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Association of pvc genes expression with Biofilm formation in Clinical Isolates of Pseudomonas aeruginosa

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Abstract

PvcABCD are cluster of genes found in Pseudomonas aeruginosa. The research was designed to examine the relationship between the pvc genes expression and cupB gene, which plays a crucial role in the development of biofilm, and rhlR, which regulates the expression of biofilm-related genes, and to investigate whether the pvc genes form one or two operons. The aims were achieved by employing qRT-PCR technique to measure the gene expression of genes of interest. It was found that out of 25 clinical isolates, 21 isolates were qualified as P.aeruginosa. Amongst, 18(85.7%) were evaluated as biofilm producers, 10 (47.6%), 5 (23.8%), and 3 (14.2%) were evaluated as strong, moderate and weak producers respectively, while, 3 (14.2%) were considered as a non-biofilm forming isolate. The pvcA and pvcB were shown to be over-expressed (>2) fold in all biofilm-producer isolates, similar to that observed in cupB and rhlR, while pvcC and pvcD showed to be down-regulated (<0.5) fold in these isolates. These findings imply that the pvc genes are organized into two operons, pvcAB, and pvcCD, and genes involved in biofilm formation are regulated by pvcAB operon. This is the first study in Iraq to investigate these genes.

Keywords: Antimicrobial Resistance, Biofilm formation, *cup*B, Gene expression, *pvc* genes, RT-qPCR, *rhl*R.

Introduction

Pseudomonas aeruginosa (PA) is one of the most common nosocomial pathogens in Iraqi hospitals that can cause serious infections with fatal complications[1, 2]. The constant misuse and abuse of antibiotics for treating the infections resulted in the widespread development of multi-drug resistant (MDR) strains and complicated medical therapy against *P. aeruginosa*[3-5]. *P. aeruginosa* possesses a large genome that provides the bacteria with a wide range of virulence factors, one of them is Biofilm. Biofilms are complex structures where communities of bacteria are embedded in the extracellular matrix. Biofilms hold a significant role in healthcare-associated infections (HAI). According to the National Institutes of Health (NIH), biofilm formation is linked to 65% and 80% of all microbial and chronic illnesses, respectively[6].

Pseudomonas aeruginosa's *cup* (chaperone/usher pathways) gene clusters encode fimbrial structures involved in cell-to-cell aggregation and the

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development of biofilms. Different kinds of adhesins are assembled by the *cup* gene clusters. These adhesions may enable *P. aeruginosa* to adapt quickly and colonize a wide range of surfaces[7, 8].

The differential expression of *cup* adhesive structures is mediated by complex regulatory networks, which may include two-component regulatory systems for monitoring environmental stimuli. *P. aeruginosa* possesses two main Quorum Sensing systems *las* and *rhl*, the *rhl* system has been reported to involve in *P.* aeruginosa biofilm formation[8, 9].

The *P.aeruginosa pvc*ABCD gene cluster is associated with biofilm formation through positively regulating the expression of different *cup* genes by the production of Paerucumarin a novel isonitrile-functionalized cumarin. (Derived from *P. aeruginosa* cumarin) paerucumarin enhances the expression of the chaperon/usher pathway (*cup*) proteins in a pathway that includes ptxR[7, 10, 11].

This study sought to understand the relationship between *pvc* genes and *cup*B and *rhl*R genes in different biofilm-forming phenotypes clinical isolates of *P.aeruginosa.* This investigation was carried out using RT-qPCR to measure the expression levels of mentioned genes. Simultaneously determining whether the *pvc* consists of one operon *pvc*ABCD [12] or two operons *pvc*AB and *pvc*CD.

Materials and Methods

Bacterial Isolates and Molecular identification

This study was carried out on 25 isolates of *P. aeruginosa* which were obtained from various wards in Baghdad's hospitals including, Burn wards, ICUs, Dialysis centers, and urology departments from October 2021 until February 2022. The identification of isolates was done by molecular analysis; after the DNA was extracted using Wizard® Genomic DNA Purification Kit (Promega, USA), 16S rRNA gene sequence analysis was performed with the following conditions: initial denaturation at 95 ℃ for 15 minutes as the first stage of amplification, followed by the 2nd stage of amplification with denaturation at 95.0℃ for 15 seconds, annealing at 60.0℃ for 30 seconds repeated for 45 cycles[13]. This program was performed using Mastercycler® nexus- PCR Thermal Cycler (Eppendorf, Germany).

To accurately confirm the identification of all isolates, qRT-PCR technique was employed to detect *gyr*B gene following the program that consists of two stages: the first stage of initial denaturation for 15 minutes at 95℃, and the second stage of denaturation for 15 seconds at 95 ℃, and annealing for 1 minute at 60 ℃[14] the second stage was repeated for 50 cycles. The program was performed using Rotor-gene Q (Qiagen, Germany). The primers used in this study are listed in Table.1

Biofilm production assay

To evaluate the quantity of biofilm formation in isolates, a modified microtiter plate approach was employed following the method reported by Lima et al[15]. All isolates were cultured on nutrient agar for 24 hours at 37˚C. Then a Single-colony was inoculated in Brian heart infusion broth (BHI) supplemented with 50 g/L sucrose; the broth was then incubated at 37˚C for 24 hours.

-A 200 µl of the bacterial suspension was transferred in triplicates on 96-well polystyrene plates (Nunc, Denmark). The negative control was BHI broth without bacterial inoculum, while the positive control was the PA01 strain since this strain is recommended as the positive control for biofilm assays.

-After transferring the suspensions to the plates, the plates were then incubated at 37℃ for an additional 24 hours, and wells were washed three times with (PBS) Phosphate-buffered saline.

-Methanol was used to fix the bacterial biofilm that had attached to the wells for 15 minutes. And crystal violet solution was used to stain the biofilm for 5 minutes.

-ELISA reader (Biorad, UK) was used to measure the absorbance of the stained biofilm at a wavelength of 570 nm. The average of the optical density (OD) readings for the three wells was used

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to determine the optical densities (ODi) for each isolate. This value was then contrasted with the optical density value of the negative control (ODc). According to Stepanović et al[16], the isolates were divided into the following 4 groups based on the findings of this comparison.

ODi≤ODc=negative; ODc<ODi<2x ODc=weakly positive; 2x ODc< ODi<4x ODc=moderate positive; 4x ODc<ODi=strongly positive.

Antimicrobial Susceptibility Profile

The Kirby-Bauer method was used to evaluate the antimicrobial susceptibility of *P. aeruginoas* isolates for the following six antibiotic groups: 1- Flouroquinolones[Ciprofloxacin (CIP/ 5 µg), Norfloxacin (NOR/ 10 µg), Levofloxacin (LEV/ 5 µg), 2- Aminoglycoside [Gentamicin (CN/10 µg), Tobramycin(TOB/10 µg), Amikacin (AMK/30 µg)], 3- Carbapenem [Imipenem (IPM/ 10 µg), Meropenem (MEM/10 µg), 4- Monocyclin [Aztreonam (ATM/ 30 µg), 5- Cephems or cephalosporin Cefepime (FEP/ 30 µg), Ceftazidime (CAZ/ 30 µg), 6- Beta- lactamase inhibitor combination agents Piperacillin* Tazobactam (TZP/ 100/10 µg)]. The method was performed on Muller-Hinton agar (Bio lab, Hungary) where the inhibition zone of the antibiotic was calculated, to interpret the susceptibility results. The Clinical and Laboratory Standards Institute (CLSI)[17] guidelines were followed according to the manual's instructions, Resistance (R), susceptibility (S), and intermediate (I)were used to describe the susceptibility data.

RNA extraction and cDNA synthesis

The bacterial colonies were suspended in TRIzol reagent and the total RNA was extracted using TransZol up plus RNA kit (TransGen/ Biotech/ China) as directed by the manufacturer. To measure the purity and concentration of isolated RNA, NanoDrop2000c (Thermo Fisher Scientific, USA) was used. As for the complementary DNA (cDNA) synthesis it was performed in a thermocycler (Applied Biosystem, USA) where 4 µl of template RNA was reversely transcribed using EasyScript® One-Step gDNA Removal and cDNA Synthesis SuperMix (TransGen, Biotech. China) the prepared cDNA was used to perform the RT-PCR amplification.

Primers Design

The oligonucleotides used in this study were designed by Asmaa M. Salih Almohaidi using the Primer-BLAST tool (https://www.ncbi.nlm.nih.gov/tools/primer- blast/) where *cup*B, *rhl*R primers were designed especially for this study, while *pvc*B, *pvc*C and *opr*D were modified to better serve the purpose of this study from the reference sequence[7, 18]. All the primers used in this study were synthesized by Alpha DNA Ltd (Canada) and stored at -23°C (Table1).

Table 1. Sequences of primers used in this study

Gene	Primer Sequence	annealing Tm ˚C	Product Size	Reference
16S rRNA	**F**-GCACTTTAAGTTGGGAGGAA	60	144bp	13
	R-CTTTACGCCCARTRAWTCCG			
*gyr*B	**F**-CCTGACCATCCGTCGCCACAAC	60	220bp	14
	R-CGCAGCAGGATGCCGACGCC			
*pvc*A	**F**-CCTGTCGCTGTCGTTCCT	56	143bp	7
	R-CCTGGTAGGCGCTGATGT			
*pvc*B	**F**-ATTCCCCATCCTGCGTT	58	212bp	Designed by the third author
	R-CGTGCAACAGGGTCAGG			
*cup*C	**F**-AAGGCGATCCACGAGATG	52	187bp	Designed by the third author

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	R-CGAAGAACACAACAGCGA			
*pvc*D	**F**-CCTCTCGGCCCTGCTT	52	158bp	7
	R-TCGGGTAGCTGCGGTTC			
*cup*B	**F**-CATCGCCTTCGATCAGGTCA	58	129bp	Designed by the third author
	R-GGGATCTTCGTGGTGTTGGT			
*rhl*R	**F**-TCCTGGAAAAGGAAGTGCG	56	147bp	Designed by the third author
	R- CCCCGTAGTTCTGCATCTG			
*opr*M	**F**-CCATGAGCCGCCAACTGTC	58	205bp	47
	R-CCTGGAACGCCGTCTGGAT			
*opr*D	**F**-CTACCGCACAAACGATGAAGG	60	154bp	Designed by the third author
	R- GCCGAAGCCGATATAATCAAACG			

Real-Time Quantitative PCR (RT–qPCR)

The identified *P.aeruginosa* isolates were subjected to real-time quantitative PCR (RT-qPCR), to evaluate the expression levels of *pvc* genes (*pvc*A, *pvc*B, *pvc*C, *pvc*D) and *cup*B, *rhl*R genes. The expression was investigated using TransStart® Top Green (TransGen, Biotech. China) as per the manufacturer's instructions. Each PCR tube had a reaction mixture of the following components: 10 µL of the 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 30sec; denaturation at 94°C for 5 sec.; annealing (refer to Table 1) for 15 sec; and extension at 72°C for 20 sec these steps were repeated for 40 cycles. PAO1 was used as a reference strain since many studies considered it as a standard strain[15], while *opr*D and *opr*M were used as housekeeping genes. The reactions were carried out in triplicate, and the expression levels for the genes were examined using the mean ct value

Statistical analysis

Statistical analyses were performed using the SPSS Statistics software V 17.0 (SPSS Inc., Chicago, IL, USA). The expression values of all the data are displayed as the average ± standard error (SD). Student's t-test was used for the statistical analysis, and significance levels were chosen at *p 0.05 and **p 0.01.

Results

Isolates molecular identification

In this study 16S rRNA and g*yr*B genes were used to identify *P. aeruginosa* clinical isolates. A conventional PCR technique was used to detect the 16S rRNA gene in 25 clinical isolates. All the tested isolates were positive for 16S rRNA 25/25 (100%) as seen in Fig.1, while real-time quantitative PCR (RT-qPCR) was used to detect *gyr*B gene in 25 clinical isolates (that tested positive for 16S rRNA). The results for this test showed that 21 out of the tested 25 isolates were positive for *gyr*B gene 21/25 (84%) (Values ranged from16 to ~22cycles), these 21 isolates were the ones selected to carry on with the study.

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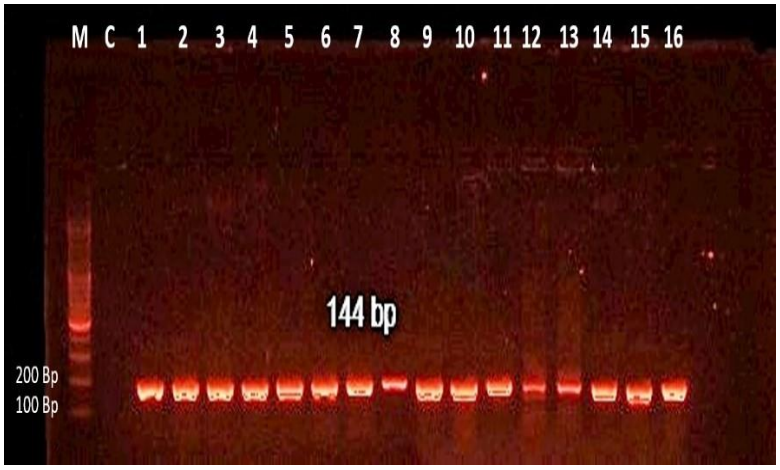

Figure 1. Amplified 16S rRNA products. Electrophoresed on 1.5 % agarose for 1.5 h. with 70 volt. The product size was 144 pb. Lane M: DNA Ladder; lane C: Negative control; lane 1 to 16: tested isolates

Biofilm Assay:

The ability of 21 *P. aeruginosa* isolates to generate biofilms was examined. Of the biofilm-producing phenotypes, 18 (85.7%) of the *P. aeruginosa* isolates were considered biofilm producers: 10 (47.6%) were qualified as strong, 5 (23.8%) were considered intermediate, and 3 (14.3%) were qualified as weak biofilm producers, while 3 (14.3%) were unable to produce biofilm (Table. 2).

Table 2. Biofilm formation phenotype in different isolates

Pattern of biofilm producer, no. (%)				**Total**
Non-producer	Weak producer	Moderate producer	Strong producer	
3	3	5	10	21
14.3%	14.3%	23.8 %	47.6%	100%

Antimicrobial Susceptibility:

Figure 2 provides a summary of the susceptibility profile of *P.aeruginsa* isolates toward 12 antimicrobial agents. Following the resistance interpretative criteria used for antimicrobial drugs as it has been previously described, the highest levels of susceptibility were to Gentamicin (80.9%), Tobramycin (78.19%), Levofloxacin(76.1%), Cefepime (71.4%), Amikacin and Ceftazidime (66.6%),) while the Meropenem (52.3%) and Imipenem (47.6%).According to Table. 3, there was a relationship between the patterns of biofilm production and antibiotic susceptibility profile. Among 21 isolates, analysis of the different resistance phenotypes showed that 14 (66.7%) were MDR isolates and 7(33.3%) were sensitive to the antibiotics used. Out of 14 MDR isolates, 13/ 14(92.9%) were biofilm-producer isolates, including 8 belonged to the strong biofilm producers group, 3 belonged to the moderate biofilm producers group and 2 belonged to the weak biofilm producers group, only 1/14(7.1%) MDR isolates was non- biofilm producers. The ability to form biofilms was substantially more likely to have the resistance phenotype MDR.

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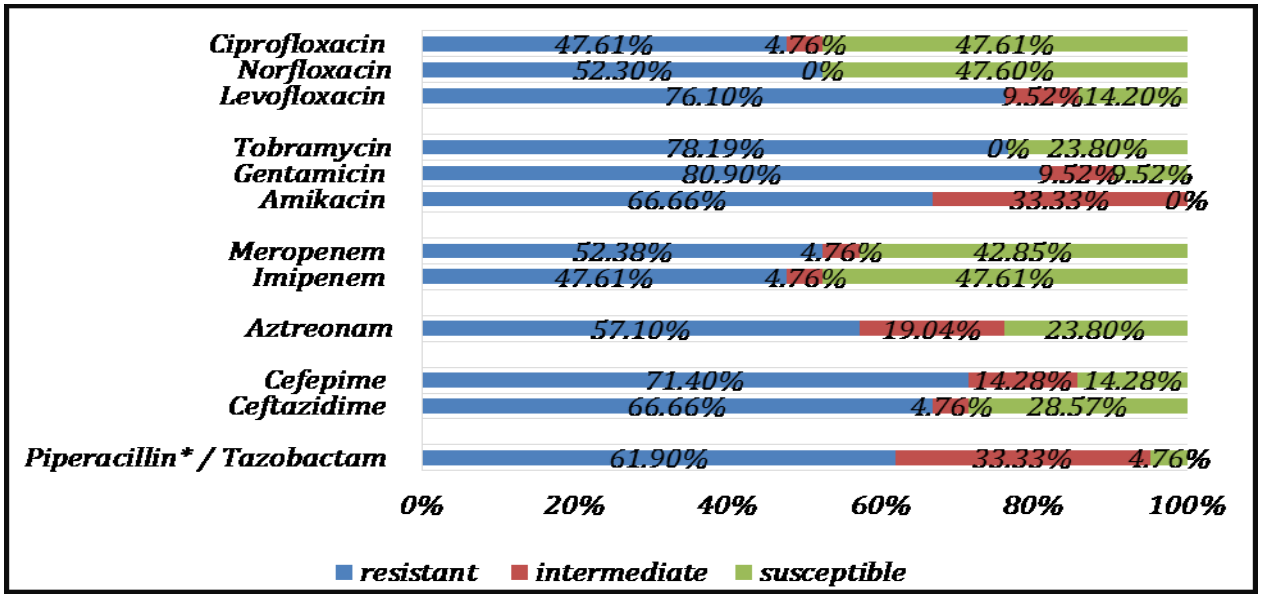

Figure 2. *P. aeruginosa* isolates resistance to different antibiotic group.

Table 3. Represents the correlation of biofilm formation and antimicrobial pattern

Phenotypic pattern of biofilm	No. of isolates	Phenotypic pattern of Antimicrobial Susceptibility	
		MDR	Susceptible
Strong	10	8(80%)	2(20%)
Moderate	5	3(60%)	2(40%)
Weak	3	2(66.7%)	1(33.3%)
Non biofilm	3	1(33.3%)	2(66.7%)
Total	21	14(66.7%)	7(33.3%)

Gene expression analysis:

In this study, RT-qPCR was performed for 8 genes with a significant expression between the clinical isolates and the reference strain, this includes two housekeeping genes. The RT- qPCR results showed that *pvc*A, *pvc*B, *cup*B genes were over-expressed >2.0 fold in 85.7% of isolates (strong, moderate and weak biofilm-forming isolates) (Fig.3).

While *pvc*C and *pvc*D showed down-regulation <0.5 fold in all isolates 100%. Fig.3 and *rhl*R only showed over- expression in two groups (strong and moderate) over 71% of the isolates.

The difference in the expression levels is visualized as an array in the amplification curve, where the expression level of *pvc*AB operon was continued and passed the threshold line in the early cycle, whereas a delay in the expression of *pvc*CD operon was observed.

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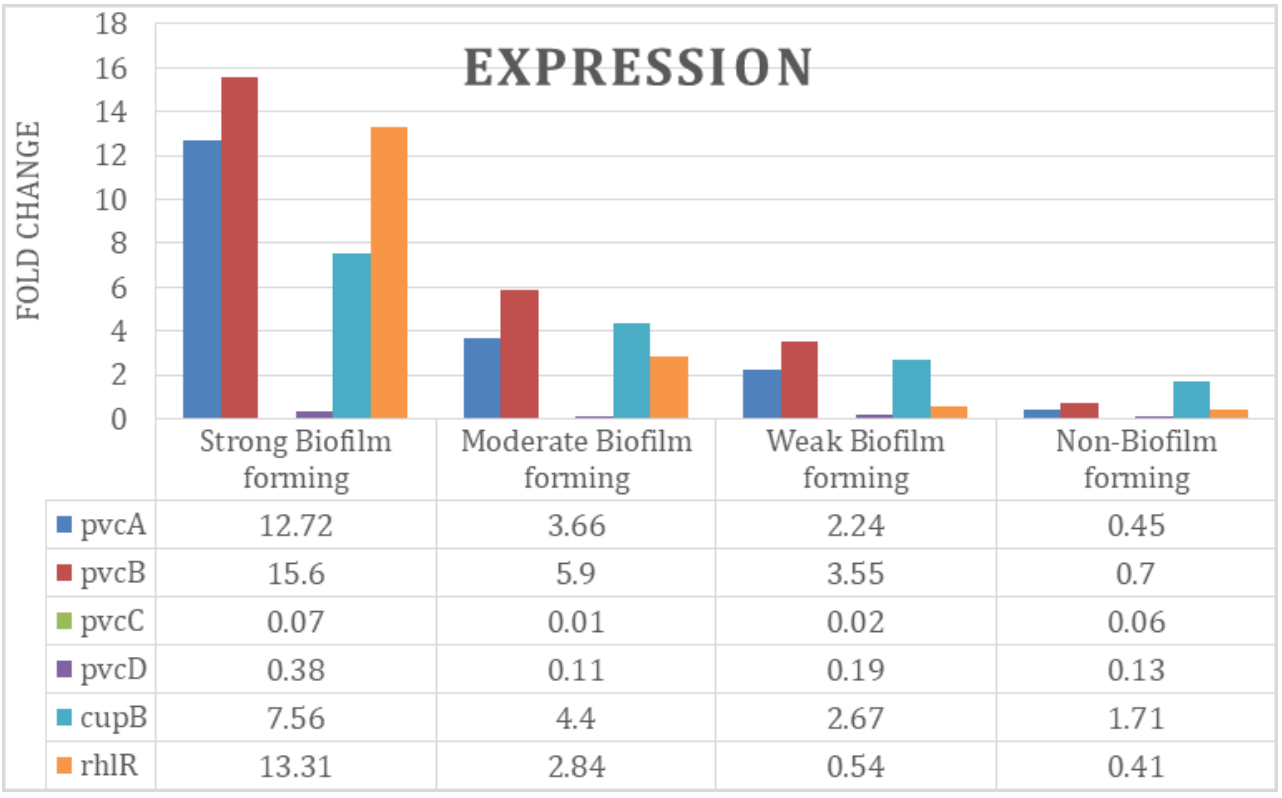

Figure 3. The fold change gene expression of the genes used in this study for biofilm forming groups.

Student t-test statistical analysis revealed significant differences in the expression of *pvc*A, *pvc*B, *cup*B, and *rhl*R compared to *pvc*C, *pvc*D (Table. 4).

Table 4. The statistical analysis of gene expression with references strain

Group	*pvc*A	*pvc*B	*pvc*C	*pvc*D	*cup*B	*gyr*B	*rhl*R
isolates	6.33 ±5.43	5.71 ±2.10	0.051 ±0.02	0.168 ±0.05	5.11 ±3.05	8.01 ±7.22	7.15 ±5.66
Reference strain	1 ±0.00	1 ±0.00	1 ±0.00	1 ±0.00	1 ±0.00	1 ±0.00	1 ±0.00
t-test	4.02 **	2.79 **	NS	NS	2.81 **	4.63 **	3.69 **
p-value	0.0081	0.0076	0.561	0.447	0.702	0.0001	0.0001

** Highly significant = p≤0.01, * Significant = p≤0.05, NS= non-significant

Table 4, shows the expression of *pvc*A gene in the tested isolates displayed over-expression (6.33±5.43) of the gene compared to the reference strain. Such over-expression is also seen in *pvc*B (5.71±2.10), *cup*B (5.11±3.05) and *rhl*R (7.15±5.66) when comparing the tested isolates with the reference strain. Unlike *pvc*C and *pvc*D which are down-regulated when compared to the reference

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strain with the expression of (0.051±0.02) for *pvc*C and (0.168±0.05) for *pvc*D.

Table 5. The statistical analysis of gene expression with Biofilm grouping

Biofilm forming groups	Fold Change in Gene expression						
	*pvc*A	*pvc*B	*pvc*C	*pvc*D	*cup*B	*rhl*R	*gyr*B
Strong Biofilm	12.72 a	15.6 a	0.07	0.38	7.56 a	13.31 a	15.6 a
Moderate biofilm	3.66 b	5.9 b	0.01	0.11	4.4 ab	2.84 b	1.34 b
Weak biofilm	2.24 b	3.55 b	0.02	0.19	2.67 b	0.54 b	0.77 b
Non biofilm	0.45 b	0.70 b	0.06	0.13	1.71 b	0.41 b	0.76 b
LSD value	5.693 *	6.188 *	0.0723 NS	0.349 NS	4.92 *	5.026 *	5.362 *

** Highly significant =p≤0.01, * Significant =p≤0.05, non-significant p>0.05

The different letters in the same column means there are significant differences.

As noticed in Table 5, the statistical comparison between current genes expression and the biofilm formation ability. The results showed a significant increase in gene folding for *pvc*A, *pvc*B, and *cup*B, *rhl*R genes that were synergistic in a compatible way with increasing the strength of biofilm for present isolates. *pvc*AB may up-regulat c*up*B while *pvc*CD may not.

Discussion

The results for 16S rRNA sequence identified all samples as *P.aeruginosa*. The sensitivity of 16S rRNA gene is 100% which agrees with the results submitted by Al-Taai et al and Al-Tememe[19,20], while other study[21] recorded 96% sensitivity. As for the results of *gyr*B gene, about 21 isolates 84% harbored g*yr*B gene this finding may suggest that the *gyr*B gene is a good candidate for *P. aeruginosa* iden tification besides 16S rRNA[22-24].

The sequences of the *gyrB* gene in *P. aeruginosa* and other species of *Pseudomonas* exhibited a larger divergence than the 16S rRNA genes, despite the fact that it is the most common gene for genetic identification and characterization of bacteria. The rationale is that the rate of gyrB molecular evolution is higher than that of 16S rRNA and that it is transmitted horizontally[21]. The *gyr*B, as housekeeping genes have already been used in bacterial phylogenetic analysis and identification, is a better candidate target than 16S rRNA for the identification of the *Pseudomonas* species[25].

P.aeruginsa with its exceptional ability to adhere to many surfaces by forming a biofilm, sets the biofilm to be one of the most important virulence factors of the bacteria. A quantitative approach was regarded as the gold standard to assess the development of biofilms. In the current investigation, biofilm formation was observed for 18 out of 21 *P. aeruginosa* isolates obtained from clinical samples 85.7%. Previous researchers found that biofilm development occurred in 93.4%, 77.5%, 83.7%, and 100% of the cases studied,

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respectively[26-29]. However, they varied in their capacity to form biofilms. This is also applying to local studies, where researchers observed biofilm formation ability in 70%, and 100% respectively of the local clinical *P.aeruginsa* isolates[30, 31]. Of the 18 total isolates, 47.6% had a strong capacity to do so, while significant isolates displayed a moderate 23.8% and weak 14.3% capacity. Similar data was reported[27, 29, 32]

In 2017, *P. aeruginosa* was recognized by WHO as a priority pathogen for the development of new antimicrobial agents[33]. The current results of antimicrobial susceptibility test demonstrate a high degree of resistance to Aminoglycosides (Gentamicin 80.9%, Tobramycin 78.1% and Amikacin 66.6%, this result agreed with the researchers that recorded higher resistance to the Aminoglycosides [34, 35]. Additionally, the findings demonstrate that the isolates were beta-lactam antibiotics resistant, particularly Meropenem 52.4% and Imipenem 47.6%, which are Carbapenems. These findings line up with the data provided by Ratajczak et al [29]. Imipenem-resistant strains were 52.72 % of *P. aeruginosa* isolated from infections, according to research done by the International Nosocomial Infection Control Consortium (INICC) [36]. A Globule rise in the number of Carbapenem resistance among Gram-negative bacilli[29].

Overall, these findings are consistent with local studies, which illustrated a high rate of resistance to Ceftazidime, Amikacin, Ciprofloxacin, Piperacillin, Levofloxacin, and Gentamycin, with the ability to form biofilms. Which become substantially more likely to have the resistance phenotype MDR, with 13/18 (72.2%) of the isolates acquiring this phenotype[21, 37, 38] Table.3.

Similar results have been reported by other studies, which found a link between the MDR phenotype and *P. aeruginosa* isolates' capacity to produce a biofilm[39, 40]. Another study in Iraq revealed a high ability (90.7%) to form biofilm in clinical MDR *P. aeruginosa* isolates, and the biofilm producers were classified into three categories (strong, moderate, and weak) according to the biofilm analysis[38]. Unlike other studies, which found that 100% of the isolates with MDR phenotype were strong biofilm producers[29]? While in Brazil, a group of researchers discovered that 51.6% of *P. aeruginosa* isolates produced biofilms that were non-MDR, and 48.4% were MDR[27]. When *P. aeruginosa* is the etiological component in an illness, multiple drug resistance and biofilm development may make therapy challenging[29].

The expression of genes such as *cup* cluster, quorum sensing systems (*Las*R-*Las*I system and *Rhl*R-*Rhl*I system) play a significant role among the regulatory systems of virulence factors for bacterial pathogenicity, including biofilm formation[41]. The expression of *rhl*R gene in Table.4 was high (7.15 fold), as this gene is required for biofilm formation, whereas, it showed down-regulated (< 1.0 fold) in the weak and non-biofilm producer isolates. Previous studies suggested that these isolates' inability to build biofilm was caused by conformational changes in quorum sensing (RhlR) proteins mediated by mutations in the *rhlI/rhl*R system[27, 42]. However, the present results go with the results of another investigation which showed a strong connection between the ability to create biofilms and the presence of pertinent genes[2, 43].

Based on the current study's findings, *P. aeruginosa* isolates both contained and expressed the gene related to biofilm formation and were thought to have significant levels of expression with a significant compatible level of gene folding with strength of the biofilm. The expression of the *cup*B gene was low (1.7fold) in the non-biofilm producer isolates, compared to the reference strain employed as a positive control. The *cup*B gene was discovered in all biofilm producer isolates >2.0 fold, including strong 7.5 fold, moderate 4.4 fold, and weak 2.6 fold. These results are consistent with a study that revealed 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], it was also mentioned that 31.03% of *P. aeruginosa* isolates had the biofilm gene (*psl*A) while none of these isolates tested positive phenotypically for the production of biofilms in Congo red agar and microtiter plate experiments[44]. According to the present study's findings, MDR P. *aeruginosa* (n =

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13) and non-MDR P. *aeruginosa* (n = 5) were both biofilm-producing and carrying the *cupB* biofilm gene (Table 3). This may lead to the initial misconception that biofilm development is unrelated to antibiotic resistance for the isolates that generated biofilm and harbored the *cup*B gene but weren't MDR. It's important to note that all of the isolates used in current investigation were tested for antimicrobial susceptibility as planktonic cells rather than as biofilms. Thus, quorum sensing, which confers the MDR phenotype, and several mechanisms of biofilm and its architectural features such as glycocalyx matrix, outer membrane structure and growth rate, persistent cell formation, genetic adaptation, and stress responses, were not involved in the test[45].

The current study demonstrated the upregulation in quorum sensing genes linked to potential virulence and the rise in biofilm biomass, as a risk factor for the emergence of extremely multi-resistant bacteria. The results of RT-qPCR showed that *pvc*A and *pvc*B, genes were presenting an over-expression (>2.0 fold) in three groups (strong, moderate and weak biofilm forming isolates). While *pvc*C and *pvc*D were down-regulated <0.5 fold in all the isolates. These results are non-compatible with other research results which reported that a deletion mutation of the *pvc*A gene reduces the expression of *cup*B *cup*C about 17-fold and 5-fold respectively, compared to *cup*A gene which was only reduced by 2.2- fold[7]. This demonstrates the huge effect of *pvc*A on *cup*B gene clusters.

According to present study findings which showed different pattern of gene expression level, a new question arises, that is the *pvc* Genes Operon consist of One or Two operons? With their Effects on Biofilm Formation Genes in *P. aeruginosa* Clinical Isolates

A previous study suggested that the *pvc* genes form one operon[12], while a computer analysis proposed that the *pvc* genes consist of *pvc*AB operon and *pvc*CD operon (data not shown) [7]. This was determined by measuring the levels of gene expression for all *pvc* genes simultaneously. The levels of expression for *pvc* genes were then compared to the levels of *cup*B expression, which encodes fimbrial structure which aids in the biofilm formation, and the *rhl*R gene expression quorum sensing gene that regulates the biofilm production in *P.aeruginosa*. Since operons are a feature of prokaryotic genomes and allow for the functionally related genes to be regulated, transcribed and translated simultaneously[46,47]. The drastic difference in the expression of *pvc* genes was highly support the possibility that *pvc* genes form two operons *pvc*AB and *pvc*CD. The results of this study conflict with previous results that presented by other authors who have also tested this possibility[7].

The sequence can form a stem-loop structure in the 51-bp intergenic region between *pvc*B and *pvc*C, therefore it is possible that there are two operons rather than one[10]. This sequence is most likely to have an attenuating effect on the expression of the *pvc*C and *pvc*D genes from a promoter upstream of *pvc*A[10]. The following model Fig.4 is proposed to explain current findings, on the basis of the presented results, the current study suggests that *pvc*A alongside *pvc*B enhances the biofilm formation by enhancing the expression of the *cup* gene cluster

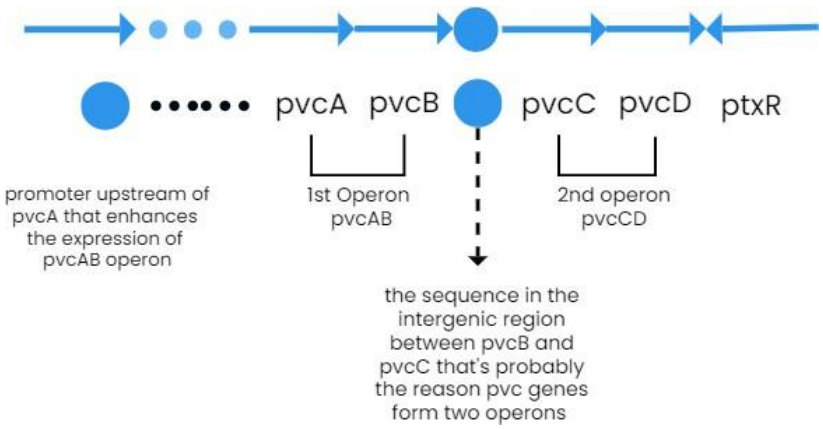

Figure 4. Diagram of the proposed *pvc* operons

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Conclusion

Current findings indicate that *gyrB* gene is a good candidate for *P.aeruginosa* identification besides 16S rRNA and there was a relationship between the patterns of biofilm production and antibiotic susceptibility profile. The ability to form biofilms was substantially more likely to have the resistance phenotype MDR. And pvc genes constitute two operons and *pvc*AB operon plays the role of enhancing the expression of *cup* genes and thus promotes biofilm formation. The data state that *pvc*CD operon is down-regulated in biofilm-forming isolates which denies its involvement in increasing biofilm intensity.

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Authors' Declaration

- Conflicts of Interest: None.
- We hereby confirm that all the Figures and Tables in the manuscript are ours. Furthermore, any Figures and images, that are not ours, have been included with the necessary permission for re-publication, which is attached to the manuscript.
- Authors sign on ethical consideration's approval.
- Ethical Clearance: The project was approved by the local ethical committee in University of Baghdad.

Authors' Contribution Statement

STA and AMSA designed the experiments, AMSA designed the primers. NAA performed the experiments, NAA, STA and AMSA analyzed the data. NAA, STA and AMSA participated in drafting and revision of the manuscript. All authors read and approved the final manuscript.

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العلاقة بين جينات pvc والغشاء الحيوي في العزلات السريرية لبكتريا الزائفة الزنجارية

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الخلاصة

*pvc*ABCD هي مجموعة جينات متواجدة في بكتريا الزائفة الزنجارية, تم تصميم البحث لأختبار العلاقة بين تعبير جينات *pvc* مع جين *cup*B, الذي يلعب دور مهم في تطوير الغشاء الحيوي, وجين *rhl*R, الذي ينظم تعبير الجينات المرتبطة بالغشاء الحيوي, والتحقق من ما اذا كانت جينات *pvc* تكون مشغل واحد أو اثنان. تم تحقيق أهداف البحث عن طريق استخدام تقنية RT-qPCR لقياس التعبير الجيني للجينات المذكورة. وجد من بين 25 عزلة سريرية تم تشخيصها 21 عزلة تعود الى بكتريا الزائفة الزنجارية, حيث كانت 18(85.7%) عزلة مكونة للغشاء الحيوي: 10 (47.6%), 5(23.8%), 3(14.2%) كانت قوية , متوسطة وضعيفة التكوين للغشاء الحيوي على التوالي. بينما 3(14.2%) عزلات كانت غير مكونة للغشاء الحيوي. أظهرت مستويات التعبير الجيني لجينيّ *pvc*A, *pvc*B فرط في التعبير (2 < fold) في جميع العزلات المكونة للغشاء الحيوي. تم الحصول على نتائج مماثلة لجينيّ *cup*B, *rhl*R . بينما أظهرت نتائج تعبير جينيّ *pvc*C,*pvc*D نقص في التعبير 0.5> fold في هذه العزلات.تشير هذه النتائج الى أن جينات *pvc* منظمة في مشغلين *pvc*AB و *pvc*CD . وكانت الجينات المرتبطة بتكوين الغشاء الحيوي تنظم من قبل مشغل *pvc*AB. هذه هي الدراسة الأولى في العراق التي تتحقق من هذه الجينات.

الكلمات المفتاحية: مقاومة المضادات, الغشاء الحيوي, جين *cup*B, التعبير الجيني, جينات RT-qPCR , *pvc* , جين *rhl*R.