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Investigation of biofilm formation ability and Assessment of *cupB* and *rhlR* Gene Expression in Clinical Isolates of *Pseudomonas aeruginosa*

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Abstract: *Pseudomonas aeruginosa* forms structured communities known as biofilms, are normally associated with the transition from an acute to a chronic infection, making the biofilm one of the bacteria's most effective virulence factors. This study was designed to investigate the biofilm formation ability to the identified clinical isolates using microtiter plate assay, and assess the gene expression of the biofilm related genes *cupB* and *rhlR* by employing the RT-qPCR. 102 samples was collected from clinical sources and after the identification 21 clinical isolates were confirmed to be *Pseudomonas aeruginosa* forming (20.5%) of the total sample size. The biofilm formation assay revealed that 18 out of the tested 21 isolated were biofilm producer 10 (47.6%), 5 (23.8%), 3 (14.2%) were strong, moderate and weak respectively, while 3(14.2%) were non-biofilm forming isolates. The folding levels for *cupB* was presenting an over-expression in all biofilm forming groups as well as the non-biofilm forming group, as for the *rhlR* expression it was found to be over-expressing in both strong and moderate biofilm forming group, while it showed low-expression in weak and non-biofilm forming groups. According to this data the *cupB* gene expression was found to be more directly connected to the intensity of the biofilm formed by the isolates compared to *rhlR*.

Key words: Biofilm Formation, Gene Expression, RT-qPCR.

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Introduction

The opportunistic pathogen *Pseudomonas aeruginosa* has a wide range of virulence factors, one of the most important factors is biofilm formation, *P.aeruginosa* can form biofilms in a variety of abiotic environments such as soil and water, as well as biotic environments such as burn wounds, bedsores, cystic fibrosis patients' lungs (1). Biofilms are cell aggregates integrated in a matrix of extracellular polymer substances (EPS) such as proteins, exopolysaccharides, nucleic acids, and lipids (2). The formation of biofilms is a complex process that requires multiple stages which begins with planktonic bacteria attaching to the surface to form a monolayer, followed by clonal growth/aggregation leading to the formation of microcolonies, maturation to form mushroom-shaped structures, and dispersal(3).

Cup (chaperone usher pathway) fimbriae are one type of appendage involved in *P. aeruginosa* biofilm formation (4). Cup fimbriae are essential for the early stages of biofilm development, especially in cell-to-cell interaction and microcolony formation(5). Three types of *cup* fimbriae are produced by *P. aeruginosa*: *cupA*, *cupB*, and *cupC*, the

products of these genes clusters can encode an usher, a chaperone, and at least one fimbrial subunit (6).

The *cup* fimbria have been showcased to participate in various stages of biofilm formation on different surfaces, Transcriptome analysis revealed the *cupA*3 and *cupB*5 genes are regulated by quorum sensing (QS) in biofilms produced by *P. aeruginosa*, implying that the of *CupA* and *CupB* expression is being regulated by QS during biofilm formation (7,8)

Pseudomonas aeruginosa uses three organizational systems (LasR/I, RhlR/I, and PQS systems) to exert control over cellular and metabolic processes involved in the generation of virulence factors such as iron chelators, extracellular proteases, motility, response to host immune signals and efflux pump expression (9,10) and to regulate biofilm formation These signaling systems regulate 10% of the *P. aeruginosa* transcriptome genome (11).QS-regulated genes in *P.aeruginosa* have specialized promoter regions to which the regulatory protein-AHL complex binds (12). In *P. aeruginosa*, QS has been proposed as a global mechanism that regulates virulence factor expression and biofilm development (13). The Rhl system was discovered to be activated during the biofilm development maturation stage, suggesting that it may be crucial for the survival of bacterial cells within biofilms under anaerobic conditions. (14).Despite the fact that the QS and *cup* genes promote biofilm formation(3). The study sought to determine the relationship between the virulence gene (*cupB*) and the QS gene (*rhlR*). After assessing the isolates' ability to form biofilms, the gene expression of *cupB* and *rhlR* was measured using qRT-PCR.

Materials and methods

1- Sample collection and Isolates identification

From October 2021 to February 2022, 102 clinical specimens were collected from various wards at AL-Yourmok Teaching Hospital and AL-Shaikh Zaied Hospital, including burn wards, ICUs, Dialysis centers, and urology departments. The samples included Burn swabs (n=30), wound swabs (n=19), urinary tract infections (n=29), and catheter tips (n=24) that were collected from patients of both genders and different ages. According to (15). Bacteria were identified using morphological features on selective media Cetrimide agar, macroscopic assessment of bacterial colonies, conventional biochemical tests.

2- Molecular identification of *Pseudomonas aeruginosa* using quantitative real time PCR technique by *gyrB* gene detection

To confirm the identification, the qRT-PCR technique was used to detect the presence of the *gyrB* gene using primers specific for this gene (table 1), as follows: Total RNA was extracted from the suspected *P.aeruginosa* isolates using the Trans Zol Up Plus RNA Kit (TransGen, Biotech. China) according to the manufacturer's protocol. Following the manufacturer's protocol, using the EasyScript® One-Step gDNA Removal and cDNA Synthesis SuperMix Kit (TransGen, Biotech. China) the total RNA was reversely-transcribed to complementary DNA (cDNA). The cDNA was synthesized and used in the qRT-PCR reaction to detect the *gyrB* gene

and identify *P. aeruginosa* isolates (Table 1).

Table (1): Sequences of primers used in this study

Gene	Primer	Annealing Tm ˚C	Product size	Reference
cupB	F-CATCGCCTTCGATCAGGTCA R-GGGATCTTCGTGGTGTTGGT	58	129bp	Designed by the third author
oprM*	F-CCATGAGCCGCCAACTGTC R-CCTGGAACGCCGTCTGGAT	58	205bp	[11]
oprD*	F-CTACCGCACAAACGATGAAGG R-GCCGAAGCCGATATAATCAAACG	60	154bp	Designed by the third author
gyrB	F-CCTGACCATCCGTCGCCACAAC R-CGCAGCAGGATGCCGACGCC	60	220bp	[6]
rhlR	F-TCCTGGAAAAGGAAGTGCG R- CCCCGTAGTTCTGCATCTG	56	147bp	Designed by the third author

***= Housekeeping genes**

3-Biofilm formation assay

The *P. aeruginosa* isolates that were positive for *gyrB* gene then underwent the Quantitative method MPA (Microtiter plate assay) to assess their ability to produce biofilm. The assay was followed step by step as it was prepared by (16). The isolated colonies were inoculated using Brian heart infusion broth (BHI) with sucrose added at the concentration of 50 g/ L (as an energy source to fuel the growth and division); the broth was then incubated at 37C for 24 hours. Then 200 µl of the bacterial suspension was transferred in triplicates on 96 well polystyrene plates (Nunc, Denmark). The negative control was BHI broth devoid of bacterial inoculum, while the positive control was the PA01 strain. The 96-well plates were then incubated at 37C for an additional 24 hours. The wells were three times rinsed with (PBS) Phosphate-buffered saline solution after the incubation. Methanol was used to preserve the bacterial biofilm that had attached to the wells for 15 minutes crystal violet solution was used to stain it for 5 minute. Using an ELISA reader (Biorad, UK), at a wavelength of 570 nm, the stained biofilm's absorbance was measured. By averaging the OD readings for the three wells, the optical densities (ODi) for the isolates were determined. This value was then contrasted with the optical density value of the negative control (ODc). According to (17). The isolates were divided into the following 4 groups based on the findings of this comparison: strong, moderate, weak and non-biofilm producers.

4- Estimation of gene expression of *cupB* and *rhlR* using qRT-PCR

The qRT-PCR and livak method was applied to measure the target genes *cupB* and *rhlR* expression with *oprM* and *oprD* as the housekeeping genes to neutralize the mRNA levels using PAO1 as reference strain. The investigation was done using TransStart® Top Green kit (TransGen, Biotech. China) as per the manufacturer's instructions. Each of the reaction tube contains 10 µL of master mix; 3 µL of cDNA; 1 µL of each primer; and 5 µL of nuclease-free water in a final volume of 20 µL. The qRT-PCR was carried out using Rotor-gene Q Real-time PCR System (Qiagene, Germany) and the program for the prepared reactions started with an initial

denaturation step at 94°C for 30s; denaturation 94°C for 5 sec.; annealing 58°C (for *oprM* and *cupB*), 60°C (for *oprD*), 56°C (for *rhlR*) for 15 sec; and extension 72°C for 20 sec these steps were repeated for 40 cycles. The reactions were carried out in triplicate, and the expression level for the isolates was examined using the delta *CT* method.

Results and discussion

1- Isolation and identification of *Pseudomonas aeruginosa*

A total of 102 clinical specimens were collected from two sources: skin samples [burn and wound] and urine samples [UTI and catheter], 25 (24.5%)/102 samples were positive for *P. aeruginosa* (Table.2). *P. aeruginosa* isolates were recognized by the standard microbiological techniques include colony characteristic on cetrimide agar, gram stain and oxidase test. The bacterial colonies appeared bright greenish especially under UV light because most of the bacteria produce pyocyanin dye with greenish color, these isolates are gram negative rod-shaped bacterium and positive for oxidase.

As a method for conforming the identification, qRT-PCR was employed to detect and measure the levels of expression for *gyrB* gene in 25 isolates. The same table recorded the results of *gyrB* gene test, the isolation rate of *P. aeruginosa* was 20.5% (21/102) of samples and the positive results for *gyrB* were found in 21 (84%)/25 tested isolates, where all isolates from (catheter, burns) were shown positive results so the *gyrB* was expressed in theses isolates, while four isolates from wound and UTI were negative in this test thus the *gyrB* gene not amplified and the fluorescence curve still remained below the threshold line. The results of amplification of *gyrB* gene illustrates in (figure 1) the evidence of this amplification was determined by the curves that passed the threshold line using 45 cycles (CT values ranged from16 to ~22 cycles). Unlike our finding that 84% of isolates contain gyrB gene, another study (18) where the authors register that 92% of the isolates were *gyrB* positive. This percentage is a proof that *gyrB* is a good target for identifying the bacteria. The study by(19) mentioned that the significantly improved divergence values in *gyrB* phylogenetic analysis compared to *16S rRNA*, makes the *gyrB* a useful alternative to *16s rRNA* in regards *P. aeruginosa* identification.

The ratio of distribution of 21 *P. aeruginosa* isolates among specimen sources was as follows: burns (33.3%), UTI (17.2%), wounds (15.8%), and catheters (12.5%). The current study found out that *P. aeruginosa* was the most common bacteria in burns, which is consistent with previous research (20, 21). *P. aeruginosa* is still a serious cause of infection and high septic mortality rate in burn patients' Long hospitalization time in the burn unit. In addition, *P. aeruginosa* urinary tract infections were the 2nd most frequent source in our samples, and patients of urine/catheter isolates were surprisingly, the most likely of any group to have received only non-antipseudomonal treatments before sample collecting. This finding confirms the widely held belief that *P. aeruginosa* is not the most common cause of UTIs. As for the most common prescribed antimicrobial agent before the catheter isolates collection it was Ciprofloxacin. (22,23). The most common factor that naturally leads the host to these infections is urinary catheterization. Catheter-associated UTI is the main cause of nosocomial

infection, compensating for 40% of all nosocomial infections (24).

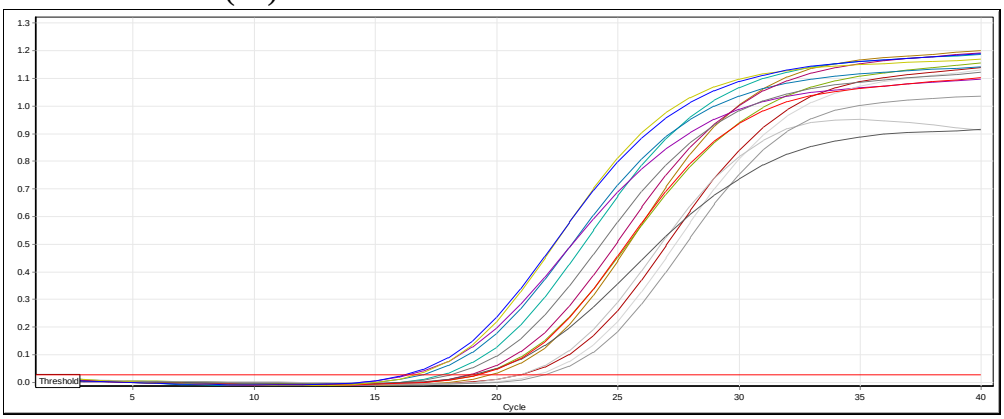

Figure (1): The RT-qPCR amplification plot for *gyrB* gene which indicates the positive results for the tested isolates.

Table (2): Distribution of bacterial isolates according to a source of isolate

Sources of specimens		Specimens number	Number of (+ve) isolates detected by standard microbiological methods	Number (%) of (+ve) isolates detected by qRT-PCR
Skin Swabs	Burns	30	10 (33.3%)	10(33.3%)
	wounds	19	5 (26.3%)	3(15.8%)
Urine	UTI	29	7 (24.1%)	5(17.2%)
	Catheter	24	3 (12.5%)	3(12.5%)
Total		102	25(24.5%)	21(20.5%)

2- Biofilm formation:

The biofilm formation ability for the *gyrB* positive isolates was assessed by using an effective method for detecting the biofilms which is the quantitative micro-titer plate assay. The results shows that in total 18/21(85.7%) of the isolates has the ability to form biofilm of which [10 (47.6%) generated strong biofilm; 5 (23.8%) formed moderate biofilm and 3 (14.3%) shaped weak biofilm], while 3 (14.3%) of the strains lacked the potential to produce biofilms (Table 2). Same as our results (25, 26) indicated that 83.75% and 86.5% respectively isolated organisms produced biofilm, in contrast to (27) that reported 68% of the isolates formed biofilm. In other study (28) 77.5% of isolates formed biofilm. This is also apply on a local studies conducted by the authors of (29-33) where they register a biofilm formation ability in 70%, 86.6%, 85% 95.4% and 100% respectively of the local clinical *P. aeruginosa* isolates.

Table (3): Biofilm formation among of *P. aeruginosa* isolates

Number and source of samples		Pattern of biofilm producer, no. (%)			
		Non-producer	Weak producer	Moderate producer	Strong producer
Skin Swabs	Burns, 10	3 (30%)	1 (10%)	3 (30%)	3 (30%)
	Wounds, 3	0 (0.0%)	1 (33.3%)	1 (33.3%)	1 (33.3%)
Urine	UTI, 5	0 (0.0%)	1 (20%)	1 (20%)	3 (60%)
	Catheters, 3	0 (0.0%)	0 (0.0%)	0 (0.0%)	3 (100%)
Total, 21		3 (14.3%)	3 (14.3%)	5 (23.8 %)	10 (47.6%)

Obviously, a significant variability in biofilm forming capacity was observed among the study isolates, such variability could be related the different

genetic makeup of these isolates and to the gene expression of their biofilm genes as well as quorum sensing genes. Biofilm development supports bacterial survival and is one of the most essential virulence determinants of *P. aeruginosa*. Once established, biofilms are extremely difficult to eliminate and serve as effective barriers to antimicrobial agents (34). *P. aeruginosa* biofilms are especially important in skin burns because they cause chronic wounds that take a long time to heal. (35).

In the current study, the difference in the source of the samples may be the important reasons for the variation in the results. The investigated isolates were studied for the clinical source of isolation and their capacity to produce biofilm as a correlation of the obtained results, all isolates (100%) obtained from wounds, UTI and catheter tips were able to produce biofilm followed by 70% from burns. On the other hand, all strains isolated from catheter tips (3 in total) dominated only strong biofilm-generating ability while Bacterial isolates isolated from UTI were noticed to be (60%) strong biofilm producers, followed by (20%) moderate and weak biofilm producers. Similarly, isolates isolated from wounds exhibited a strong, moderate and weak biofilm producers (33.3%), while out of 10 isolates isolated from burn, 30% showed a strong and moderate biofilm producers followed by (10%) strains were weak biofilm-forming ability and 30% of isolates isolated from burn showed non- producer biofilm

Biofilms are a major issue in burns, accounting for 60% of burn-related mortality. Biofilms are also an issue in wounds and patients who use medical devices and urinary catheters (36)**.** Biofilm formation on the catheter tube is facilitated by multiple factors, including the plastic surface of the tube, the lack of host defense mechanisms and the accumulation of urine. *P. aeruginosa* can adhere to different surfaces in the form of a biofilm surrounded by a slime layer, which may shield it from potentially toxic elements Slime has the potential to encapsulate organic materials in the environment, but other possible scenarios can also explain for microbial availability in biofilms. The findings show that catheters containing plastic as a basic polymer promote the survival of *P. aeruginosa* (37).

3- Gene expression of Target genes:

In the present investigate the gene expression for the virulence gene *cupB* and the quorum-sensing regulator *rhlR* was assessed using RT-qPCR. According to (38) genes are considered differentially expressed if they show a fold-change of at least 1.5. Based on this statement the gene expression pattern of *rhlR* gene was analyzed in *P. aeruginosa* isolates (Table 4). The expression of *rhlR* gene was high (7.15 fold), and because this gene required for biofilm forming, this justify the increase level of *rhlR* expression gene in the biofilm forming groups (>1 fold), it was 13.3 fold and 2.8 fold in strong and moderate biofilm forming isolates respectively whereas their expression in the weak and non-biofilm producer isolates were down regulated (< 1.0 fold) 0.5 fold and 0.4 fold respectively. Figure.2 demonstrate the amplification curve of *cupB* and *rhlR* genes, previous studies suggested that these isolates' inability to build biofilm was caused by conformational changes in quorum sensing (*rhlR*) proteins brought on by mutations in the *rhlI/rhlR* system (39, 40). However, the results of another investigation showed a strong connection between the ability to create

biofilms and the presence of pertinent genes (41,2).

Table (4): The statistical analysis of the gene expression results

Target gene	Isolates	Reference strain	t-test	P-value
cupB	5.11 ±3.05	1±0.00	2.81**	0.702
rhlR	7.15 ±5.66	1 ±0.00	3.69 **	0.0001
gyrB	8.01±7.22	1±0.00	4.63 **	0.0001
**** Highly significant =(P≤0.01), * Significant =(P≤0.01), NS= non-significant**				

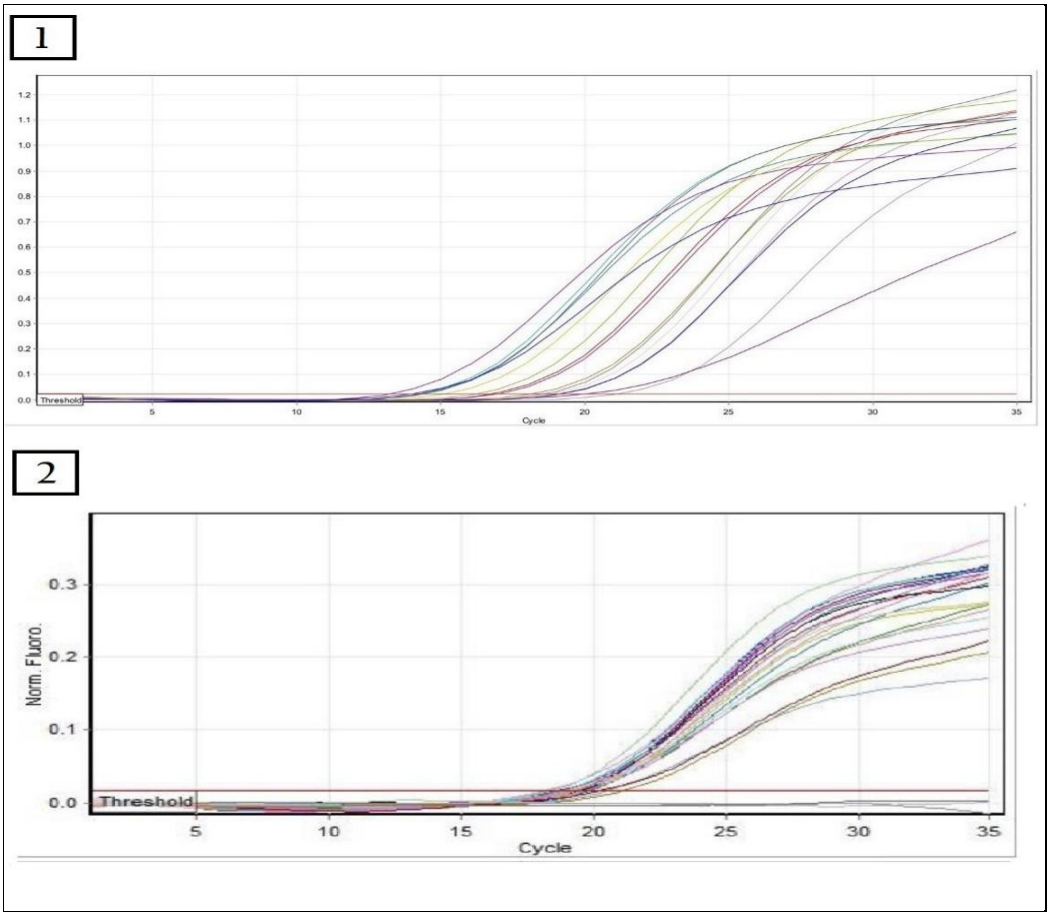

Figure (2): The amplification curve plot in RT-qPCR for both 1) *rhlR* gene 2) *cupB* gene

Based on the study's findings *P. aeruginosa* isolates both generated and contained the gene related with biofilm formation were thought to have significant levels of expression. The expression of the *cupB* gene was low (1.7 fold) in non-biofilm producer isolates compared to the reference strain employed as a positive control in the test. The *cupB* gene was expressed in all biofilm producer isolates (>2.0 fold), including strong 7.5 fold, moderate 4.4 fold, and weak 2.6 fold (fig.3). This could be the outcome of certain point mutations in the quorum sensing genes (40). One other prospect is the presence of multiple strains of *P. aeruginosa* at the site of infection, which leads to QS gene expression defection (40). These results are consistent with a study where the authors stated that the existence of biofilm genes without biofilm production might be caused by chromosomal changes in various regulatory systems, which would

influence the synthesis of functional biofilm related proteins (44).

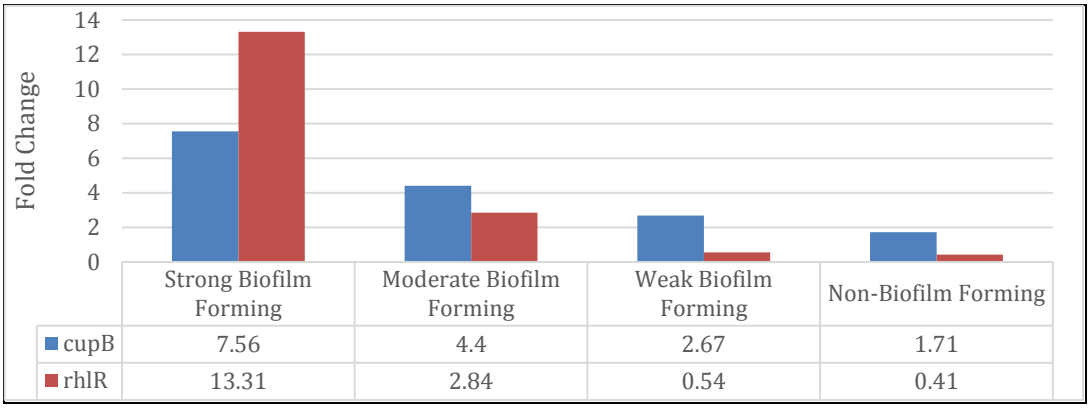

Figure (3): The fold change gene expression of the virulence and QS genes used in this study for every different biofilm forming group.

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