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Study of Siderophore Genes Expression of *Pseudomonas aeruginosa* Isolated from Wound Infections

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Abstract: *Pseudomonas aeruginosa* (*P. aeruginosa*) is one of the important types of nosocomial infections, especially burns and wounds, as well as resistance to antibiotics. Siderophores are one of the virulence factors which altering iron cellular homeostasis, controlling the production of virulence factors for certain bacteria, and encouraging bacterial dispersal, they can have a direct impact on the host immune system. The aim of the work was to detect siderophore genes in a carbapenems-resistant *P. aeruginosa* isolates from wound swab samples from patients with severe wound infections. Samples were collected at three hospitals in Baghdad between November 2023 and the end of February 2024. In addition to contrasting the impact of meropenem on siderophore genes (*plcN* and *exoS*), (gene expression) in the presence of antibiotic at ranges from (16-8) µg/ml. The results of gene expression revealed that the *plcN* gene exhibited the lowest value (0.8) and the greatest value (4.43) when treated with antibiotics; similarly, the *exoS* gene showed values ranging from the lowest value (2.37) to the highest value (4.25). It was concluded that there was a correlation between *P. aeruginosa* resistance to meropenem and the expression of siderophore genes, *plcN* and *exoS*.

Keywords: Gene expression, *Pseudomonas aeruginosa*, *plcN* , *exoS.*

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Introduction

Pseudomonas aeruginosa has an intricate association with other pathogens such as *Staphylococcus aureus* that inhabit the same infection site, and it can increase the aggressiveness of those infections. (1,2). The wound infections are usually classified into ‘acute’ and ‘chronic’ even though the difference between them is not always clear (3). The *P. aeruginosa* infections typically evolve to a pattern of persistence (chronicity).

This strategy allows the microorganism to survive for long periods under the challenging selective pressure forced by the immune system and antibiotic treatment (4). *P. aeruginosa* invades multiple tissue types, causing acute and chronic infections, and utilizes different mechanisms to evade antimicrobial treatment (5). The pathogenesis of the *P. aeruginosa* is facilitated by various bacterial virulence factors that aid in adhesion, disrupt host cell

signaling pathways, and target the extracellular matrix. *P. aeruginosa* is especially notable and harmful because of its capacity to avoid immune responses and cause severe invasive illnesses, which lead to persistent infections that are very challenging to eradicate (6). The *P. aeruginosa* releases a variety of virulence factors that play a critical role in its interactions with other bacteria, resulting in infection and heightened virulence (7). Carbapenems resistant *P. aeruginosa*, is a significant challenge in clinical practice, hence complicating patient treatment, mechanism of carbapenems resistance is important because it significantly alters the efficacy of commonly used antipseudomonal agents, including ceftazidime, cefepime and piperacillin-tazobactam, as well as the newly introduced beta-lactam/beta-lactamase inhibitor combinations such as ceftolozane-tazobactam, imipenem-relebactam and ceftazidime-avibactam. The carbapenem resistance determinants carried by the *P. aeruginosa* are often encoded on plasmids, such as IncP type; class I integrons (8). Iron /siderophore uptake may play an important role siderophores, heme, and iron regulatory genes in the virulence of *P. aeruginosa* isolates collected from wound infection(9). *P. aeruginosa* possesses many antibiotic resistance and tolerance phenotypes, enabling the bacterial population to potentially survive antibiotic treatment throughout an infection. The phenotypes exhibit significant diversity, both in terms of their developmental methods and their ability to survive in the presence of antibiotics. Antibiotic tolerance has been discovered to facilitate the creation of full antibiotic resistance (10). Siderophores (SPs) are secondary metabolites produced by several organisms to obtain iron from their surroundings, enabling the cell to absorb this crucial nutrient. Organisms form soluble ferric complexes known as SPs to facilitate the uptake of ferric iron, which they have a significant affinity (11). The aim of this study is evaluate of the expression of siderophore genes (*plcN* and *exoS*) upon the exposure of antibiotic.

Materials and Methods

Collection of samples

Two hundred swabs (wound infectins), samples were collected from patients at the Baghdad hospitals (Al Kindi Teaching and Medical City and Al-yarmok Teaching Hospital) who had wound infections of both sexes and all ages. The samples were collected from the beginning of November 2023 until the February 2024.

In the biofilm formation and secretion of extracellular proteins in *P. aeruginosa* isolates. the role of

Isolation and Identification of *Pseudomonas aeruginosa*

By using sterile cotton swabs, samples were collected and then transported to laboratory immediately. The swabs were streaked on nutrient, MacConkey, blood, and Pseudomonas cetrimide agar (PCA) plates and then incubated overnight at 37^{o}C to study the morphology of colonies. colonies that are suspected as *P. aeruginosa* were processed further for Conventional biochemical tests: indole, cytochrome oxidase, catalase, motility, and citrate utilization tests. VITEK 2 system and molecular markers based on the amplification of *16S rRNA* were used to characterize and confirm the identification of

presumptive *P. aeruginosa* isolates (12).

Minimum Inhibitory Concentration (MIC) of *P. aeruginosa*

1. Minimum inhibitory concentrations were determined by the microtiter plates technique and broth dilution method against meropenem antibiotics. A loopful (1 µl) culture was streaked on Mueller–Hinton (MH) broth plates containing a concentrations of meropenem at (1024 - 2 µg/ml), next the Sub-MIC, of antibiotic was identified. One milliliter of sterile saline was transferred into a test tube, transferring a few isolated colonies from the plates to the saline tube using sterile cotton swabs. The suspension was adjusted to the McFarlandstandard, desired by the (0.5 McF) (13).
2. Wells in column A, starting from the leftmost side of the plate.
3. The antibiotic mix to the wells in the (A) column by aspirating and dispensing the solution 6-8 times. Excluding any splashing.
4. Extract a 100 µl volume from column A and transfer it to column B. to column (C), and continuing until only column (H) remains. The same set of criteria for dilution can be applied uniformly across the entire series.
5. Remove 100 µl from the (H) column.
6. Using a micropipette set to 100 µl, distribute bacteria into each well.
7. The plates Incubated at 37ºC to 24 hrs.
8. After the incubation, dye resazurin (20 µl) was added into all the wells of plate and for 30 min was incubated to see any changes of color. The MICs were determined visually in broth micro dilutions as the lowest concentrations of the solution, at which no color changed from blue to pink in the resazurin broth assay (14).

Quantitative Minimum inhibitory concentrations (MIC)

1. 100 µl of broth Mueller–Hinton to fill each well on a microtiter plate.
2. Using a 100 µl pipette, apply the suitable antibiotic solutions.

Preparation of primers

According to the previous studies were obtained specific primers for detection of the gene expression Table (1).

Table (1): Oligonucleotides sequence of primer for (*16SrRNA, ExoS* and *PlcN*) gene expression.

Gene	Oligonucleotide primer sequence (5' to 3')	Product size (bp)	Referance
exoS	**F**: CGTATGAGTCAGCAAGGGCG	**118**	**(15)**
	R: GCGATGTGGTCACTGGCTTC		
plcN	**F**: GTTATCGCAACCAGCCCTAC	**466**	**(16)**
	R: AGGTCGAACACCTGGAACAC		
16s rRNA	**F**: CATGGCTCAGATTGAACGCTG	**225**	**(17)**
	R: GCTAATCCGACCTAGGCTCATC		

Analysis of (qRT- PCR) Assay

The Qubit® 1st-Step RT-qPCR System (Qubit®- USA) was used to amplify mRNA particles. The amplification reaction was done using the master amplification reaction listed in Table (2), together with the Qubit 2nd-Step RT-PCR and the Qubit®-USA listed in Table (3). Experiments were conducted to synthesize various properties of annealing temperature and cDNA.

Table (2): Component of PCR Master Mix.

PCR Master mix reaction components		Volume
GoTaq® Green Master Mix or Master Mix		12.5 µl
Template DNA		1.5 µl
Primers	Forward	1 Ml
	Reverse	1 Ml
Nuclease - free (H_2O)		9 µL
Total volume		25 Ml

Table (3): Quantitative Real-time PCR Reaction.

Cycle step	Stages	Temp	Time
1	Initial Denaturation	95 ºC	60 sec
40-45	Denaturation Extension	95 ºC 60 ºC	15 sec 30 sec (+ plate read)
1	Melt Curve	60-95 ºC	40 min

Gene expression by delta delta Ct method (ΔΔCt)

The delta delta Ct (ΔΔCt) method is the most straightforward approach for comparing Ct values between a reference gene and a target gene in relative quantification. This method entails selecting a calibrator sample. The calibrator sample might be either the untreated sample, the sample at the optimum temperature of 37ºC, or any sample that one wishes to use for comparison with the unknown samples. Initially, the ΔCt is computed for each sample by comparing the target gene with the reference gene. This calculation is performed for both the unknown samples and the calibrator sample, as indicated by the following equation.

ΔCt = Ct target gene - Ct reference gene

The ΔCt of the unknown sample is subtracted from the ΔCt of the calibrator to produce the ΔΔCt value.

ΔΔCt = sample (Ct target - Ct reference) – control (Ct target- Ct reference)

The normalized target quantity in the sample is derived using the formula $2^{-\Delta\Delta Ct}$. This value can be employed to compare the levels of expression across various samples. The mRNA expression levels were assessed using a comparative cycle threshold (CT) method called $2^{-\Delta\Delta Ct}$. The outcome was analyzed and collected utilizing the Livak algorithm (18).

Results and Discussion

Quantitative Real-Time PCR (qRT-PCR) results

Using real-time PCR quantification, the current study used SYBR green fluorescent dye, which intercalates and identifies all double-stranded DNA, including cDNA. The amplification was expressed as a cycle threshold, or Ct value (19).

This step's essential objective was to measure the expression of the *plcN* and *exoS* genes and compare the expression of each gene in the presence and absence of the antibiotic to determine the effect on *P. aeruginosa* resistance to carbapenems (20).

The *16S rRNA* gene was chosen as housekeeping gene for this experiment because it exhibits continuous expression in the cells under investigation across a range of circumstances. In the current quantitative PCR investigation, four isolates of *P. aeruginosa* resistant to carbapenem that share both *plcN* and *exoS* genes were used. In the current study, untreated and treated samples of resistant bacteria that were grown

using antibiotics were compared to analyze the mRNA expression of the *plcN* and *exoS* genes using the quantitative RT-PCR assay (qRT-PCR). The amplification values of the genes' Ct values were listed from the quantitative RT-PCR program. Relative quantification from delta delta Ct value was used to calculate the fold change in gene expression (21).

Gene expression of *16SrRNA* by Real-Time PCR

The housekeeping gene (*16S rRNA*) whose PCR Ct value was employed in the current investigation, was the gene expression without antibiotic treatment is shown in Table (4). *16SrRNA* Ct value ranged from (10.06 to 7.82), for the resistant isolates before the use antibiotic.

Quantification of *plcN* Expression by Real-Time PCR

The isolates before treatment (B.T.) in the group had a Ct value of (14.02 to 13.34) for *plcN* before treatment. After receiving antibiotic treatment (A.T), the isolates *plcN* Ct levels ranged from (17.96 to 17.92). Table (4) indicates that there was a noteworthy variation in the average Ct values among the various study groups. After receiving antibiotic therapy, the isolates' mean Ct values were more than the target gene's mean Ct number on relacing mRNA, its higher expression, and the target gene's lowest copy number carried in mRNA isolates (22).

Table (4): Gene expression(*plcN*) before and after treatment with meropenem.

	Sample	CT *16S RNA*	CT *plcN*	ΔCT	ΔΔCT	Fold	Mean
Before treatment	1.	10.06	14.02	3.96	0	1	1.00 ±0.00
	2.	6.57	15.89	9.32	0	1	
	3.	8.27	14.21	5.94	0	1	
	4.	7.82	13.34	5.52	0	1	
After treatment	1.	13.69	17.96	4.27	0.31	0.8	2.54 ±0.28
	2.	5.78	12.95	7.17	-2.15	4.43	
	3.	14.69	18.96	4.27	-1.67	3.18	
	4.	13.21	17.92	4.71	- 0.81	1.75	
** (P≤0.01)							

Quantification of *exoS* expression by by Real-Time PCR

When the resistant isolates were treated with the group, the Ct value for *exoS* of (37.33 to 34.48). After treatment with antibiotic, the isolates' Ct levels for *exoS* ranged from (31.71 to 30.13). This indicates that the genes are found in mRNA samples in isolates. These data clearly show that the antibiotic group has the lowest copy number of target gene carried on mRNA in isolates and the largest copy number of target gene on mRNAs, indicating its increased expression. Table (5), distinction in the average Ct values illustrates that there was a noteworthy among the various study groups it can be seen that the genes are present in the mRNA samples since the mean Ct values in the isolates after antibiotic treatment were higher than the mean Ct values of the isolates before treatment and after treatment antibiotic, which was then

somewhat higher than those in the isolates. These findings clearly show that the target gene is linked to the antibiotic group, which is connected with the highest copy number on mRNAs, indicating its increased expression (23).

Table (5): Gene expression (*exoS*) before and after treatment with meropenem.

	Sample	CT *16S RNA*	CT *exoS*	ΔCT	ΔΔCT	Fold	Mean
Before treatment	**1.**	10.06	37.33	27.27	0	1	1.00 ±0.00
	2.	6.57	18.12	11.55	0	1	
	3.	8.27	38.21	29.94	0	1	
	4.	7.82	34.48	26.66	0	1	
After treatment	**1.**	5.69	31.71	26.02	-1.25	2.37	2.79 ±0.35
	2.	6.42	16.91	10.49	-1.06	2.08	
	3.	4.49	32.34	27.85	-2.09	4.25	
	4.	4.77	30.13	25.36	-1.3	2.46	
**** (P≤0.01)**							

Fold gene expression of *plcN* and *exoS*

The results of the study related gene *plcN* are displayed in Table (4). The bacteria in the samples were mean ± SD (1±0) when they were left untreated, and mean ± SD (2.54± 0.28) when they were treated with an antibiotic from the meropenem. At a dose of 16 µg/ml for sample (1) and 8 µg/ml for samples (2,3 and 4) respectively, is the sub MIC antibiotic. By employing real-time PCR to compare the fold of the *plcN* gene expression with samples that were not treated with antibiotics, the results showed that there was an overexpression of the gene. The study findings revealed to gene *exoS* that the bacteria in the untreated samples had a mean ± SD (1±0) and a mean ± SD (2.79 ± 0.35) when treated with an antibiotic from the meropenem. Were for sample (1), the sub MIC antibiotic was 16 µg/ml; for samples (2,3 and 4), it was 8 µg/ml. The *exoS* gene's fold of expression is depicted in Table (5). This work used real-time PCR to demonstrate that the gene was overexpressed by comparing it to isolates that did not get antibiotic treatment. And by comparing the results with *P.aeruginosa* isolates that were not given meropenem treatment, these results were supported by study that showed difference in the fold of gene expression when *P.aeruginosa* isolates were treated with meropenem (24).

Conclusion

The current study was concluded that the most local clinical isolates of the *P. aeruginosa.* had high resistance percentage to meropenem, that considered a powerful antibiotic was applied for treatment of wound infections that caused by *P. aeruginosa*. The present study was found that the gene expression fold was for both genes (*plcN* and *exoS*) after treatment with meropenem were led to increased gene expression for both genes, which links these genes and bacterial resistance.

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