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Quantifying the expression levels of *mexA* and *mexB* genes in response to the efflux pump inhibitor PAβN in ciprofloxacin-resistant *Pseudomonas aeruginosa* isolated from urinary tract infections

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Abstract. *Urinary tract infections (UTIs) are among the most prevalent bacterial infections worldwide, occurring in both community and healthcare settings. Pseudomonas aeruginosa, a Gram-negative opportunistic pathogen, is one of the five most significant nosocomial bacteria and a major contributor to UTIs. This study aimed to quantify the expression levels of efflux pump genes mexA and mexB in response to the efflux pump inhibitor phenylalanine-arginine β-naphthylamide (PAβN) in ciprofloxacin-resistant P. aeruginosa isolates.*

Methods. Fifty urine specimens were collected from UTI patients at various hospitals in Baghdad. Specimens were directly cultured by streaking on differential media. Five ciprofloxacin-resistant P. aeruginosa isolates were identified, with resistance confirmed using the disk diffusion method for antibiotic susceptibility. The broth microdilution method was employed to determine the minimum inhibitory concentration (MIC) of ciprofloxacin (CIP) alone and in combination with PAβN to assess PAβN's inhibitory activity. RNA was extracted and purified from the bacterial isolates, followed by reverse transcription and quantitative PCR to evaluate the expression of efflux pump-related genes. The expression levels of mexA and mexB were measured in the presence of the tested compounds using quantitative PCR.

Results. Antibiotic susceptibility testing revealed that the isolates were resistant to nearly all antibiotics tested, except piperacillin-tazobactam, which was effective against 64% of the isolates. None of the five selected isolates showed sensitivity to ciprofloxacin. The MIC for ciprofloxacin ranged from 31.25 to 62.5 mg/L, while the sub-MIC in the presence of PAβN was significantly reduced, ranging from 7.81 to 15.62 mg/L. The expression levels of mexA and mexB genes decreased significantly in three of the five isolates when exposed to PAβN and ciprofloxacin compared to ciprofloxacin alone, with expression levels reduced from 1.319 to 0.574, 0.159 to 0.008, and 194.0 to 4.9, respectively. However, two isolates exhibited overexpression of these genes.

Conclusions. The presence of PAβN significantly reduced ciprofloxacin MICs in most ciprofloxacin-resistant P. aeruginosa isolates in vitro. The expression levels of mexA and mexB genes decreased in most isolates when PAβN was used in combination with ciprofloxacin, suggesting that PAβN could enhance the efficacy of ciprofloxacin. These findings indicate that PAβN may be a promising adjunctive antimicrobial agent for treating UTIs caused by resistant P. aeruginosa.

Keywords: *Pseudomonas aeruginosa, efflux pumps, mexA gene, mexB gene, phenylalanine-arginine β-naphthylamide, multidrug resistance, urinary tract infections.*

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Кількісне визначення рівнів експресії генів *mexA* та *mexB* у відповідь на інгібітор ефлюксних насосів PAβN у ципрофлоксацин-резистентних ізолятах *Pseudomonas aeruginosa*, виділених у пацієнтів з інфекцією сечової системи

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Резюме. *Інфекції сечової системи (ІСС) є однією з найпоширеніших бактеріальних інфекцій у світі. Pseudomonas aeruginosa, грамнегативний опортуністичний патоген, є одним із п'яти найзначніших нозокоміальних бактерій і основним чинником ІСС. Це дослідження мало на меті кількісно оцінити рівні експресії генів ефлюксних насосів mexA та mexB у відповідь на інгібітор ефлюксних насосів фенілаланін-аргінін β-нафтиламін (PAβN) у ципрофлоксацин-резистентних ізолятах P. aeruginosa.*

Методи. П'ятдесят зразків сечі було зібрано від пацієнтів з ІСС у різних лікарнях Багдада. Зразки безпосередньо культивували шляхом нанесення на диференційні середовища. Було ідентифіковано п'ять ципрофлоксацин-резистентних ізолятів P. aeruginosa, резистентність яких підтверджена методом дискової дифузії для визначення чутливості до антибіотиків. Метод мікродилюції в бульйоні використовували для визначення мінімальної інгібуючої концентрації (MIK) ципрофлоксацину (CIP) окремо та в комбінації з PAβN для оцінки інгібуючої активності PAβN. РНК екстрагували та очищали з бактеріальних ізолятів, після чого проводили зворотну транскрипцію та кількісну ПЛР для оцінки експресії генів, пов'язаних із ефлюксними насосами. Рівні експресії mexA та mexB вимірювали в присутності досліджуваних сполук за допомогою кількісної ПЛР.

Результати. Тестування на чутливість до антибіотиків показало, що ізоляти були резистентними до майже всіх протестованих антибіотиків, за винятком піперациліну-тазобактаму, який був ефективним проти 64% ізолятів. Жоден із п'яти відібраних ізолятів не виявив чутливості до ципрофлоксацину. MIK для ципрофлоксацину варіювала від 31,25 до 62,5 мг/л, тоді як суб-MIK у присутності PAβN значно знижувалася, варіюючи від 7,81 до 15,62 мг/л. Рівні експресії генів mexA та mexB значно знизилися в трьох із п'яти ізолятів при дії PAβN і ципрофлоксацину порівняно з ципрофлоксацином окремо, зі зниженням рівнів експресії з 1,319 до 0,574, з 0,159 до 0,008 та з 194,0 до 4,9 відповідно. Однак два ізоляти показали надмірну експресію цих генів.

Висновки. Присутність PAβN значно знизила MIK ципрофлоксацину в більшості ципрофлоксацин-резистентних ізолятів P. aeruginosa in vitro. Рівні експресії генів mexA та mexB знизились в більшості ізолятів при комбінованому використанні PAβN із ципрофлоксацином, що свідчить про те, що PAβN може підвищувати ефективність ципрофлоксацину. Ці результати вказують на те, що PAβN може бути перспективним допоміжним антимікробним агентом для лікування ІСШ, спричинених резистентними P. aeruginosa.

Ключові слова: *Pseudomonas aeruginosa, ефлюксні насоси, ген mexA, ген mexB, фенілаланін-аргінін β-нафтиламін, множинна резистентність, інфекції сечовивідних шляхів.*

Introduction. Urinary tract infections (UTIs) rank among the most prevalent bacterial infections worldwide, occurring in both community and healthcare settings. The primary microorganisms implicated in UTIs include *Escherichia coli, Pseudomonas aeruginosa, Proteus mirabilis, Klebsiella pneumoniae*, and *Streptococcus faecalis*, alongside various other microorganisms, including fungi and certain viruses [1, 2].

Pseudomonas aeruginosa is a gram-negative, opportunistic pathogen and is recognized as one of the five most significant nosocomial bacteria globally. It is a rod-shaped bacterium measuring 1–5 μm in length and 0.5–1.0 μm in width, non-spore-forming, and motile via one or two polar flagella [3]. This aerobic bacterium thrives in diverse environments, including soil, plants, and mammalian tissues. It can persist on medical devices and various surfaces by utilizing essential adhesion components such as flagella, pili, and biofilms.

P. aeruginosa is a major contributor to antibiotic resistance and hospital-acquired infections, commonly found on hospital floors, in operating rooms, and on surgical instruments. It is capable of surviving disinfectants and certain sterilization processes [4, 5]. This pathogen is often associated with healthcare-related infections such as ventilator-associated pneumonia (VAP), intensive care unit infections, central line-associated bloodstream infections, surgical site infections, urinary tract infections, burn wound infections, keratitis, and otitis media [6].

The increased mortality rate among patients suffering from these infections is largely attributed to *P.*

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aeruginosa's adaptability to environmental changes and its ability to rapidly develop resistance to antibiotics [7, 8]. The growing incidence of nosocomial infections caused by multidrug-resistant (MDR), extensively drug-resistant (XDR), and pan-drug-resistant (PDR) strains of *P. aeruginosa* poses a significant challenge to antimicrobial therapy [9, 10].

Infections caused by *P. aeruginosa* are difficult to treat due to both intrinsic and acquired resistance mechanisms. Intrinsic resistance mechanisms include reduced outer membrane permeability, inducible β-lactamase production, and the activity of multidrug efflux systems [11]. *P. aeruginosa* possesses several multidrug efflux systems—MexAB-OprM, MexCD-OprJ, MexEF-OprN, and MexXY-OprM—which are major contributors to drug resistance in clinical isolates [12]. Based on this, the current study focuses particularly on these systems.

Among these, MexAB-OprM is regarded as the most crucial efflux pump due to its ability to expel a wide range of antibiotics, including fluoroquinolones, chloramphenicol, and certain β-lactams [13, 14].

Numerous natural compounds have shown the ability to inhibit efflux pumps, notably Phenylalanine-Arginine β-Naphthylamide (PAβN), a peptidomimetic compound recognized as a potent inhibitor of RND efflux pumps in gram-negative bacteria [15]. PAβN works through competitive inhibition, being preferentially expelled by efflux pumps instead of antibiotics, thus allowing antibiotics to accumulate inside bacterial cells. Additionally, PAβN diminishes quorum sensing (QS) signaling molecules without affecting bacterial viability and effectively inactivates virulence factors such as elastase, protease, pyocyanin, and bacterial motility [16].

The rise of antibiotic-resistant microorganisms has spurred research into novel therapeutic combinations that protect antibiotics from degradation by resistant bacterial enzymes [17]. Therefore, the present study aims to inhibit efflux pumps using chemical agents known as efflux pump inhibitors (EPIs), such as PAβN, to enhance the efficacy of antibiotics and increase the susceptibility of MDR isolates.

Material and methods. *Isolation and identification of Pseudomonas aeruginosa*. Fifty urine specimens were collected from UTI patients at various hospitals in Baghdad. The specimens were streaked onto blood agar, MacConkey agar, and cetrimide agar, and incubated aerobically at 37°C for 24 hours.

Morphological identification was conducted by gram staining and macroscopic examination. Confirmation of *P. aeruginosa* was achieved using the VITEK 2 Compact system and molecular detection methods [18].

Antibiotic susceptibility testing (Disc diffusion method). The Kirby-Bauer disc diffusion method was employed to assess the antibiotic susceptibility of bacterial isolates [19]. The inhibition zones were measured in millimeters and interpreted following the Clinical and Laboratory Standards Institute (CLSI, 2023) guidelines. The antibiotics tested included: Piperacillin (100 µg), Ceftazidime (30 µg), Cefepime (30 µg), Ticarcillin-clavulanate (75/10 µg), Piperacillin-tazobactam (100/10 µg), Aztreonam (30 µg), Imipenem (10 µg), Meropenem (10 µg), Gentamicin (10 µg), Amikacin (30 µg), Levofloxacin (5 µg), and Ciprofloxacin (5 µg).

Estimation of Minimal Inhibitory Concentration (MIC) by broth microdilution method. The MIC of ciprofloxacin against P. aeruginosa was determined using the microtiter broth dilution method with resazurin dye, following CLSI guidelines [20].

- Bacterial suspensions were adjusted to 0.5 McFarland standard, then diluted in Mueller-Hinton broth to achieve an inoculum of approximately 1.5×10^5 CFU/ml.
- Ciprofloxacin solutions (1–512 µg/ml) were prepared from a 1024 µg/ml stock solution.
- Following 24 hours of incubation, 20 µl of resazurin dye was added to each well and incubated for 30 minutes.
- MIC values were visually determined as the lowest concentration where no color change (from blue to pink) was observed.

Assessment of the synergistic effect of the efflux pump inhibitor PaβN. The synergistic effects of PAβN and ciprofloxacin were evaluated by broth microdilution. Bacterial suspensions were incubated with ciprofloxacin in the presence and absence of 50 µg/ml PAβN. A 2 µL aliquot of the 5 mg/mL PAβN stock was added to each well simultaneously with the antibiotic.

MexA and MexB genes expression. Five *P. aeruginosa isolates* (PA8, PA20, PA44, PA45, and PA50) were selected based on their source (UTI patients), efflux pump activity, strong biofilm formation, and resistance to ciprofloxacin.

- Planktonic cells were cultured overnight in Mueller-Hinton broth at 37°C.
- The effects of ciprofloxacin alone and ciprofloxacin + PAβN on MexA and MexB expression were assessed at sub-MIC concentrations.
- RNA was extracted using the Direct-zol™ RNA MiniPrep Kit (ZYMO RESEARCH, USA) following the manufacturer's protocol.
- Gene expression was quantified by qPCR using 16S rRNA as the housekeeping gene.
- Reactions and thermocycler conditions are detailed in Tables 1 and 2.
- Fold changes in gene expression were calculated using the ΔΔCT method [21].

Molecular methods such as PCR are powerful tools for diagnosing most pathogenic microorganisms responsible for urinary tract infections [22–26].

Table 1

Components of qRT-PCR used in MexA and MexB gene expression analysis

Component	Volume (µL)	Final Concentration
TAKARA qPCR Master Mix (2X) Universal	10.0	2X
Forward Primer	0.4	0.2 µM
Reverse Primer	0.4	0.2 µM
Nuclease-Free Water	Up to 20.0	—
Template DNA	—	1 pg – 100 ng

Table 2

qRT-PCR thermocycling conditions used in this study

Step	Temperature (°C)	Time	Cycle(s)
Enzyme Activation	95	5 min	1
Denaturation	95	20 sec	40
Annealing	60	20 sec	
Extension	72	20 sec	

Result. Isolation of Pseudomonas aeruginosa. Among the urine samples collected, Pseudomonas aeruginosa was isolated from 28% of the samples, while the remaining 72% were negative for this bacterium.

Antibiotic sensitivity of Pseudomonas aeruginosa. The antibiotic susceptibility of P. aeruginosa to selected antibiotics is shown in Table 3. In general, the isolates exhibited resistance to most antibiotics tested, with the exception of piperacillin-tazobactam (TZP), which was effective against 64% of the isolates.

Table 3

Antibiotic sensitivity of Pseudomonas aeruginosa to selected antibiotics

Antibiotic	Resistant	Intermediate	Sensitive
Piperacillin	44 (88%)	4 (8%)	2 (4%)
Piperacillin-tazobactam	10 (20%)	8 (16%)	32 (64%)
Ticarcillin-clavulanate	46 (92%)	4 (8%)	0 (0%)
Amikacin	23 (46%)	0 (0%)	27 (54%)
Gentamicin	26 (52%)	1 (2%)	23 (46%)
Aztreonam	24 (48%)	5 (10%)	21 (42%)
Cefepime	28 (56%)	0 (0%)	22 (44%)
Ceftazidime	29 (58%)	2 (4%)	19 (38%)
Ciprofloxacin	27 (54%)	2 (4%)	21 (42%)
Levofloxacin	29 (58%)	2 (4%)	19 (38%)
Imipenem	25 (50%)	1 (2%)	24 (48%)
Meropenem	26 (52%)	0 (0%)	24 (48%)

Determination of ciprofloxacin MIC. The MIC of ciprofloxacin against P. aeruginosa was determined using the 96-well microtiter plate method. The MIC ranged from 62.5 mg/L to 125 mg/L, while sub-MIC values ranged from 31.25 mg/L to 62.5 mg/L.

Determination of Ciprofloxacin + PAβN MIC. When combined with the efflux pump inhibitor PAβN, the MIC of ciprofloxacin was significantly reduced, ranging from 31.25 mg/L to 15.62 mg/L. The sub-MIC concentrations were between 15.62 mg/L and 7.81 mg/L. These results are summarized in Table 4.

Table 4

MIC and sub-MIC of ciprofloxacin alone and in combination with PAβN

Isolate No.	ID	Ciprofloxacin MIC (mg/L)	Sub-MIC (mg/L)	CIP + PAβN MIC (mg/L)	Sub-MIC (mg/L)
1	8	125	62.5	31.25	15.62
2	20	125	62.5	31.25	15.62
3	44	125	62.5	31.25	15.62
4	45	125	62.5	31.25	15.62
5	50	125	62.5	15.62	7.81

MexA and MexB gene expression following treatment with ciprofloxacin and ciprofloxacin + PaβN. Five isolates were used to evaluate the expression of *MexA* and *MexB* genes under treatment with ciprofloxacin alone and in combination with PAβN. Expression levels are presented in Tables 5 and 6, respectively.

Table 5

Expression levels of MexA gene following ciprofloxacin and ciprofloxacin + PAβN treatment

No.	Isolate ID	Sub-MIC CIP	Sub-MIC CIP+PAβN	Control ΔΔCt	Fold Change (Control)	ΔΔCt CIP	Fold Change CIP	ΔΔCt CIP+PAβN	Fold Change CIP+PAβN
1	8	62.5	15.62	0	1.0	-0.4	1.32	0.8	0.57
2	20	62.5	15.62	0	1.0	-1.5	2.8	-4.4	21.1
3	44	62.5	15.62	0	1.0	2.65	0.16	6.85	0.008
4	45	62.5	15.62	0	1.0	-7.6	194.0	-2.3	4.9
5	50	62.5	7.81	0	1.0	0.19	0.9	-0.87	1.8

Table 6

Expression levels of MexB gene following ciprofloxacin and ciprofloxacin + PAβN treatment

No.	Isolate ID	Sub-MIC CIP	Sub-MIC CIP+PAβN	Control ΔΔCt	Fold Change (Control)	ΔΔCt CIP	Fold Change CIP	ΔΔCt CIP+PAβN	Fold Change CIP+PAβN
1	8	62.5	15.62	0	1.0	-5.55	46.85	-3.35	10.20
2	20	62.5	15.62	0	1.0	-1.0	2.0	-1.6	3.0
3	44	62.5	15.62	0	1.0	2.95	0.13	4.8	0.036
4	45	62.5	15.62	0	1.0	-6.5	90.5	1.5	0.3
5	50	62.5	7.81	0	1.0	0.67	0.6	-0.96	1.9

Figure 1 shows PCR results for 16S rRNA, MexA, and MexB.

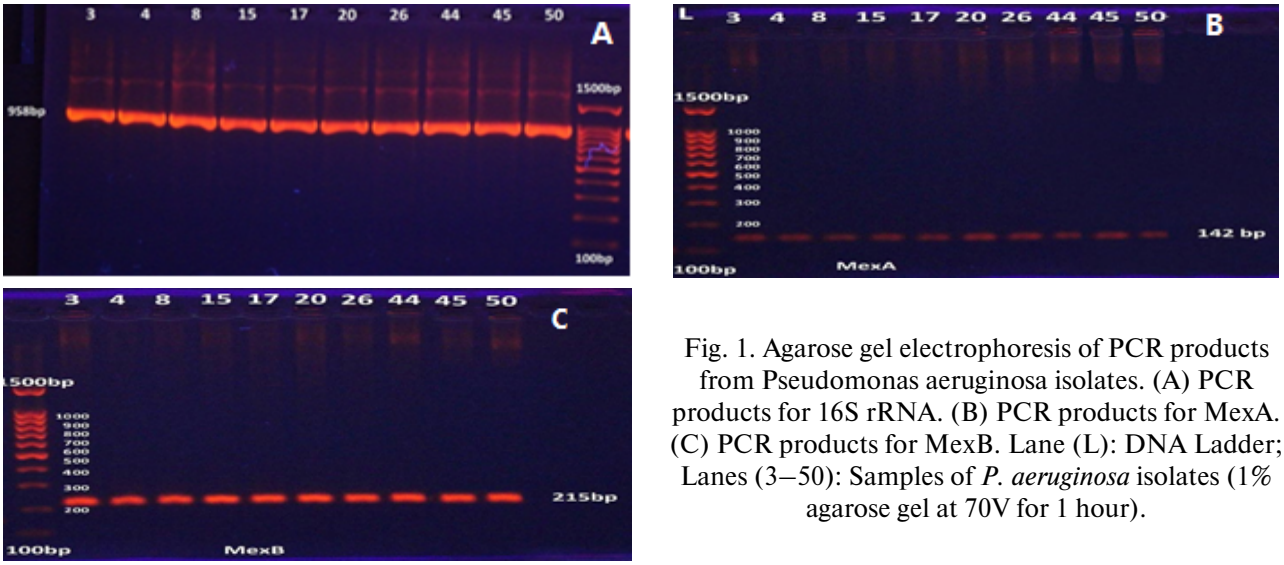

Fig. 1. Agarose gel electrophoresis of PCR products from Pseudomonas aeruginosa isolates. (A) PCR products for 16S rRNA. (B) PCR products for MexA. (C) PCR products for MexB. Lane (L): DNA Ladder; Lanes (3–50): Samples of *P. aeruginosa* isolates (1% agarose gel at 70V for 1 hour).

Discussion. *Isolation and identification of Pseudomonas aeruginosa*. The result of the isolation of *P. aeruginosa* showed that 28% of the total samples were positive. This finding aligns with several other studies that reported similar percentages of positive isolates, such as Mohamed et al. (2019) [27], who recorded 27%. However, our findings differ from those of Boushra et al. (2024) [28], who reported a higher percentage of 40%. This variation may be attributed to geographic, climatic, hygienic, and social factors [29].

Antibiotic sensitivity. Antibiotic sensitivity testing was performed using the Kirby-Bauer method. Our isolates appeared to be resistant to nearly all antibiotics tested, except Piperacillin-tazobactam, which was effective against 64% of the isolates. Notably, none of the five selected isolates in our study showed any sensitivity to ciprofloxacin, as shown in Table (3). The results were compared with other studies conducted both within and outside Iraq. These studies showed inconsistent results, which may be attributed to differences in clinical specimens, population-related social factors, and levels of exposure to antimicrobial agents, as reported by Ahmed Hasan et al. (2020) [30, 31].

Determination of MIC and sub-MIC. MIC determination was performed using the 96-well microtiter plate method for ciprofloxacin and its combination with PaβN. The MIC for ciprofloxacin alone ranged from 62.5 mg/L to 125 mg/L, indicating complete resistance of the isolates to ciprofloxacin. The sub-MIC values ranged from 31.25 mg/L to 62.5 mg/L, representing concentrations at which the bacteria could still grow [32, 33].

When ciprofloxacin was combined with PaβN, both the MIC and sub-MIC values decreased significantly. MIC values ranged from 31.25 mg/L to 15.62 mg/L, while sub-MIC values ranged from 15.62 mg/L to 7.81 mg/L. This suggests that the combination was more effective at inhibiting bacterial growth. These findings indicate that PaβN can suppress virulence factors, especially efflux pumps, in resistant *P. aeruginosa* strains, thereby reducing resistance and enhancing the effectiveness of ciprofloxacin [15, 16].

MexA and MexB gene expression. Gene expression data presented in Tables (5) and (6) showed that PaβN significantly reduced the expression levels of *MexA* and *MexB* genes in most isolates ($P \leq 0.05$). This supports the idea that combining ciprofloxacin with PaβN can reduce bacterial resistance and confirms the relationship between the overexpression of these genes and resistance to ciprofloxacin alone [16].

Our findings showed generally low MexA and MexB expression among the resistant isolates, which contrasts with the results of Abdolhosseini et al. [34], who did not use an efflux pump inhibitor. The reduction in gene expression in our study may support the hypothesis of synergism between PaβN and ciprofloxacin [35], indicating a potential bactericidal effect of the combination against *P. aeruginosa* [36].

Interestingly, isolates 2 and 5 exhibited different patterns in gene expression fold changes, suggesting possible mutations in regulatory genes that affect efflux pump expression, as suggested by Hadir et al. [37]. Due to a lack of references regarding such mutations, we recommend further research on isolates with this behavior.

Many recent studies have explored alternative antimicrobial strategies using chemical, biological, and physical approaches. Examples include the use of methanolic extracts of *Cladophora glomerata* and *Spirulina platensis* as antimicrobial agents [38, 39], *Lactobacillus acidophilus* supernatants against *P. aeruginosa* [26], essential oils of *Mentha spicata* to inhibit *Proteus mirabilis* biofilms [40], nickel oxide nanoparticles [41], nano-zirconium oxide particles combined with selected antibiotics against *E. coli* and *K. pneumoniae* [42], and even the application of audible sounds and magnetic fields on methicillin-resistant *Staphylococcus aureus* [43].

This study faced several limitations. First, the time constraints for sample collection affected the outcomes. Second, there were delays in receiving the PaβN compound from the supplier. Third, although real-time PCR was used to analyze gene expression, the reliance on conventional PCR and electrophoresis for some procedures limited precision.

Conclusions. In conclusion, the addition of PaβN led to a significant reduction in ciprofloxacin MICs in most ciprofloxacin-resistant *P. aeruginosa* isolates in vitro, indicating its potential as a promising antimicrobial adjuvant. The gene expression analysis revealed a clear decrease in *MexA* and *MexB* expression when ciprofloxacin was combined with PaβN in most tested isolates. This suggests that additional regulatory factors, such as mutations or plasmid loss, may influence gene expression in the presence of PaβN. Our study raises important questions about antibiotic combination therapies and highlights the need for further research to enhance antimicrobial strategies.

Ethical statement. This study was approved by the Ethics Commission of the Institute of Genetic Engineering and Biotechnology for Postgraduate Studies, University of Baghdad, Iraq (Reference No. 30 / 7289, dated 30/11/2023).

Conflicts of interest. The authors declare no conflicts of interest.

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Data availability statement. The datasets used and analyzed during the current study are available from the corresponding author upon reasonable request.

Authors' contributions. Both authors contributed equally to the development of the research plan, statistical analysis of results, and manuscript writing.

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