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Analysis of oprM gene expression and its role in multidrug resistance of Pseudomonas aeruginosa

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Abstract

Pseudomonas aeruginosa is a significant opportunistic pathogen characterized by high antibiotic resistance, often linked to efflux pump systems. *P. aeruginosa* can acquire antimicrobial resistance via efflux pumps, which are specialized transport proteins that expel diverse substrates from the interior of the bacterial cell to its exterior. This study aimed to investigate the expression of the *oprM* efflux pump gene and its association with antibiotic resistance in clinical isolates of *Pseudomonas aeruginosa*. Out of 160 clinical specimens collected from patients suffering from various infections, 107 isolates (66.87%) were identified as *P. aeruginosa*. Molecular detection confirmed the presence of the *oprM* gene in 96% (24 out of 25) of the selected isolates. Furthermore, gene expression analysis via RT-qPCR demonstrated an increased expression of the *oprM* gene in two extensively drug-resistant (XDR) *P. aeruginosa* isolates following treatment with sub-inhibitory concentrations (sub-MIC) of ceftazidime and ciprofloxacin. These findings highlight the role of the *oprM* efflux pump in the adaptive resistance mechanisms of *P. aeruginosa*. During this study, a total of 160 clinical specimens were collected from patients aged 5–65 years who were suffering from burns, wounds, otitis media, or urinary tract infections. Specimens were obtained from four hospitals in Iraq between 11/9/2025 and 30/12/2025. The isolates were initially identified through microscopic examination and cultural characteristics on selective and differential media, followed by confirmation using the VITEK-II system. The minimum inhibitory concentrations (MIC) of various antibiotics were determined for all *P. aeruginosa* isolates. Detection of the efflux pump gene *oprM* was carried out using PCR amplification with specific primers. Two *P. aeruginosa* isolates that carried the *oprM* gene and exhibited an extensively drug resistant (XDR) profile were selected to assess the effect of highly resistant antibiotics specifically the β-lactam ceftazidime and the fluoroquinolone ciprofloxacin on *oprM* gene expression after determining the sub-MIC values for each antibiotic. The results showed that, out of 160 collected specimens, 107 isolates (66.87%) exhibited characteristic morphological features and biochemical reactions consistent with *Pseudomonas aeruginosa*. Molecular detection of the *oprM* efflux pump gene revealed its presence in 24 out of 25 isolates (96%) obtained from burn, wound, otitis media, and urinary tract infections. Gene expression analysis demonstrated an increased expression of the *oprM* gene in the two XDR *P.*

aeruginosa isolates following treatment with sub-MIC concentrations of the antibiotics ceftazidime and ciprofloxacin.

Keywords: *Pseudomonas aeruginosa*, XDR, efflux pump(*oprM*), CAZ, CIP resistance

Introduction

Pseudomonas aeruginosa is a widely distributed Gram-negative opportunistic pathogen and is considered among the three most common bacterial species associated with a broad range of human infections, It can cause a variety of community-acquired infections[1], including keratitis associated with contaminated contact lenses, ear infections such as otitis externa and chronic suppurative otitis media, as well as skin and soft-tissue infections, diarrhea, and urinary tract infections[2].

The nosocomial pathogen is also responsible for a range of hospital-acquired infections, including burn and postoperative wound infections, bloodstream infections and septicemia, ventilator-associated pneumonia and catheter-associated urinary tract infections[3].The pathogenicity of the bacterium depends on several virulence factors, which are generally classified into three main categories: surface-associated structures, secreted virulence products, and factors involved in cell-to-cell interactions. The surface structures of *P. aeruginosa* including lipopolysaccharides, flagella, type IV pili, phospholipids, and outer membrane protein F play a crucial role in facilitating bacterial adhesion and colonization of host tissues[4].

The extracellular virulence factors including elastase A, elastase B, alkaline protease, exotoxin A, exoenzyme S, and phospholipase C contribute to the disruption of host cell signaling pathways and enhance bacterial invasion, ultimately leading to the development of widespread infections[5]. Bacterial cell-to-cell communication is regulated through a mechanism known as quorum sensing[6]. Infections caused by *P. aeruginosa* have become increasingly difficult to eliminate due to the bacterium ability to develop resistance to multiple classes of antibiotics including (β-lactams, aminoglycosides, and fluoroquinolones). This resistance arises through several mechanisms involving intrinsic, acquired, and adaptive strategies that enable the organism to withstand the effects of most antimicrobial agents[7]. *Pseudomonas aeruginosa* exhibits a high degree of intrinsic resistance to many antimicrobial agents largely due to its limited outer membrane permeability the activity of multiple efflux pump systems and the production of antibiotic-inactivating enzymes such as β-lactamases[8].

Efflux pumps are bacterial transport proteins that function to expel various substrates from the interior of the cell to the external environment[9]. Due to their long standing presence in natural environments bacteria have developed the ability to survive within their ecological niches and protect themselves from harmful substances produced either by competing microbial species or by the host. These substances include antimicrobial compounds, reactive oxygen species (ROS), and toxic byproducts generated during metabolic degradation processes[10]. Efflux pumps are classified into five major superfamilies: (i) the primary active transporters represented by the ATP-binding cassette (ABC) family; (ii) the secondary active transporters including the major facilitator superfamily (MFS); (iii) the multidrug resistance (MDR) subfamily; (iv) the multidrug and toxin extrusion (MATE) family; and (v) the resistance–nodulation–cell division (RND) family[11]. Antibiotic extrusion via multidrug efflux systems belonging to the RND superfamily is one of the most prevalent mechanisms of drug resistance in *P. aeruginosa*. The RND multidrug efflux system is composed of three components: the inner cytoplasmic membrane transporter, the periplasmic membrane fusion

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protein (MFP), and the outer membrane protein, which together facilitate the transport of antibiotics from the cytoplasm to the extracellular environment[12]. In *P.aeruginosa* the resistance nodulation cell division family (RND) type included four efflux pump systems which are MexAB- oprM, MexCD-OprJ, MexEF-OprN and MexXY- oprM [13].

Materials and Methods

Study design and sample collection:

A total of 160 clinical samples were collected from patients aged 5–65 years suffering from burns, wounds, middle ear infections, and urinary tract infections at four hospitals in Iraq (Al-Yarmouk Teaching Hospital, Al-Karkh General Hospital, Baghdad Teaching Hospital, and Al-Diwanya Hospital) between September 11, 2025, and December 30, 2025. Samples from burns, wounds, and middle ear infections were obtained using sterile swabs from the infected sites, whereas urine samples were collected in sterile containers from patients with urinary tract infections. Swab samples were initially cultured on nutrient agar and MacConkey agar plates and incubated at 37°C overnight. Subsequent identification involved Gram staining, biochemical testing, and final confirmation using the VITEK-2 system.

Determination of the minimum inhibitory concentration (MIC) for antibiotics:

The minimum inhibitory concentration (MIC) of ceftazidime and ciprofloxacin was determined against two extensively drug-resistant (XDR) *P.aeruginosa* isolates (PA7 and PA28). The broth microdilution method was employed according to the Clinical and Laboratory Standards Institute (CLSI) guidelines (2025). The assay was performed in sterile 96-well microtiter plates. For ciprofloxacin, a stock solution (2000 µg/ ml) was used to prepare a two-fold serial diluton from (1000 µg/ ml to 0.58 µg/ ml) across 12 wells. A similar procedure was repeated for ceftazidime; dilutions prepared starting from a 1.000.000 µg /ml stock solution to achieve a final concentration rang from (500,000-0.24) µg/ ml. Each well was initially filled with 100 µl of sterile Muller Hinton Broth (MHB). Following the dilution process, 100 µl was removed from the 12th well in order to keep 100 µl remaining in the 12th well before inoculation with the bacterial isolates. Each test was repeated three times to ensure reproducible results. To achieve bacterial suspensions of approximately (1.5×10^8 CFU/ml), the isolates were cultured in (MHB) at 37 °C overnight, and the turbidity adjusted to 0.5 standardized using the McFarland standard which corresponds to OD of 0.08-0.13 at a wave length of 600 nm. After normalization, 100 µl of the standardized bacterial suspension was added to every well containing antibiotics. Each plate contained both a positive control Muller Hinton Broth inoculated with bacteria (no antibiotic) and a negative control only Muller Hinton Broth (neither bacteria nor antibiotics). Plates were covered and incubated at 37 °C for 24 hours; the minimum inhibitory concentration (MIC) for each antibiotic was defined as the lowest concentration of the antibiotic that produced no visible bacterial growth, results were interpreted according to CLSI (2025).

Molecular study:

1-DNA Extraction and Purification

Due to practical resource constraints, it was not feasible to perform molecular testing on all 107 positive isolates. Therefore, a representative subset of 25 *P. aeruginosa* isolates that exhibited the most prominent multidrug-resistant (MDR) phenotypes across the different clinical sources was selected for molecular detection of the *oprM* gene. Genomic DNA was obtained according to the manufacturer's directions, from twenty-five isolates using the Wizard Genomic DNA Purification Kit (Promega, USA; Madison) and was subsequently verified for quality and integrity via gel electrophoresis on a 1% agarose gel. Extracted DNA was visualized using Ethidium bromide and UV light , then measured concentration and purity by a Nanodrop spectrophotometer with an indication of high-quality genomic DNA for further molecular investigations obtained through purity ratios ranging from 1.8-2.0. Extracted genomic DNAs were stored at -20°C until used as templates for PCR.

2-PCR Amplification and Molecular Detection

PCR was used to determine the presence of the *oprM* efflux pump gene in 25 *P.aeruginosa* isolates. Specific primers (Table 1) were constructed using Primer3Plus (USA) to amplify this gene from the isolates DNA. The PCR reaction mixture is described in the text, and the conditions were optimized. The PCR results were then examined using gel electrophoresis to confirm amplification.

3-RNA Purification

The sub-inhibitory concentrations (sub-MIC) were determined as exactly one-half (1/2) of the established MIC values for each antibiotic. Consequently, the sub-MIC of ceftazidime was set at 16 µg/ml for both isolates, while the sub-MIC of ciprofloxacin was set at 2 µg/ml for isolate PA7 and 4 µg/ml for isolate PA28. For gene expression induction, the isolates were inoculated into Mueller-Hinton broth containing these specific sub-MIC concentrations and incubated at 37°C for 24 hours (overnight exposure) to trigger adaptive stress responses before the bacterial cells were harvested for total RNA extraction. The RNA was extracted from two XDR *P.aeruginosa* isolates (ps7) and (ps28) after treatment with sub-MICs of CAZ and CIP antibiotics using the TransZol Up Plus RNA kit procedure and the purity and concentration of total RNA extracted for both two treated isolates and for control were evaluated by using a nanodrop spectrophotometer that measured the absorbance at the (260/280nm ratio).

4-Synthesis stage of cDNA

The GoScript® Reverse Transcription System was used to create first strand cDNA from up to 500 ng of poly(A) RNA - 5 µg of total RNA.

5-Gene expression analysis

The expression of the efflux pump gene (*oprM*) was determined using quantitative real-time PCR (qRT-PCR) amplification and quantification utilizing cDNA synthesis. The qRT-PCR was carried out using the PerfectStart® Green qPCR SuperMix kit. The relative expression of the *oprM* gene was normalized against the *16S rRNA* housekeeping gene, which served as the internal reference. The specific primers utilized for the amplification were: *16S rRNA* (F:5'-CTGGCCTTGACATGCTGAGA-3', R:5'-TCACCGGCAGTCTCCTTAGA-3') yielding a 183 bp product, and *oprM* (F:5'-CCATGAGCCGCCAACTGTC-3', R:5'-CCTGGAACGCCGTCTGGAT-3') yielding a 180 bp product.

6-Setup Reactions and Thermal Cycling Procedures

The qRT-PCR reactions were executed in a final volume of 20 µl per isolate. Each reaction mixture comprised 1 µl of cDNA template, 10 µl of 2× PerfectStart® Green qPCR SuperMix, 0.4 µl of each forward and reverse primer (10 µM), and 8.2 µl of nuclease-free water. The three-step thermal cycling profile was programmed for an initial denaturation at 94°C for 30 seconds, followed by 40 cycles of denaturation at 94°C for 30 seconds, annealing at 60°C for 30 seconds, and extension at 72°C for 30 seconds.

Table (1): Primers for efflux pump genes.

Name of gene	Primer sequence (5´- 3´)	Product size (bp)	References
oprM	F: CCATGAGCCGCCAACTGTC	180	Pourakbari *et al*., 2016
	R: CCTGGAACGCCGTCTGGAT		

Results and Discussion

Out of 160 clinical specimens collected, 107 isolates (66.87%) were definitively identified as *Pseudomonas aeruginosa* based on their distinct morphological and biochemical profiles. Distribution analysis indicated that burn infections had the highest prevalence rate at 32.5%, followed by wound infections (18.75%), urinary tract infections (8.4%), and otitis media (6.87%). Conversely, the remaining 53 isolates (33.12%) consisted of various other pathogenic taxa, including *Serratia marcescens, Klebsiella pneumoniae*, *Escherichia coli*, *Proteus mirabilis*, *Burkholderia pseudomallei*,as shown in figure(1).

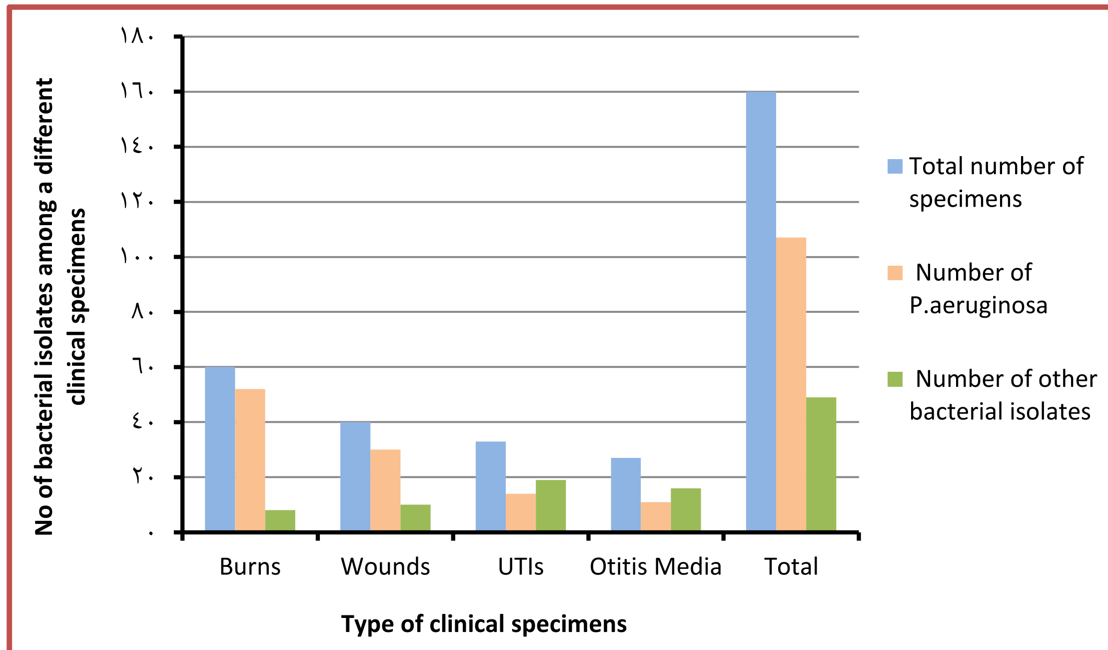
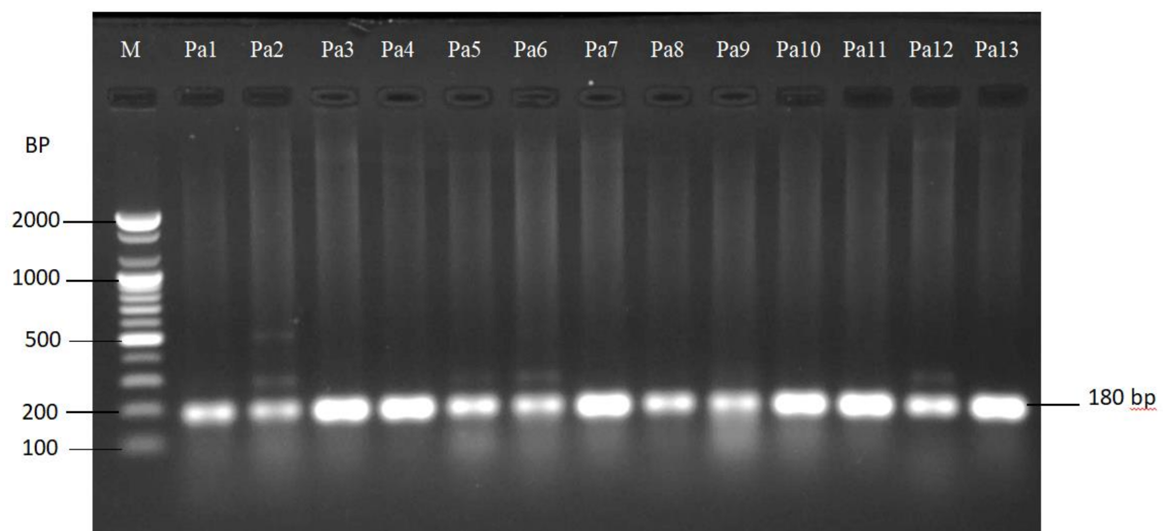


Figure (1): The distribution of bacterial isolates among a diverse clinical cases.

The findings of bacterial isolation in the present study are consistent with those reported in a local investigation conducted by Ibrahim[14]. In that study, 101 clinical specimens were collected from various cases in Baghdad. Among these, 73 isolates (72.28%) were identified as *Pseudomonas aeruginosa*. The distribution of isolates was as follows: 36 (49.31%) were recovered from burn infections, 19 (26.06%) from wound infections, 8 (10.95%) from urinary tract infections, 6 (8.21%) from bloodstream infections, and 4 (5.47%) from cases of otitis media. The remaining isolated bacteria were belong to Proteus mirabilis, *Acinetobacter baumannii*, and *Staphylococcus aureus*. The elevated isolation rate of *Pseudomonas aeruginosa* from burn infections observed in the present study is consistent with the findings reported by Hateet[15]. In that study, 105 bacterial isolates were obtained from burn patients admitted to Misan Hospital. *P. aeruginosa* was identified as the predominant pathogen, accounting for 21 isolates (20%). This was followed by *Staphylococcus aureus* with 17 isolates (16.14%), *Enterobacter spp*. with 17 isolates (16.19%), *Proteus vulgaris* with 14 isolates (13.33%), *Proteus mirabilis* with 11 isolates (10.47%), *Escherichia coli* with 8 isolates (7.6%), *Klebsiella pneumoniae* with 7 isolates (6.6%), and *Staphylococcus lentus* with 5 isolates (4.76%).The higher isolation ratio for *P.aeruginosa* from burns wound infections may be due to destroy the protective skin layer, disrupting the natural skin barrier and depressing the immune responses make the body vulnerable to a wide range of possible infections[16]. Microbial infection following burns, in which a major portion of the skin is injured and an extremely dangerous consequence may be occurred and its frequently a primary reason for a high mortality in these patients[17]. The burn wound surfaces contains a significant quantity of a protein-rich environment that provides a suitable habitat for bacterial colonization and multiplication[18].

The present study demonstrated that two *Pseudomonas aeruginosa* isolates (Ps7 and Ps28) exhibited minimum inhibitory concentration (MIC) values of 32 µg/ml for ceftazidime antibiotic, and these findings were supported by the antibiotic disc diffusion assay, which showed comparable resistance profiles. Furthermore, the MIC values of ciprofloxacin were determined for both isolates, where isolate Ps7 showed MIC of 4 µg/ml, while isolate Ps28 demonstrated a MIC value of 8 µg/ml, reflecting differences in the degree of antimicrobial susceptibility between the tested isolates. The result of MIC was a nearly similar to a study conducted by Alkhulaifi, which revealed that isolates, of *pseudomonas aeruginosa* from burn infections in Basra/ Iraq have MIC values for ceftazidime resistance at a concentration of (32 -64µg/ ml) and ciprofloxacin (16-4) [19]. Also the results match the findings of Bite, who revealed that's the MIC inhibited growth of *P. aeruginosa* at concentrations of (32-4 µg/ ml) for ceftazidime and (32-0.03 µg/ ml) for ciprofloxacin[20] . *Pseudomonas aeruginosa* exhibits resistance to ceftazidime, a β-lactam antibiotic, through a combination of mechanisms. These include various mutations that impact multiple cellular pathways, which together contribute to an increase in the minimum inhibitory concentration (MIC) [21]. Mutants created experimentally had the highest frequency of sequence differences predicted to influence PBP4 function, which is encoded by dacB. Furthermore, dacB mutations are a prominent cause of ceftazidime resistance in *P. aeruginosa* and have a considerable impact on the MIC.[22]. *P. aeruginosa* develops resistance to fluoroquinolones like ciprofloxacin primarily through chromosomal changes in the quinolone resistance-determining regions (QRDR) of the *gyrA*, *gyrB, parC*, and *parE* genes as well as enhanced development of efflux pumps. These modifications lower the antibiotic's intracellular concentration or reduce its capacity to bind to DNA gyrase and topoisomerase IV, which raises MIC values[23]

The present study revealed that 25 *Pseudomonas aeruginosa* isolates were positive for the *oprM* gene, representing a prevalence rate of 96%. The amplification products were verified by comparing their molecular size with a 100 bp DNA ladder and by analyzing the electrophoretic band patterns of the detected genes in the examined isolates. The expected amplicon size for the *oprM* gene was 180 bp. Moreover, the DNA template appeared as a distinct single band under UV light as shown in Figure (2).



Figure(2): Amplification of *oprM* gene of MDR *P.aeruginosa* isolates fractionated on 1% agarose gel electrophoresis stained with Eth.Br. Lane M: 100bp ladder marker. Lane 1-13 resemble 180bp PCR products

Two extensively drug-resistant (XDR) *Pseudomonas aeruginosa* isolates obtained from burn infections and carry the *oprM* gene were selected for gene expression analysis. Reverse transcription quantitative PCR (RT-qPCR) was employed to determine the relative expression level of the target gene in comparison with the housekeeping gene *16S rRNA*, which served as the internal reference gene. Total RNA was first extracted from the selected isolates using the TranZol Up Plus RNA Kit according to the manufacturer's instructions, and the extracted RNA was subsequently reverse-transcribed into complementary DNA (cDNA) for amplification. The concentration and purity of the extracted RNA were assessed using a NanoDrop spectrophotometer, yielding values ranging from 44 to 155 ng/µl. Relative gene expression levels were calculated based on the comparative Ct (ΔΔCt) method, which is widely used for relative quantification in real-time PCR assays[24].

In this study, two extensively drug-resistant (XDR) *Pseudomonas aeruginosa* isolates (PA7 and PA28) were exposed to ciprofloxacin and ceftazidime following determination of the minimum inhibitory concentrations (MICs), and sub-MIC levels were subsequently selected for each isolate. The sub-MIC of ciprofloxacin was established at 4 µg/ml for isolate PA28 and 2 µg/ml for isolate PA7. In contrast, ceftazidime demonstrated a uniform sub-MIC value of 16 µg/ml for both isolates. This study aimed to evaluate the association between exposure to sub-MIC levels of these antibiotics and the expression of the efflux pump gene (*oprM*).The current study of relative gene expression analysis revealed that's the most frequently overexpressed *oprM* gene, had fold changes ranging from (2.55 – 2.17) for CAZ and (3.37- 5.34) for CIP when compared to the reference gene *16SrRNA*, which used as the control as shown in table(2). The quantitative real-time PCR analysis demonstrated a significant upregulation of the *oprM* gene in both extensively drug-resistant (XDR) *P. aeruginosa* isolates following antibiotic exposure compared to the untreated controls. Specifically, for isolate PA7, the expression of *oprM* exhibited a 2.55-fold increase after ceftazidime (CAZ) treatment and a 3.37-fold increase after ciprofloxacin (CIP) treatment. For isolate PA28, the magnitude of upregulation was 2.17-fold and 5.34-fold following exposure to ceftazidime and ciprofloxacin, respectively. Overall, the *oprM* gene expression fold changes ranged from 2.17 to 2.55 for ceftazidime and 3.37 to 5.34 for ciprofloxacin.

Table (2): *oprM* gene expression in *pseudomonas aeruginosa* (Isolate7,28) before and after treated with sub- MIC concentrations of CAZ and CIP antibiotic

Gene expression of *oprM* (Isolate 7)

Treatment	*16S rRNA*	*oprM*	ΔCT	ΔΔCT	Folding
Control	14.375	24.547	10.172	0.000	1

PA7+ 2 subMIC of CIP	14.616	23.036	8.420	-1.75194	3.37
Control	15.099	26.938	11.839	0.000	1
PA7+ 16 subMIC of CAZ	14.558	25.047	10.489	-1.350	2.55

Gene expression of *oprM* (Isolate 28)

Treatment	***16S rRNA***	***oprM***	**ΔCT**	**ΔΔCT**	**Folding**
Control	12.981	25.818	12.83683	0	1
PA28+ 4 subMIC of CIP	15.980	26.401	10.42085	-2.41598	5.34
Control	17.093	28.027	10.93353	0	1
PA28+ 16 subMIC of CAZ	19.654	29.472	9.8176	-1.11593	2.17

The result of gene expression in this study is consistent with earlier findings by Pourakbari[25], who found that's sixteen XDR *P.aeruginosa* isolates treated with CAZ and CIP have overexpression of the *oprM* efflux pump gene at the folding range (2.1–6.4). Also this result matches the findings of Goli[26], which showed that's treated *P.aeruginosa* with CIP have overexpressed the *oprM* efflux pump gene. The *oprM* gene in *Pseudomonas aeruginosa* encodes an outer membrane protein that is an integral part of the *MexAB- oprM* multidrug efflux pump system. This protein provides a channel in the outer membrane that permits harmful chemicals and antibiotics to leave the bacterial cell. The *MexAB- oprM* pump increases resistance to several antibiotics, including fluoroquinolones and β-lactams. Overexpression of this system lowers the intracellular concentration of drugs and increases multidrug resistance[27]. *Pseudomonas aeruginosa* exposure to sub-inhibitory concentrations (Sub-MIC) of antibiotics may serve as an environmental signal that initiates adaptive responses, such as the overexpression of efflux pump genes like *mexAB-oprM* by increasing antibiotic efflux, decreasing intracellular drug accumulation and raising resistance levels this reaction improves bacterial survival, In Gram-negative bacteria sub-MIC exposure has been demonstrated to alter gene expression and encourage resistance mechanisms[28].

A limitation of the present study is the small sample size utilized for relative gene expression analysis, which was restricted to two extreme XDR isolates. While these isolates served as a robust proof-of-concept to demonstrate the molecular induction of *oprM* under antibiotic stress, further large-scale investigations with a larger cohort are warranted to fully validate and generalize these preliminary findings.

Conclusion

In conclusion, a clear correlation was established between the exposure to sub-MIC concentrations of ceftazidime and ciprofloxacin and the significant overexpression of the *oprM* efflux pump gene in XDR *P. aeruginosa* clinical isolates. The magnitude of this genetic upregulation ranged from 2.17 to 2.55-fold for ceftazidime and 3.37 to 5.34-fold for ciprofloxacin, highlighting the vital role of the *oprM* outer membrane protein in the bacteria's adaptive resistance machinery under antimicrobial stress.

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Author's Declaration

- We hereby confirm that all the Figures and Tables in the manuscript are original and have been created by us.
- We have obtained ethical clearance for our study from the local ethical committee at [Al-Nahrain University/College of biotechnology]. This approval underscores our commitment to ethical research practices and the well-being of our participants.
- We declare that there are no conflicts of interest related to this work.

Author's Contribution Statement

Yaqeen I. Mohammed: Performed the study, conducted the research, collected and analyzed the data, interpreted the results and drafted the manuscript.

Bushra H. Saleh: Supervised the study, provided scientific guidance throughout the research process and critically reviewed the manuscript.

Arwa S. Mohamed: contributed to the research process and assisted in manuscript preparation.

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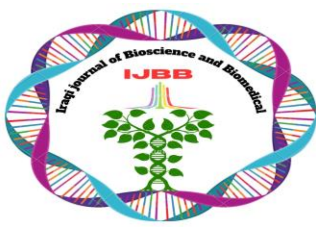

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