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# Effectiveness of imipenem stress on upregulation of *phzM* expression encoding pyocyanin production by *Pseudomonas aeruginosa* clinical isolates

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**ABSTRACT**

Hitherto, studies on the expression of phzM gene that plays a role in pyocyanin pigment production by *Pseudomonas aeruginosa* isolates under imipenem stress had not been conducted. Hence, the assessment of imipenem stress on the expression of phzM gene is the main focus of contemporaneous study. Thirty *P. aeruginosa* isolates were obtained from the Department of Biology at the College of Science, University of Baghdad. Isolates were previously diagnosed as *P. aeruginosa* by Vitek-2 system. Investigation of biofilm formation capability was conducted using the microtiter plate method. Furthermore, phzM was ascertained in *P. aeruginosa* isolates using the conventional PCR technique. while, qRT-PCR technique was undertaken to check the expression of phzM in presence and absence of imipenem. Results revealed that out of 30 isolates, 7(23.33%) formed strong biofilm, thirteen (43.33%) generated a moderate biofilm and ten (33.33%) produced a weak biofilm. Significant reduction in the effect of imipenem on biofilm formation was achieved phenotypically, and genotypic results indicated that imipenem stress upregulated the phzM gene expression. So, imipenem stress induces phzM gene, which is one of the genes that encodes pyocyanin production.

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## Introduction

*Pseudomonas aeruginosa* infections in cases with immunosuppression and other comorbidities are often hard to treat especially in those who were exposed to inappropriate therapy or were infected with pathogens that are characterized with multiple drug resistance. which, in turn, resulted in a demand for discovering new antibiotics (Al Jader & Ibrahem 2022, Horcajada et al. 2019; Del Barrio et al. 2020, Witwit et al. 2024, Mohamed et al. 2025). A variety of extracellular pigments can be produced by the genus *Pseudomonas,* and the most important one is known as phenazines. Production of the compound that is blue green phenazine known as pyocyanin pigment is the most characteristic feature of *P. aeruginosa* (Abdali & Al-Attar 2020).

Phenazines are known as redox-active heterocyclic compounds that have toxic impact on living cells by their interaction with oxygen to fabricate reactive oxygen species (ROS) like superoxide ($O2^{-1}$), hydrogen peroxide ($H_2O_2$), and hydroxyl radical (HO·) (Dietrich et al. 2006; Younis & Faisal 2024; Montelongo-Martínez et al. 2022). Pyocyanin stain, which is usually manufactured by *P. aeruginosa,* can be considered as an principal virulence factor which resulted in a great destruction to lung and airways. Extrametabolic pathway is involved in order to manufacture pyocyanin from phenazine -1- carboxylic acid. phz*M* and phz*S* genes encode two enzymes that have a crucial role in this pathway (Wang et al. 2020). Pyocyanin, that has a close redox potential to menaquinone can also be employed as a reversible dye.

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The Chemical structure resemblance to flavin adenine dinucleotide, isoalloxazine and flavoproteins ellicit its biological activity (Ohfuji et al. 2004). A valuable indication on biofilm formation and uptaking of iron had been reported by pyocyanin under low oxygen conditions, that in turn, can elevate the resistance of metals mainly silver (Abdelaziz et al. 2023). From one of the previous studies, it was found that sub-MIC of antibiotics resulted in an unexpected ascendancy on the pyocyanin-producing genes expression (Younis & Faisal 2024). According to our knowledge, the effects of the imipenem stress (at sub-MIC) on the expression of phenazine-specific methyl transferase *phzM* gene had not been determined.

Accordingly, the premier goal of present study is to estimate the impact of imipenem stress on biofilm formation and *phzM* gene expression that plays a cardinal role in pyocyanin pigment manufacturing.

## Materials and Methods

### *Microorganisms*

Bacterial isolates represent 30 *P. aeruginosa* strains were obtained from Department of Biology, College of Science, University of Baghdad. These were previously diagnosed by Vitek-2 system.

### *Biofilm formation assay*.

Tested isolates were cultured in tryptone soy broth (HiMedia) and overnight incubation at 37˚C was performed. 100 µl of the bacterial inoculum of bacteria was added to 2 ml of sterilized normal saline in order to reach a turbidity that was adjusted to the McFarland standard of no. 0.5. Trypton soya broth with 1% glucose was added to the flat-bottom microtiter plates in a volume of 180 µl, and then, bacterial suspension was put down in three wells of the plate in a volume of 20 µl. A negative control was done by adding only tryptone soy broth to six wells. Plate was covered with the supplied lid and incubated at 37°C overnight. After incubation, removal of the bacterial culture from wells was performed and non-adherent cells were pulled out by washing 2-3 times with distilled water. Afterwards, 200 µl of absolute methanol (10 min) was accomplished to fix the adherent bacteria. then washing and air‑drying of the wells was accomplished. All assays were done in triplicates. Crystal violet at a concentration of 0.1% (200 µl ) was included to wells for 15 minutes for biofilm staining. Washing (2-3 times) by distilled water was conducted to remove the excess of the stain. The amount of stain that was extricated by adding 33% glycial acetic acid (HiMedia) (200 µl) in each well was quantified by measuring the optical density 630nm via a microplate reader (Naves et al. 2008; Ibtissem et al. 2013). The categories of all isolates are based on the ODc value as illustrated in Table 1.

**Table 1** Classification of bacterial adhesion on microtiter plates according to Zhao & Liu (2010).

| Mean OD (630 nm) | Biofilm intensity |
|---|---|
| OD≤ODc | No biofilm formed |
| 2ODc>OD>ODc | Weak biofilm |
| 4ODc>OD>2ODc | Moderate biofilm |
| OD>4ODc | Strong biofilm |

### *Determination of Imipenem Minimum Inhibitory Concentration (MIC)*

The Method of broth dilution was applied to determine MIC. Double serial dilutions (from 1024 to 0.5 µg/ml) of imipenem (ACS DOBFAR S.P.A., Italy) were prepared form stock solution (100 mg/ ml) in test tubes using Mueller Hinton broth as a diluent. The results were compared with standard breakpoints values according to CLSI (CLSI 2024).

### *Molecular Studies*

### *Detection of phzM gene*

The polymerase chain reaction (PCR) was used to confirm the existence of *phzM* gene in 7 strains that showed strong biofilm former. Brain heart infusion broth was inoculated with bacteria overnight at 37°C. The Presto™ Mini gDNA kit (Geneaid/Taiwan) for DNA extraction was utilized. Concentrations of DNA were calculated using Quantus™ Fluorometer (Promega/ United states) that ranged from 96 to106 ng/ul. Extracted DNA was stored at-20ºC until use. The primer used in detection *phzM* gene was designed according to software of Genious prime2023.1.1 which relies on accession number. CP026155 of GenBank is listed in Table-2. PCR premix, primers and DNA were combined in a PCR tube to a final volume of 25 µl. Specifically, from each primer 1 µl, the premix 12.5µl and template DNA 2µl were added to the tube. The remaining volume was filled with sterile deionized water. Under the following conditions, amplification was accomplished on a Master thermocycler gradient PCR (Eppendorf, Germany): an initial denaturation step at 92°C for 3min; denaturation step of 30 cycles at 92°C for 10sec. Afterward, annealing step at 58°C for 30sec, an extension at 72°C for 1 min., and a final extension step at 72°C for 3 min. The products of PCR were separated by gel electrophoresis (1% agarose), with a DNA ladder of about 100 bp as a reference (Green & Sambrook 2019). The primers used in current study can be illustrated in Table 2.

### *RT-qPCR of the phzM gene*

Pure Kit of GENEzol™ TriRNA(Geneaid/Taiwan) had been employed to extract RNA from four isolates of *P. aeruginosa* with different MIC values low, middle and high. In order to assess the gene expression of *phzM* gene, *Fbp* gene expression was utilized as an internal control. Primers of these genes were listed previously in Table 2. RT-qPCR was performed using qPCR soft 3.4 - © by Analytik JenaAG (Analytikjena, Germany) and undertaken on the KAPA SYBER FAST ONE-STEP qPCR kits (Kapa Biosystems, USA) using ethidium bromide dye, fluorescent dye. A volume of 20 µl of a total reaction mixture was assembled by the addition of Luna Universal One-Step Reaction Mix (10µl), each primer (0.8µl), 1µl of Luna WarmStart® RT Enzyme Mix (Reverse transcriptase), RNA template (variable) complete with nuclease free water. A melting curve was generated with temperatures ranging from 60°C to 95°C; each 15 seconds, elevating by 0.5°C every 15 seconds. Gene threshold cycle(Ct) was normalized against the *Fbp* gene Ct amplified from the corresponding sample. Calculation of fold change was according to the $2^{-\Delta\Delta Ct}$ method (Livak & Schmittgen 2001).

**Table 2** Primers used in the present study.

| Target gene | Primer sequences (5´-3´) | Reference |
|---|---|---|
| *phzM* | F- GGCCTTCGAGATCTTCCAGG | Primer was designed |
| | R- GAACTCCTCGCCGTAGAACA | |
| *Fbp* (HKG) | F-CCTACCTGTTGGTCTTCGACCCG | Anderson *et al*. (2008). |
| | R- GCTGATGTTGTCGTGGGTGAGG | |

### *Statistical analysis*

The data were investigated and charted by Graph Prism version 10. The proportion and their frequencies were checked by applying the Chi-square test to investigate significant comparisons between percentages of bacterial isolates. A one-sample T-test was conducted to calculate the value significance, mean and standard error of MIC and fold rate for gene expression. Results are considered significant when *p*-values ≤ 0.05.

## Results

### *Biofilm Formation*

The results revealed that out of thirty isolates, seven (23.33%) isolates were strong biofilm producers, thirteen (43.33%) isolates were moderate and ten (33.33%) isolates were weak biofilm former. Tested strains differed in their capabilities to produce biofilm ranging from an $OD_{630}$ of 0.083 to an $OD_{630}$ of 0.393. In the case of biofilm degree estimation, microplate reader was used to determine the absorbancy at 630nm. Figure 1 illustrates the distribution of *P. aeruginosa* isolates stratified by biofilm formation with a calculated probability for the Chi-Square test.

### *Detection of the phzM gene.*

Depending on the results of biofilm formation, only strong biofilm producers (7 isolates) were examined for the existence of the *phzM* gene via using the conventional PCR technique. By virtue of the present findings, All 7 (100%) isolates harbored this gene with its 120bp in their genomic material as appeared in figure 2.

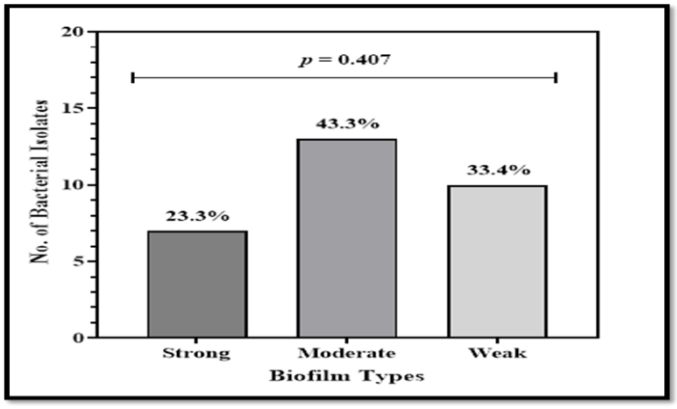


**Fig 1.** Distribution of *P. aeruginosa* isolates stratified the biofilm former; *p*: probability for Chi-Square test.

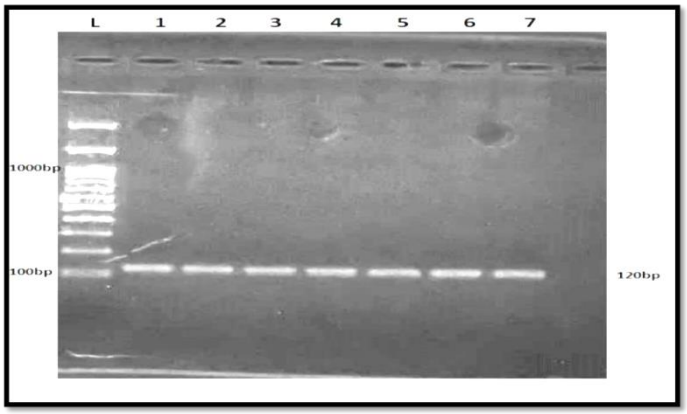


**Fig 2.** Agarose gel (1%) analysis of the *phzM* gene in *P. aeruginosa* isolates, run at 5 V/cm for 1 h, stained with ethidium bromide and visualized under UV. Lane M: 100 bp DNA ladder; lanes 1–7: DNA from different isolates.

***Estimation of minimal inhibitory concentration of imipenem***

Results exhibited that the MIC values ranged between 256 to 0.5 µg/ml. Four isolates out of seven were selected for testing of the *phzM* gene expression according to low, middle and high MIC values. P18 has the lowest MIC that was 0.5 µg/ml, P19 and P26 had an MIC of 128 µg/ml, and P20 had the highest one in MIC that reached 256µg/ml.

***Imipenem stress against strong-biofilm P. aeruginosa isolates***

Imipenem stress was tested against strong-biofilm *P. aeruginosa* isolates in 96‑well flat bottoms polystyrene microtiter plates. The results obtained showed that 5 isolates (P18, P19, P20, P23, P26) out of 7 lost their ability to adhere on the polystyrene microtiter plate, while the remaining isolates P21 and P30 showed weak biofilm formation under imipenem sub-MIC. Results showed a highly significant decline in the formation of biofilm after imipenem addition ($p$<0.0001). Effect of imipenem stress on strong-biofilm former can be revealed in Table 3 and Figure 3.

**Table 3** Minimum inhibitory concentration (MIC) of imipenem against strong-biofilm producers' taxa of *P. aeruginosa*.

| Isolate no. | MIC (µg/ml) | Sub-MIC (µg/ml) |
|---|---|---|
| P18 | 0.5 | 0.25 |
| P19 | 128 | 64 |
| P20 | 256 | 128 |
| P21 | 128 | 64 |
| P23 | 256 | 128 |
| P26 | 128 | 64 |
| P30 | 64 | 32 |
| T-test; p-value | T= 3.88; p = 0.008 ** | |

To detect the effect of imipenem on *phzM* expressiom in the tested isolates, it was added at a sub-MIC to the overnight cultures. Results approved that imipenem addition at sub-MIC resulted in upregulation of *phzm* gene in inclusive tested isolates ranging from 0.54 to 5.71. Thence, and in apart due to the imipenem antibacterial activity, these results showed that this carbapenem antibiotic increased the gene expression of *phzM* by 7.889, 3.138, 32.67 and 32.44-fold in isolates P18, P19, P20 and P26 respectively. Results can be summarized in Table 4. the differences between the values of fold change of the tested isolates were statistically nonsignificant. The mean of fold change is 19.03 and and the standard deviation is 15.73.

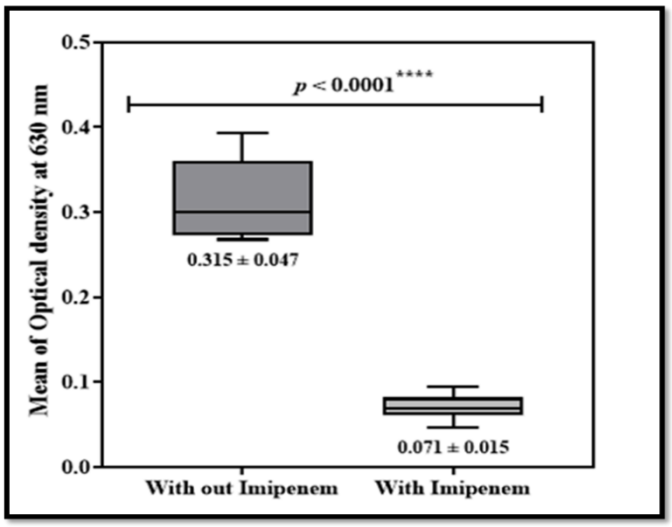


**Fig 3.** Effect of imipenem stress on strong-biofilm former *P. aeruginosa* isolates(with imipenem) and results of the optical density without imipenem; $p$: due to probability for T-test.

**Discussion**

Biofilm in persistent infections is thought to be a major clinical challenge. The biofilm production can be detected phenotypically and several methods are available for its detection; however, the microtiter plate method is the most accurate assay (Vestweber et al. 2024; Atshan et al. 2012; Gad et al. 2009). Therefore, in this study, the potential of the chosen isolates to produce biofilm was assessed by using microtiter plates. Results revealed that from thirty isolates, seven (23.33%) isolates were strong biofilm producers, thirteen (43.33%) isolates were moderate and ten (33.33%) isolates revealed weak biofilm former. A wide range of isolates capabilities to create biofilm was exhibited ranging from $OD_{630}$ 0.083 to $OD_{630}$ 0.393. Eventhough current results showed that the moderate biofilm former isolates showed the highest percentage (43.3%) and the strong biofilm former isolates showed the lowest percentage (23.3%) between the total isolates (as in figure 1), but there was no significant differences between strong, moderate and weak biofilm former isolates ($p$ = 0.407).

Establishment of biofilm is a guard mechanism of bacteria that shield it from defenses of the host and achieves resistance to standard antibiotic therapy (Croes et al. 2009). Therefore, developing an effective pharmaceutical material that have the ability to promote the treatment of biofilm bacterial infections became an urgent need. Nowadays, some nanoparticles can facilitate the curing of some these infections. A local study found that the biofilm inhibition percentage was 80% towards *P. aeruginosa* by using nanoparticles of chitosan and

**Table 4** Fold change of phzM gene in imipenem stress existance that was recorded for each isolate.

| Bacterial isolates | ΔCt Without imipenem | ΔCt With imipenem | ΔΔCt | Fold change ($2^{-\Delta\Delta Ct}$) |
|---|---|---|---|---|
| P18 | 8.69 | 5.71 | -2.98 | 7.889 |
| P19 | 6.53 | 4.88 | -1.65 | 3.138 |
| P20 | 5.57 | 0.54 | -5.03 | 32.672 |
| P26 | 8.14 | 3.12 | -5.02 | 32.446 |
| Statistical analysis | | | | p = 0.094, NS |

palladium (CH/Pd NPs) (Hamid et al. 2024). Recently, a study revealed that bacteriocin had an antibiofilm capability (ALattar et al. 2024).

By virtue of the present findings, all the stronger biofilm formating (100%) isolates harbored *phzM* gene with its 120bp in their genomic material. This can be explained by the possibility that there could be a correlation between the biofilm production propensity of these isolates according to biofilm responsible genes and the pyocyanin production genes, as both phenomena are considered to be virulence factors. They are compatible to those of a contemporary study which detected *phzM* in all collected strains (Wang et al. 2023). In contrast, a previous study found that 4.1% of *P. aeruginosa* lacked bands of this gene (Nowroozi et al. 2012).

The results manifested that the range of MIC was 256 to 0.5 µg/ml. P18 had the lowest MIC value which was 0.5 µg/ml, P19 and P26 appeared MIC of 128 µg/ml, while P20 was the highest one in MIC value that reached to 256µg/ml. Although, strong biofilm former showed the lower percentage between the chosen isolates, neverthelss, a highly significant difference was recorded among them for imipenem sensitivity (p = 0.008). Results showed that the mean of MIC for these seven isolates was (137.2143), Std. Deviation was (93.56453). Carbapenems, such as imipenem, are cosidered to be the most effective anti-pseudomonal therapies. In patients with multi-β-lactam-resistant *Pseudomonas* infections, these carbapenems are often used as a last resort (Kazeminezhad et al. 2017). According to current results, isolates showed variable susceptibility to imipeneme, whereas all *P. aeruginosa* isolates were 100% sensitive for imipenem as was reported by Yaseen and Ahmed (2023) (Yaseen & Ahmed 2023).

The results obtained showed that 5 (71.42%) isolates (P18, P19, P20, P23, P26) out of 7 isolates, suffer the loss of capacity to form biofilm on polystyrene microtiter plates. In comparison, the remaining P21 and P30 (28.57%) isolates exhibited weak biofilm formation under imipenem sub-MIC after all of them were strong biofilm producers without the antibiotic stress. An expected explanation for this result is that imipenem antibiotic has a high efficacy in preventing the adhesion of bacterial cells on surfaces which is in agreement with a previous study that found imipenem and pexiganan in pretreatment was heavily influenced adherence and formation of biofilm *in vitro* (Cirioni et al. 2013). On another side, inhibition of the adhesins that aid bacteria to adhere to the surfaces might be occurred (Cozen & Read 2012). In another local study, it was found that nearly 30% of *P. aeruginosa* isolates were imipenem resistants (Jaddoa et al. 2024). Results showed a highly significant decrease in biofilm formation after imipenem addition ($p$<0.0001) as evident in Figure 3.

The present results revealed that high efficacy of imipenem stress on *P. aeruginosa*. An expected explanation of the *phzM* upregulation in all tested isolates that were treated with their sub-MIC values is that the effectiveness of imipenm (eventhough in very low concentration) led to heightened recruitment of defence mechanisms by upgrades virulence genes which pyocyanin production *phzM* gene is one of them. *P. aeruginosa* exhibits this behavior in order to overcome the stressful conditions that affect its persistance in presence of antibiotic stress. Couce and Blazquez (2009) demonstrated that at low doses of antibiotics, genetic variations could be induced, and alteration of virulence genes expression may occurred. On the other hand, it was suggested that continuous exposure of antibiotics can facilitate mutations, that can impact expression of genes in regulatory regions (Couce & Blazquez 2009). Davies *et al*. (2006) found that many antibiotic sub-MIC can interfere with bacterial physiology, including virulence modification, morphology and genome stability that can results in genetic variations (Davies et al. 2006). Kumar *et al*. (2021) noted that alternative pathways can be affected by antimicrobial activity (Kumar et al. 2021). The present results are disagreed with a recent local studies that found that *phzM* expression was down-regulated after treatment with gentamicin and iron oxide nanoparticles (Karlowsky et al. 2023), in addition to another study that revealed a down regulation of the virulent factor gene *phzM* when treated with neem oil and gentamicin in combination (Ahmed & Abdul Muhsin 2024).

It was found that the current results are in accordance with that had been achieved by Younis and Faisal (2024), which revealed that cefotaxime, ampicillin or amoxiclav caused upregulation of pyocyanin producing genes related to multiple operons thereby elevating pyocyanin production (Younis & Faisal 2024) They also mentioned that Overexpression occurred in cefotaxime treatment with fold change 340.14 for *phzM*, and 280.13 for *phzS* genes (Younis & Faisal 2024). Both genes are shrouded the operon of phzA1, qscR is shrouded the phzA2 operon, that encoding orphan transcription in addition to hypothetical protein (Ahmed & Alhammer 2024). The present results agree with a previous study that observed high expression of phzS, *phzA1* and *phzM* was detected, while *phzH* revealed lower expression. Pyocyanin genes expression differences is probably because of their presence on different operons, thereby responding differently (Couce & Blazquez 2009). The present results showed that high efficacy of imipenem on *P. aeruginosa* even in very low concentrations are incompatible to a recent study found that susceptibility to imipenem was 56.5% of isolates were imipenem-susceptible (Cui et al. 2016). In our opinion, this work is the first one to determine the efficacy of imipenem sub-MIC on *phzm* pyocyanin production encoding gene. Current results suggest studying the imipenem sub-MIC effect on remaining pyocyanin production- encoding genes in additon to another *P. aeruginosa* and other bacterial genus virulence genes.

## Conclusion

Treatment of *P. aeruginosa* related infections must be taken in consideration because imipenem stress induces *phzM* gene which is one of the genes that encodes pyocyanin production, as the use of antibiotics may lead to up-regulation of virulence factors and thus assist in pathogen shedding, in particular, when they are used in concentrations below a lethal dose.

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## Ethical approval

Not applicable

## Conflict of Interest

All authors declare that they have no conflict of interest.

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