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Emerging Efflux Pump-Mediated Cefiderocol Resistance in Pseudomonas aeruginosa Clinical Isolates from Nasiriyah, Iraq A Molecular and Phenotypic Study

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ABSTRACT

Background: The thalassemia syndromes are a group of inherent hemolytic anemias characterized by the decrease or absence of the synthesis of one or more globin chains of hemoglobin. Elevated levels of NOD-like receptor protein 3 (NLRP3) and ferritin, along with oxidative stress markers such as malondialdehyde (MDA) and catalase activity, have been linked to thalassemia disease progression and prognosis. **Aim:** This study aims to investigate the relationship between serum levels of NLRP3, ferritin, MDA, and catalase activity in beta-thalassemia patients compared to healthy controls, providing insights into potential diagnostic and prognostic biomarkers. **Materials and Methods:** (75) patients with beta-thalassemia major aged 6-18 years who were selected at the Al-Zahra Teaching Hospital/ALNAJAF AL ASHRAF CITY along with (45) normal people of the same age without any history of hematological diseases, chronic diseases, acute illness, or infection. Patients who met the selection criteria were included in the study. All subjects were enrolled in this study between November 2024 and January 2025; Serum levels of NLRP3, ferritin, MDA, and Catalase were analyzed using enzyme-linked immunosorbent assay (ELISA) and spectrophotometric methods. For data collection and statistical analysis, Microsoft Office Excel 2013 and GraphPad Prism 9.2.0 were utilized. **Results:** The study found elevated levels of NLRP3 in thalassemia patients (7.006 ± 1.014) compared to the healthy group (1.35 ± 0.2868), with a P value of 0.0003. Ferritin levels were also higher in patients (1576 ± 85.09 ng/ml) compared to the healthy group (73.1 ± 13.67 ng/ml), with a P value of <0.0001. MDA levels increased in patients (0.157 ± 0.01964) compared to the healthy group (0.06813 ± 0.0149), with a highly significant P value of 0.0007. In contrast, catalase activity was significantly lower in the patient group (0.5321 ± 0.07339) than in the healthy group (0.8746 ± 0.1104), with a P value of 0.0129. These findings suggest that an increase in NLRP3, ferritin, and MDA levels and a decrease in catalase activity are associated with an increased risk of thalassemia disease. **Conclusion**: Elevated NLRP3, ferritin, and MDA levels, alongside reduced catalase activity, are associated with thalassemia disease progression. These biomarkers could serve as valuable tools for early detection and monitoring therapeutic responses in thalassemia patients.

Keywords: *Pseudomonas aeruginosa*; cefiderocol; efflux pump; resistance; Iraq.

1. Introduction

Pseudomonas aeruginosa is Gram-negative bacterium that has converted one of main causes of hospital-acquired infections worldwide [1]. It is commonly gained from patients with burn, urinary tract infections, pneumonia, and bloodstream infections, especially individuals with weakened immune systems [2,3]. One of the most characteristic features of P. aeruginosa is outstanding capability to avoid

multiple mechanisms of antimicrobial agents. This organism has a large and adaptable genome, efficient regulatory systems, and many inherent and acquired resistance apparatuses that allow it to continue in hospital environments and significantly restrict available treatment routes [4].

In recent years, reports of multidrug-resistant (MDR) as well as extensively drug-resistant (XDR) P. aeruginosa strains has increased dramatically, posing a significant global health threat. Recognizing the severity of this problem, World Health Organization has recorded carbapenem-resistant P. aeruginosa such a dangerous bacterium that urgently requires new treatment strategy [5,6]. Cefiderocol, Cephalosporin siderophores was recently established to direct the growing problem of carbapenem-resistant Gram-negative infections [7].

Interestingly, this antibiotic has a unique mode of action: it uses a "Trojan horse" style by mimicking natural siderophores and utilize bacterial iron uptake systems to cross the outer membrane. Once internal, cefiderocol binds to penicillin-binding proteins and inhibits the synthesis of cell wall, ultimately leading to bacterial death [8]. This mechanism binds to the classical activity of a β-lactam antibiotic with iron-mediated transport that was expected to overcome many of the resistance barriers typically found in Gram-negative pathogens. Initial clinical studies and surveillance data supported this expectation, showing high efficacy of cefiderocol against MDR P. aeruginosa, such as isolate resistant to carbapenems and colistin [9,10].

Despite early success of cefiderocol, resistance to it has already been documented in different parts of the world. Multiple mechanisms have been proposed, such as mutations in siderophore receptor genes, altered β-lactamase expression, and enhanced efflux pump activity [11]. Among these, efflux systems are considered one of the most powerful resistance strategies in P. aeruginosa because they aggressively expel antibiotics from the cell, lower intracellular drug concentrations [12]. The MexAB-OprM efflux pump plays a major task in resistance to several antibiotic classes, like β-lactams, fluoroquinolones, and aminoglycosides. Overexpression of this system or mutations in its regulatory genes (mexR, nalC, and nalD) could reduce sensitivity to cefiderocol [14,15].

Investigations to explore the relationship between efflux pumps and cefiderocol resistance remain limited. Most existing studies start from Europe, East Asia, and North America, leaving significant regional knowledge gaps—especially in the Middle East [8,16]. In Iraq, antimicrobic resistance have raised challenge due to extensive misuse of antibiotics. Also, insufficient infections control policies, and limited availability for novel agents. While cefiderocol recently present in certain medical centers, little is known about level of resistance among local P. aeruginosa isolate or the molecular strategies that can aid to reduced sensitivity.

Understanding this process is necessary for controlling effective infection and antibiotic stewardship efforts. Identifying specific resistance factors such as efflux pump overactivity can help refine therapeutic approaches and delay the spread of resistance to this valuable last-line antibiotic.

For these reasons, the current work was conducted to examine cefiderocol resistance in clinical P. aeruginosa samples that obtained from Nasiriyah General hospital in Iraq. By merging phenotypic susceptibility testing with molecular detection and sequencing of efflux pump genes and their regulators, this work provides the first molecular evidence from the region regarding the role of efflux pumps in cefiderocol resistance.

2. Materials and Methods

2.1. Bacterial isolates

A total of 125 non-duplicate clinical isolates of *P. aeruginosa* were obtained from patients known to various wards of the General Hospital in Nasiriyah, Iraq, between January and August 2025. The isolates were collected from several clinical specimens, including urine, sputum, wound swabs, and blood cultures. Initial identification was performed using standard microbiological techniques, including Gram staining, observation of colony morphology, and routine biochemical tests. For further characterization, the isolates were inoculated on Tryptic Soy Agar (TSA), MacConkey agar, and blood agar to examine growth characteristics, lactose fermentation, and hemolytic activity, respectively as shown in **Figure 1.** Species confirmation was confirmed by VITEK 2 automated identification system (bioMérieux, France).

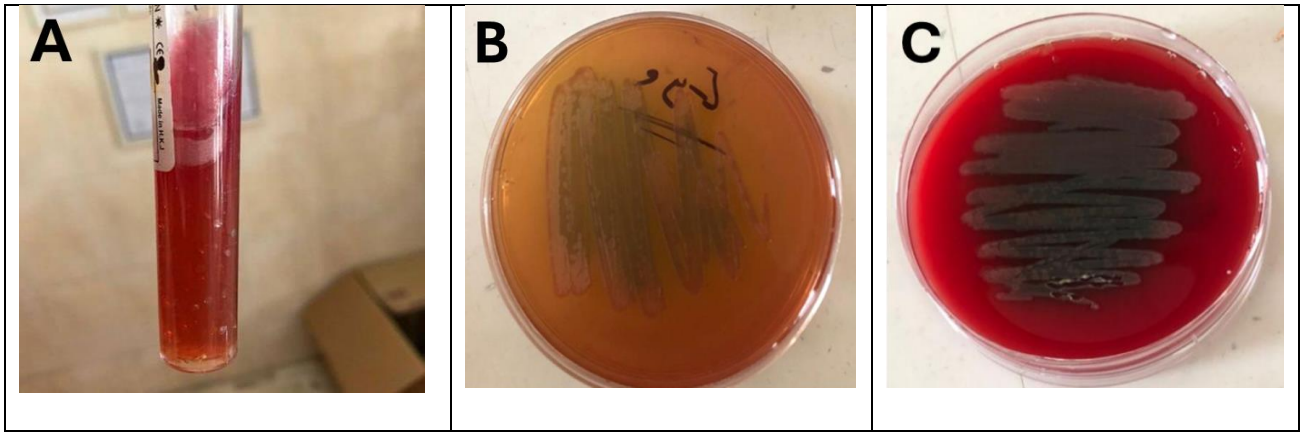

Figure 1. Colony morphology of P. aeruginosa on different culture media.

Representative isolates of P. aeruginosa showing:

(A) Tryptic Soy Agar (TSA) — smooth, pigmented colonies

(B) MacConkey agar — non-lactose fermenting colonies appearing colorless

(C) Blood agar — β-hemolytic.

2.2. Antimicrobial susceptibility testing

Minimum inhibitory concentrations (MICs) of cefiderocol and selected comparator antibiotics was detected using the broth microdilution method depending on the guidelines of the Clinical and Laboratory Standards Institute [17]. Iron-depleted cation-adjusted Mueller–Hinton broth (ID-CAMHB; Thermo Fisher Scientific, USA) was used for cefiderocol testing, as recommended. Pseudomonas aeruginosa ATCC 27853 (American Type Culture Collection, Manassas, VA, USA) was employed as the quality control strain. Interpretation of results was carried out following CLSI breakpoints.

2.3. Efflux pump inhibition assay

To estimate the importance of efflux pumps in cefiderocol resistance, MIC testing was repeated in the existence of the efflux pump inhibitor phenyl-arginine-β-naphthylamide (PAβN; Sigma-Aldrich, USA) at a final concentration of 25 μg/mL. This concentration was selected based on previous studies demonstrating its effectiveness in inhibiting resistance-nodulation-division (RND) efflux pumps in P.

aeruginosa without affecting bacterial growth [18,19]. A decrease of fourfold or greater in the MIC in the presence of the inhibitor was considered indicative of efflux-mediated resistance, as described in earlier reports [20]. All tests were performed in triplicate, and control wells without the inhibitor were included to ensure result accuracy.

2.4. DNA extraction and PCR amplification

Genomic DNA was extracted from overnight cultures of Pseudomonas aeruginosa utilizing a commercial DNA extraction kit (Qiagen, Germany) after the manufacturer's instructions. Polymerase chain reaction (PCR) assays were carried out to detect efflux pump genes (mexA, mexB, oprM) and their regulatory genes (mexR, nalC, nalD). Primers were designed based on previously published sequences [21–23] and synthesized by Macrogen (Korea) as shown in Table 1. PCR amplifications were performed in a 25 µL reaction mixture containing 12.5 µL of 2× PCR Master Mix (Promega, USA), 1 µL of each primer (10 µM), 2 µL of DNA template, and 8.5 µL of nuclease-free water. The amplification protocol included an initial denaturation at 95°C for 5 min, followed by 35 cycles of denaturation at 95°C for 30 s, annealing at 55–60°C for 30 s, and extension at 72°C for 1 min, with a final extension at 72°C for 7 min. PCR products on 1.5% agarose gels, stain them with ethidium bromide (0.5 µg/mL), and visualized under ultraviolet light using a gel documentation system (Bio-Rad, USA).

Table 1. Primers used for amplification of efflux pump and regulatory genes in P. aeruginosa

Gene	Primer sequence (5′–3′)	Product size (bp)	Reference
mexA	F: CGACATCGTCACGTTCAAGTR: CAGGTAGCCGACCTTGATGA	880 bp	[21]
mexB	F: GGTTTCGGCGATGATGTTCTR: GCTGGCTGAGGATGATGTTG	450 bp	[21]
oprM	F: TCGCTGCTGTCGATGATGTTR: CGTAGTAGCGGTTGGTGATG	330 bp	[21]
mexR	F: ATGGCTGAAGGCGTTCTGTAR: CGTCTCGATGTTGATGGTGA	420 bp	[22]
nalC	F: TCGACCGACCTGATGAACATR: CGGCTTGGTGTTGTTGTTGT	320 bp	[22]
nalD	F: GCGACATCGAAGGCGATTTR: TGCTTCCAGGTCGATGATGA	310 bp	[23]

2.5. Sequencing and analysis

Representative PCR products were purified using a commercial PCR purification kit (Qiagen, Germany) and sequenced with an automated sequencer (Applied Biosystems, USA). The acquired sequences were aligned and compared against reference sequences in the National Center for Biotechnology Information (NCBI) database to identify mutations associated with efflux pump overexpression. Sequence analysis and multiple alignments were performed using MEGA version 11 software to detect nucleotide changes and predict potential functional impacts.

2.6. Statistical analysis

GraphPad Prism version 10 (GraphPad Software, San Diego, CA, USA) Was used to look at the data we obtained. Associations between cefiderocol resistance, efflux pump activity, and gene mutations were confirmed using either the chi-square test or Fisher's exact test, depending on the data distribution. Differences were measured statistically significantly at a p-value of less than 0.05.

3. Results

3.1. Distribution of clinical isolates

There were 125 distinct *P. aeruginosa* isolates gained from patients admitted to the General Hospital in Nasiriyah, Iraq, between January and August 2025. The isolations were recovered from urine (42%), wound swabs (28%), sputum (18%), and blood cultures (12%) **Table 2.**

Table 2. The quantity and proportion of isolates of P. aeruginosa from various clinical specimens at the General Hospital, Nasiriyah, Iraq (January–August 2025).

Specimen type	Number of isolates (n)	Percentage (%)
Urine	53	42
Wound swabs	35	28
Sputum	23	18
Blood cultures	14	12
Total	**125**	**100**

3.2. Cefiderocol Susceptibility

Antimicrobial susceptibility testing showed that 18 out of 125 isolates (14.4%) exhibited reduced susceptibility to cefiderocol. With a median MIC of 1 μg/mL, the minimum inhibitory concentration (MIC) values varied from 0.25 to 8 μg/mL **Table 3**.

Table 3: Cefiderocol susceptibility of P. aeruginosa clinical isolates (n = 125) from the General Hospital, Nasiriyah, Iraq

MIC (μg/mL)	Number of isolates (n)	Percentage (%)
0.25	40	32
0.5	37	29.6
1	30	24
2	10	8
4	5	4
8	3	2.4
Total	**125**	**100**

3.3. Efflux Pump Activity

The efflux pump inhibitor PAβN was used to assess the role of efflux pumps in cefiderocol resistance. In the existance of the inhibitor, a fourfold or greater reduction in MIC was observed in 12 of the 18 resistant isolates (66.7%) as shown in **Figure 2**, indicating that efflux pump activity plays a significant role

in reducing cefiderocol susceptibility.

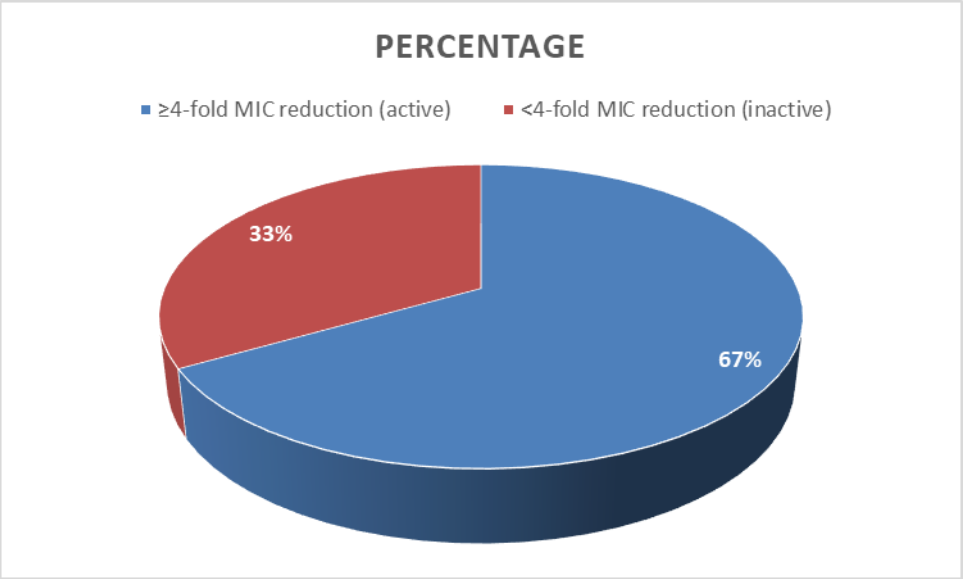

Figure 2. Efflux pump activity in cefiderocol-resistant P. aeruginosa isolates.

The pie chart shows the percentage of isolates demonstrating ≥4-fold reduction in MIC in the presence of the efflux pump inhibitor PAβN (active efflux) versus isolates showing <4-fold reduction (inactive efflux).

3.4. Efflux Pump Gene Detection and Regulatory Mutations

Polymerase chain reaction (PCR) assays confirmed that all 125 P. aeruginosa isolates carried the core efflux pump genes mexA, mexB, and oprM, as seen in Figure 3. Further analysis of the regulatory genes revealed mutations in mexR, nalC, or nalD in 10 of the 18 cefiderocol-resistant isolates (55.6%). Sequencing selected PCR products identified specific nucleotide changes that are known to result in overexpression of the mexAB-oprM efflux system. These results suggest that mutations in regulatory genes increase efflux activity and lower cefiderocol susceptibility in certain isolates. Additional MIC testing can help confirm this link by comparing cefiderocol response between isolates with and without these mutations, as shown in Figure 4.

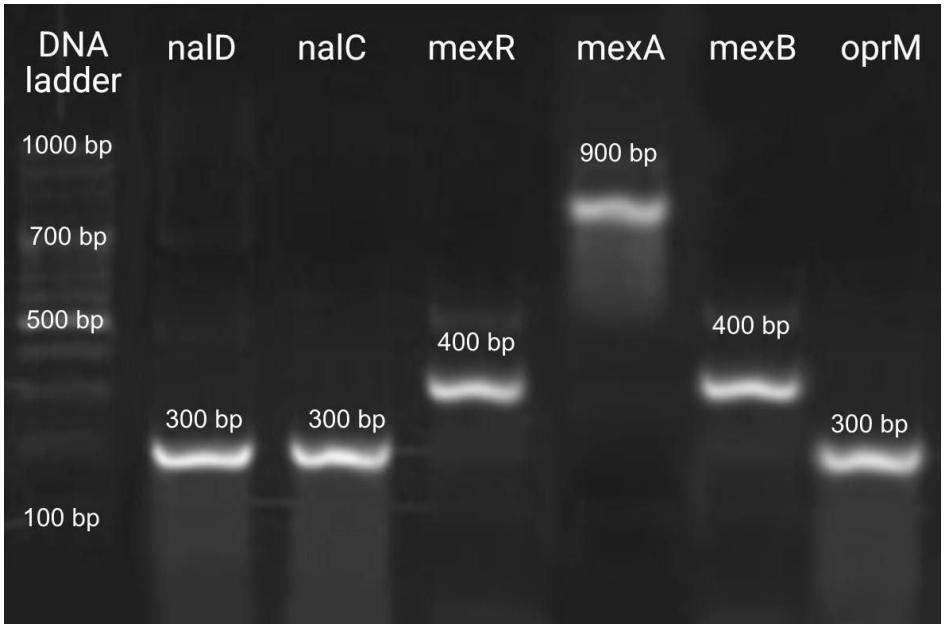

Figure 3. PCR amplification of efflux pump genes and their regulators in *P. aeruginosa*.

Lane "DNA ladder" shows the molecular weight marker. Lanes "nalD," "nalC," "mexR," "mexA," "mexB," and "oprM" represent PCR products of the corresponding efflux pump regulatory and structural genes. The observed amplicons appear at their expected molecular weights: nalD and nalC (~300 bp), mexR (~400 bp), mexA (~900 bp), mexB (~400 bp), and oprM (~300 bp).

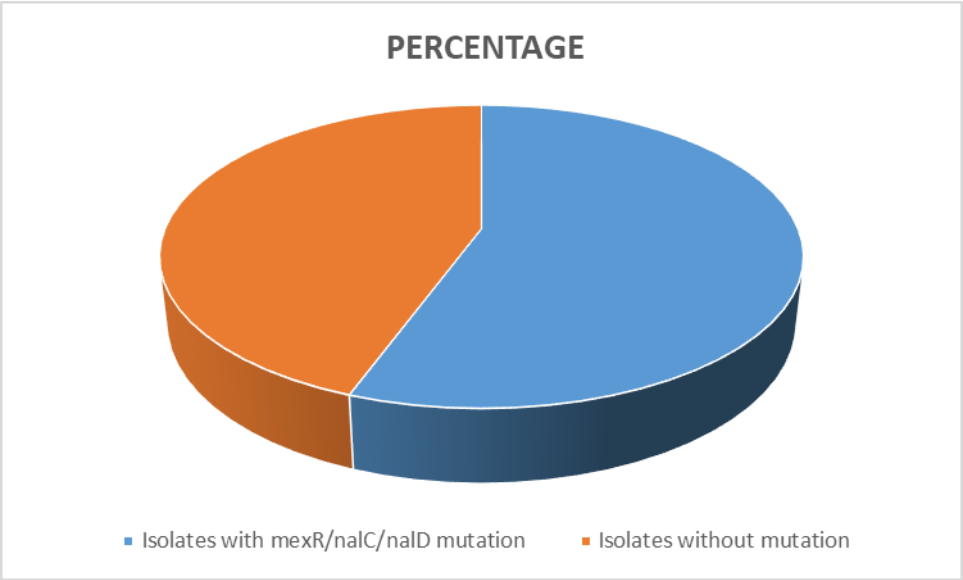

Figure 4. Comparison percentage of cefiderocol susceptibility between P. aeruginosa isolates with and without efflux regulatory gene mutations.

3.5. Correlation Between Efflux and Cefiderocol Resistance

Among the 18 cefiderocol-resistant isolates, mutations were identified in the efflux pump regulatory genes: six isolates carried mutations in mexR, three in nalC, one in nalD, and two isolates carried mutations in both mexR and nalC. Statistical analysis showed that isolates carrying any of these

regulatory gene mutations had significantly higher MIC values compared to cefiderocol ($p < 0.05$), as shown in Table 4. Isolates that also exhibited a reduced efflux-mediated MIC in the presence of PAβN exhibited the highest levelof resistance, suggesting a combined influence of genetic and phenotypic mechanism on antibiotic susceptibility, as shown in **Figure 5**.

Table 4. Relationship between efflux regulatory gene mutations and cefiderocol MIC levels in P. aeruginosa isolates

Regulatory Gene Mutation	Number of Isolates	MIC Trend to Cefiderocol	Notes
mexR	6	Significantly higher MIC	Some showed efflux inhibition effect
nalC	3	Significantly higher MIC	Some showed efflux inhibition effect
nalD	1	Significantly higher MIC	—
mexR + nalC	2	Highest MIC values	Showed reduced MIC in presence of PAβN
No mutation	6	Lower MIC	Baseline control

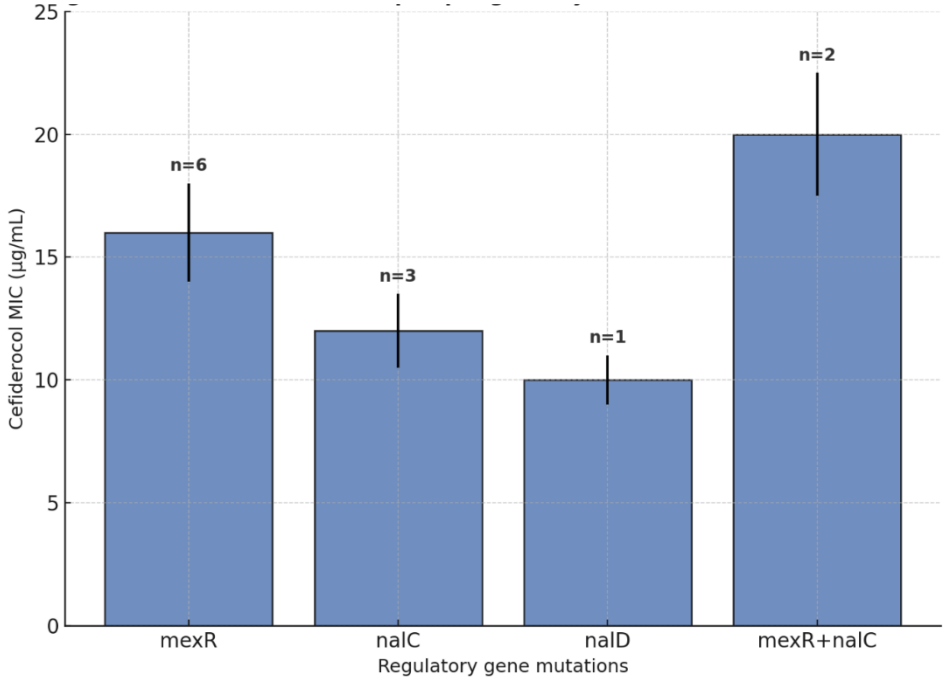

Figure 5. Distribution of efflux pump regulatory gene mutations and their association with cefiderocol resistance in P. aeruginosa isolates.

The figure illustrates the mean cefiderocol MIC values observed in resistant isolates carrying mutations in mexR, nalC, nalD, or combined mexR + nalC. Indicating the standard error of the mean (SEM) are error bars. The quantity of isolated (n) for each group is shown above the bars.

4. Discussion

This study investigated the occurrence of cefiderocol resistance among clinical isolate of P. aeruginosa from patient in Nasiriyah, Iraq. While most isolates remained susceptible, a significant proportion (14.4%) showed decreased susceptibility to the drug. This finding is concerning, as cefiderocol one of the most promising therapeutic options for treating multidrug-resistant Gram-negative bacterial infections, including carbapenem-resistant P. aeruginosa [24]. The detection of resistant isolates within a relatively short observation period suggests that resistance may develop more rapidly than expected. Efflux pump activity appears to play major role in this decreased sensitivity [25]. When isolate was tested in the presence of the efflux inhibitor phenylalanine arginine beta-naphthylamide (PAβN), two-thirds of cefiderocol-resistant strains show a fourfold or greater decrease in minimum inhibitory limit (MIC) value, confirming the involvement of active efflux. This observation is consistent with previous studies linking reduced cefiderocol and beta-lactam efficacy to upregulation of the mexAB-oprM efflux system [26]. In this work, more than half of the resistant isolates involved mutations in the regulatory genes mexR, nalC, and nalD. These mutations have been proven to affect with the normal suppression of efflux operons. Thus, it can lead to their overexpression and increased drug export [27,28]. One isolate had a regulatory mutation and showed a clear PAβN-dependent reduction in MIC, however it also displayed the strongest resistance. This relationship suggests that both gene alteration and active efflux may interact to give the bacterium a survival benefit once exposed to cefiderocol [15]. Similar observations have been recorded in Europe and Japan, where P. aeruginosa strains overexpressing mexAB-oprM exhibited higher cefiderocol MICs even without β-lactamase production [29]. These findings indicate that efflux-based resistance can function alone or alongside other mechanisms such as reduced membrane permeability or increased β-lactamase activity. Clinically, this situation raises concern. Wider use of cefiderocol could gradually lose effectiveness if efflux-related resistance becomes more common. Continued local surveillance of resistance patterns and their molecular causes is therefore important to guide proper antibiotic use. While efflux pump inhibitors are not yet available for treatment, they may finally facilitate restoring or extending the action of cefiderocol [8,30]. This work had some limits. It involved isolates from a single hospital, so the data may not represent the situation across Iraq. Only a few regulatory genes were analyzed, while other possible factors—such as metallo-β-lactamases or porin modification—were not tested. The expression level of efflux genes was also not measured, which would have provided stronger support for their role [31]. Despite these limits, the study gives an early view of cefiderocol resistance among P. aeruginosa isolates in Nasiriyah. The findings emphasize the need for ongoing molecular observing, cautious antibiotic prescribing. In addition, further research into alternative strategies to control emerging resistance is needed [32].

5. Conclusion

Among P. aeruginosa clinical isolates, a high occurrence of efflux-associated genes (mexA, mexB, and oprM) was detected. Mutations in mexR, nalC, and nalD were significantly associated with higher cefiderocol MIC values, especially in isolates showing efflux inhibition activity. These results underline the importance of regular molecular surveillance for resistance determinants, and the part efflux-mediated mechanisms play in reducing cefiderocol efficacy. To maintain cefiderocol's clinical usefulness against multidrug-resistant *P. aeruginosa*, current monitoring and the formation of innovative therapeutic advances that aim efflux regulation are needed.

6. Limitations of the Study

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Conflict of interest statement: The author announces that there is absence conflicts of interest related to this study.

Funding Statement: The author states that this study was carried out without external financial support from governmental, commercial, or not-for-profit organizations.

Data Availability Statement: All data generated or analyzed during this study are included in this published article. Additional information can be made available from the corresponding author upon reasonable request.

Ethical Approval Statement: This study was conducted using discarded clinical specimens that were collected as part of routine diagnostic procedures. No additional samples were taken from patients for research purposes. All data were anonymized previously to ensure confidentiality.

Informed Consent Statement: For this investigation, patient permission was not required because only discarded clinical specimens were used. No additional interventions were performed, and all data were anonymized to protect patient confidentiality.

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