pale greenish and lactose non-fermenter colonies when cultured on macConkey agar plates, while it appeared they appeared as large, opaque irregular colonies with *B*-hemolytic colonies when cultured on blood agar.

Colonial morphology on blood agar, macConkey agar and cetrimide agar was depended initially to identify bacterial isolates, colony shape, texture, colour and edges were examined. In addition to macroscopic characteristics; microscopic examination of a gram-stained slide was examined under a light microscope with special regard towards cell shape and arrangement. Performing conventional biochemical tests and VITEK 2 compact system were depended to complete the identification.

Biofilm Formation

Congo red method:

Bacterial ability to produce slime layer and biofilm formation was detected using Congo-red agar medium (CRA). A positive result was indicated by black colonies with dry crystalline consistency while pink colonies considered as non-slime producers. A darkening of the colonies but with the absence of a dry crystalline colonial morphology indicated an indeterminate result [8].

Tube method (Christensen's method):

Isolates were inoculated in glass test tube which contained (8 ml) BHI broth with 1% glucose and incubated at 24 h at 37°C. The sessile isolates of which biofilm forming isolates adhered to glass tubes are stained with safranin for 10 min, after planktonic cells are discharged by rinsing twice with phosphate-buffered saline (PBS). Then, safranin-stained glass test tube is rinsed twice with PBS to discharge stain. After air drying of test tube process, the occurrence of visible film lined the walls, and the bottom of the tube indicates biofilm production [9].

Microtiter plate method:

To study the ability of biofilm formation, isolates selected from different sources (2 wounds, 4 burns, 1 urine, 1 ear and 2 environmental) depending on sensitive and resistance to antibiotic aminoglycoside such as amikacin and fluoroquinolones such as ciprofloxacin. Quantification of biofilm formation by *P. aeruginosa* was assessed as described by [10].

Determination of Minimal Inhibitory Concentration (MIC) of Amikacin and Ciprofloxacin:

To determine the lowest concentration of antibiotics that inhibit the growth of the microorganism VITEK 2 compact system were used.

The MIC for each bacterial isolates was interpreted as the lowest concentration showing no growth. The results were compared with standard breakpoints values; sensitive (≤ 2 µg/ml), intermediate (4-8 µg/ml) and resistant (≥ 16 µg/ml) according to CLSI (2016).

Synergistic effect of sub-MIC of Amikacin and Ciprofloxacin on biofilm formation:

The lowest concentration of Amikacin and Ciprofloxacine that inhibit the visible growth of the microorganism was assessed by using VITEK 2 compact system. A volume 100 µl of double strength Brain heart infusion broth was added to wells from 1 to 12 of sterile 96-well U shaped-bottom polystyrene microplates. Afterwards, 50 µl of each antibiotics MIC used were mixed together to reach a total volume of 100 µl were added to wells from No.1 to No.11. All wells were inoculated with 10 µl of bacterial suspension

comparable to McFarland standard No.(0.5) (1.5×10^8 CFU/ml) except for the well No.11, which considered as negative control. Whereas well No. 12 considered as positive control. The microtiter plate was incubated at 37°C for 24 h.

Gene expression:

In order to test the synergistic effect of Amikacin and Ciprofloxacin on gene expression of *pelA* and *algD*, eppendorf tubes were used, and each tube contains double amount of the antibiotic and broth that used in the previous section and then twenty tubes were incubated under aerobic conditions at 37°C for 24 hrs. RNA were extracted by using TRIzol™ Reagent according to the protocol described by the manufacturer.

Quantitative reverse transcription-PCR (qRT-PCR):

In order to assess the gene expression of *pelA* and *algD* gene; though, results were normalized using *gyrA* (Housekeeping gene). Primers of these genes (listed in table 1) 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 used in qPCR. The reaction mixture was summarized in table (2). Moreover, after several trials, thermocycler protocol was optimized and the resultant protocol is listed in tables (3).

Table (1): Primers utilized in this study

Primer name		Sequence (5´-3´)	Product size	Reference
PelA	F	CCTTCAGCCATCCGTTCTTCT	118 bp	(Colvin et al., 2011)
	R	TCGCGTACGAAGTCGACCTT		
algD	F	ATGCGAATCAGCATCTTTGGT	1310 bp	(Stover et al., 2000)
	R	CTACCAGCAGATGCCCTCGGC		
gyrA (HouseKeeping gene)	F	CACCGCCGTGTGCTTTATG	843 bp	(Colvin et al., 2011)
	R	GGGTCTGGGCATAGAGGTTG		

Table (2): Components of qRT-PCR used in *pelA* and *algD* genes expression

Master mix components	Volume (µl)
qPCR Master Mix	5
RT mix	0.25
MgCl2	0.25
Forward primer	0.5
Reverse primer	0.5
Nuclease Free Water	2.5
RNA	1
Total volume	10
Aliquot per single rxn	9 µl of Master mix per tube and add 1µl of Template

Table (3): **qRT-PCR protocol of primers *pelA***

Step	Temperature (°C)	Duration	Cycles
Reverse transcription (RT). Enzyme activation	37°C	15 minutes	1
Initial Denaturation	95°C	5 minutes	1
Denaturation	95°C	20 seconds	40
Annealing	45°C	20 seconds	
Extension	72ºC	20 seconds	

Result and Discussion

Isolation and Identification of *Pseudomonas aeruginosa*:

Out of Eighty clinical specimens and forty environmental samples only 42 isolates were able to grow on cetrimide agar at 42˚C which is considered as primary identification for *Pseudomonas* spp., 37 isolates were identified as *P. aeruginosa* by using VITEK 2 compact system.

Biofilm Formation

Congo red method:

Only 70% of the tested isolates gave black colored colonies when cultured on Congo red agar plates. And the (Figure 1) shows the results.

Figure (1): The biofilm and slime layer production on Congo red agar plate.

Tube method:

By using tube method, visible thick film was obtained on the wall of tube and bottom of the tube. All the tested isolates were biofilm producers indicated by the appearance of thick film inside the wall of the tube indicating strong biofilm production, as shown in Figure (2).

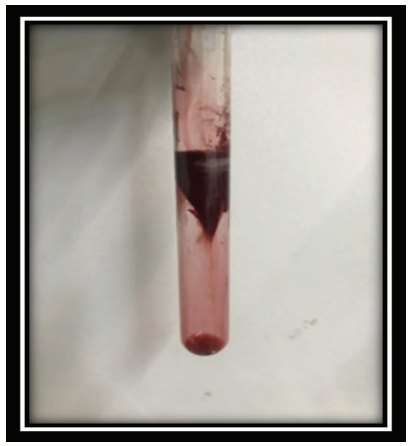

Figure (2): The biofilm production on glass tube wall by *Pseudomonas aeruginosa* after 24 hr. incubation stained with safranin by using tube method.

Microtiter plate method:

This method is considered as quantitative analysis that used for detection of biofilm formation, by using microplate reader it gives numerical value for absorption at 630 nm to determine the amount of biofilm formed through adhesion of bacteria to surfaces of microtiter plate, the absorbance represents the thickness of the biofilm, that formed on the wells surface by *P.*

aeruginosa isolates. The results of the current study obtained by using microtiter plate method indicates that all the isolates have the ability for strong biofilm production and there is no remarkable differences in biofilm thickness among the isolates, all the obtained OD values were four times larger than the OD of control.

Quantitative Real Time-PCR:

The expression of *pelA* and *algD* of *P. aeruginosa* biofilm genes were studied by relative qRT-PCR. The isolates were tested with the presence of sub-MIC of two antibiotics used, the amikacin and ciprofloxacin. Actually, ten isolates enrolled in this experiment were chosen for the reasons that it has strong biofilm intensity, it's collected from different sources (wounds, burns, urine, ear and environmental).

Gene expression of *pelA* and *algD* genes:

The treated and untreated isolates were tested genetically by using Real time PCR to realize if the *pelA* and *algD* genes were effected or not by the sub-minimum inhibitory concentration of antibiotcs. The results indicated that there is a down regulation effect in the *algD* gene and the *pelA* showed slight variation in the expression level 80% of the isolates were down regulated. While 20% were up regulated.

For treated isolates there is no gene expression for *algD* gene which is responsible for biofilm formation, so the effect were phenotypically by biofilm suppression and genetically by gene down regulation, the biofilm suppression may be due to *algD* role in β-D-mannuronic acid and α-L-guluronic acid production which has an important role in structural stability and protection of biofilm. Moreover, the *algD* gene mediates the control of alginate biosynthesis and transcription of the Alg proteins, and also is responsible for the final production of precursor GDP-mannuronic acid, the foundation molecule for polymerization and alginate synthesis. In the other hand, *pelA* gene expression revealed different expression manner, two isolates (R3 and R9) presented up regulation when treated by antibiotics, the isolate R3 from burn and the isolate R9 from environmental, while the other isolates revealed down regulation manner which has a noticeable impact on biofilm formation due to importance of *pelA* on extracellular polysaccharide formation in biofilm matrix, promoting attachment to surfaces and other cells, building and providing a scaffold to help maintain biofilm structure, and protecting cells from antimicrobials and host defenses, table (4).

Table (4): Gene expression results for *algD* and *pelA* before and after treatment with antibiotics.

Isolate number	CT gyrA before treatment	CT gyrA after treatment	CT algD before treatment	CT algD after treatment	FC algD	CT pelA before treatment	CT pelA after treatment	FC pelA
R1	29.1	33.3	28.6	-1.0	0	28.9	-1.0	0
R2	27.9	37.8	30.1	-1.0	0	29.1	37.6	0.82
R3	27.5	38.0	29.2	-1.0	0	29.9	36.3	16.53
R4	21.6	32.7	21.8	-1.0	0	24.7	35.2	0.49
R5	28.5	34.3	34.1	-1.0	0	31.0	-1.0	0
R6	24.9	32.5	25.1	-1.0	0	27.8	37.6	0.20
R7	34.1	34.7	-1.0	-1.0	0	34.0	35.6	0.50
R8	31.2	35.8	-1.0	-1.0	0	31.9	35.9	0.51
R9	24.7	33.2	25.9	-1.0	0	29.6	36.3	3.71
R10	36.7	36.9	32.2	-1.0	0	34.2	34.0	0.31

Practical experiments to estimate the gene expression of *algD* and *pelA* genes showed a difference in results between them on the one hand and between isolates on the other. The calculation method for estimating gene expression was based on a comparison between treated and untreated isolates in order to find the difference in the effect of antibiotics on the expression of these genes. Titration of the values obtained from the interaction was based on the use of *gyrA* gene values as a reference gene.

For the *algD* gene, the results showed complete inhibition of gene expression when using antibiotic treatment as opposed to untreated isolates. These results support the theory that this type of antibiotic affects bacterial cells by affecting the expression of the *algD* gene. Where the effect was the opposite kind of inhibiting gene expression.

As for the *pelA* gene, the results showed a variation in gene expression between isolates. Some isolates showed an increase in gene expression ranging from 1.3 to 16 times, and this increase may be evidence of the use of these isolates as a result of this gene in antibiotic resistance when there are no other auxiliary mechanisms. Whereas, the adverse effect on gene expression inhibition was an indication that these isolates might use auxiliary resistance mechanisms other than this gene. In a clearer sense, the effect of antibiotics on the expression of this gene is not as direct as it is an auxiliary effect on resistance mechanisms. Our results came in agreement with local study by AL-Sheikhly *et.al* in (2019) who revealed that *pelA* gene was also down regulated by the effect of antibiotics.

Ethical Clearance: The Research Ethical Committee at scientific research by ethical approval of both MOH and MOHSER in Iraq

Conflict of Interest: Non

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