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Brief Communication

Efflux-mediated ciprofloxacin and cefixime resistance in *Pseudomonas aeruginosa*

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Abstract

Pseudomonas aeruginosa is a gram-negative, motile and clinically relevant bacterium. It is a significant pathogen associated with immunosuppression, cystic fibrosis and malignancy. Infections due to multidrug-resistant *P. aeruginosa* cause greater morbidity, death rate, and increased healthcare expenses. Drug efflux is a prevalent mechanism of antibiotic resistance in *P. aeruginosa*. The objective of the study was to analyze the antibiotic susceptibility profile of a clinical strain of *P. aeruginosa* (GC14) and to identify the mechanism of antibiotic resistance. The antibiotic resistance of GC14 was tested against 19 antibiotics using an antibiotic susceptibility test. A polymerase chain reaction-based strategy was employed to detect the presence of *mexB, mexD* and *mexY* efflux pump genes. The efflux pump inhibition assay was conducted to analyze the effect of efflux pumps on antibiotic resistance in GC14, using carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP) as an efflux pump inhibitor. The antibiotic susceptibility test confirmed that GC14 is multidrug-resistant and showed resistance to tetracycline, cefixime, ciprofloxacin, erythromycin, meropenem, azithromycin, doxycycline, aztreonam, co-trimoxazole and gentamycin. The genes encoding transporter protein viz. *mexB, mexD* and *mexY* were amplified successfully from *P. aeruginosa*, revealing the presence of efflux pump genes. The data obtained from the efflux inhibition assay using CCCP showed that efflux pumps play a significant role in ciprofloxacin and cefixime resistance. The study emphasizes the importance of efflux pumps in antibiotic resistance and also confers the necessity of continuous surveillance and regular monitoring of the emergence of multidrug-resistant isolates of *P. aeruginosa*.

Key words: antibiotic resistance, carbonyl cyanide *m*-chlorophenyl hydrazone, cefixime, ciprofloxacin, efflux pumps, minimum inhibitory concentration, multi-drug resistance, *Pseudomonas aeruginosa*.
Abbreviations: CCCP, carbonyl cyanide *m*-chlorophenyl hydrazone; CFM, cefixime; CI, ciprofloxacin; MDR, multi-drug resistance; TET, tetracycline.

Introduction

Pseudomonas aeruginosa is a gram-negative, ubiquitous human pathogen listed in the ESKAPE (*Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, P. aeruginosa*, and *Enterobacter* spp.) pathogen list (Boucher et al. 2009). It causes opportunistic infections in plants and animals, including humans, mainly in immunocompromised patients and people with severe burns and cystic fibrosis (Abbas et al. 2020). The infections caused by *P. aeruginosa* have been recognized as a global healthcare concern (Rosenthal et al. 2016).

Antibiotic resistance is common in *P. aeruginosa*, and multidrug-resistant (MDR) strains are responsible for about 13% of the total infections (Nicolle 2005). Comparison of non-resistant infections showed that MDR *P. aeruginosa* infections resulted in a 70% increase in cost per patient (Morales et al. 2012). The major causative factors for the inherent MDR in *P. aeruginosa* include; low cell wall permeability, large and adaptable genome, formation of biofilms and mobile genetic elements (Lambert 2002). An important mechanism leading to drug resistance in *P. aeruginosa* is antibiotic efflux pumps, which extrude the antibiotics to the exterior of the bacterial cell and provide protection against the antibacterial compounds. Although the overexpression of the efflux system contributes to the MDR phenotype, it also targets the novel antimicrobial agents, thereby creating a significant threat in the healthcare system (Strateva, Yordanov 2009; Askoura et al. 2011). The efflux pumps categorized under the resistance-nodulation cell division family (RND family) are responsible for intrinsic drug resistance in gram-negative bacteria (Fernando, Kumar 2013). MexAB-OprM, MexCD-OprJ, MexEF-OprN, and MexXY-OprM are the important efflux systems of the RND family in *P. aeruginosa*, and it exhibits

a wide range of drug specifications (Li et al. 1998). The various factors such as high bacterial load, stationary phase growth, and low pH range seen in infections can promote the synthesis and activity of the efflux pump systems (Aeschlimann 2003).

The treatment of infections caused by *P. aeruginosa* is a great challenge due to its ability to resist a wide range of available antibiotics. *P. aeruginosa* is one of the WHO critical priority infectious agents. The role of efflux-mediated resistance in the MDR strains of *P. aeruginosa*, especially from South Kerala, is not well studied. In this context, the objective of the present investigation was to detect the presence of major efflux pump genes and to determine the efflux-mediated resistance to commonly used antibiotics, ciprofloxacin (CIP), cefixime (CFM) and tetracycline (TET).

Materials and methods

Bacterial strains and identification

P. aeruginosa, GC14 strain, was cultured from a urine sample in the State Public Health and Clinical Laboratory, Thiruvananthapuram, Kerala. The characteristic blue-green appearance of the culture due to the presence of pyocyanin indicated species identity.

Antimicrobial susceptibility testing

The antibiotic susceptibility of GC14 was assessed by the Kirby-Bauer disc diffusion assay on Muller-Hinton Agar as per the guidelines of Clinical Laboratory Standards Institute by using sterile antibiotic discs (Himedia) (Bauer et al. 1966). The *E. coli* strain (ATCC 25922) was used as a control for the antibiotic susceptibility test. A total of 19 antibiotic impregnated discs were used: azithromycin (15 µg), aztreonam (30 µg), cefepime (30 µg), cefixime (5 µg), cefotaxime (30 µg), cefpodoxime (10 µg), ceftazidime (30 µg), ceftriaxone (30 µg), chloramphenicol (30 µg), ciprofloxacin (5 µg), co-trimoxazole (25 µg), doxycycline hydrochloride (30 µg), erythromycin (15 µg), gentamicin (10 µg), levofloxacin (5 µg), meropenem (10 µg), norfloxacin (10 µg), streptomycin (25 µg), and tetracycline (30 µg). Bacterial resistance to at least one agent in three or more antimicrobial classes was considered as MDR.

Amplification of the efflux pump genes by PCR

PCR was carried out in a thermal cycler (T100 Thermal cycler, Bio-Rad) with a volume of 25 µL containing 1X reaction buffer (SRL), 10 pmol of each primer (Table 1), 100 µM of each dNTP's and 1 unit of Taq polymerase (SRL). The efflux pump genes, *mexBF, mexBR, mexDF, mexDR, mexYF* and *mexYR*, were amplified using the PCR programmed for 30 cycles consisting of denaturation of template DNA for 30 s at 94 °C, primer annealing for 30 s at 53 °C, and extension of the primers for 30 s at 72 °C. Initial denaturation was carried out for 3 min at 94 °C and final extension for 10 min at 72 °C. The PCR products were separated by 1% agarose gel stained with 0.5 µg mL^{-1} ethidium bromide. A 100 base pair ladder (SRL) was used as a molecular size standard. The agarose gel was visualized under and EZ gel documentation system (Bio-Rad). The primers were procured from G-Biosciences, USA.

Table 1. List of oligonucleotides used in the present study

Primer	Nucleotide sequence (5' to 3')	Reference
mexBF	ATCTACCGGCAGTTCTCCAT	Present study
mexBR	GGAACATCCGGTTGAACCAG	Present study
mexDF	TGGTGTTCCTGTTGCTGGT	Present study
mexDR	ATGAGGATCGCGTTCTTCG	Present study
mexYF	GGTGGAAGTGCAGAACCGC	Present study
mexYR	GGTCGGGCCAGATGCGCAT	Present study

Determination of minimum inhibitory concentration of CCCP

To determine the presence of an efflux pump mechanism, the minimum inhibitory concentration (MIC) value of the efflux pump inhibitor, carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP), was determined by microtiter broth dilution method. A stock solution of CCCP was prepared in dimethyl sulfoxide to a final concentration of 10 mg mL^{-1}. The overnight bacterial suspension was adjusted to 0.5 McFarland standard and then diluted to 1×10^6 CFU mL^{-1}. From the suspension, 100 µL was inoculated to each well and 100 µL CCCP (2048 µg mL^{-1}), prepared in Muller Hinton broth was added in the first row. A two-fold serial dilution was obtained vertically, so as to obtain 5×10^5 CFU mL^{-1} in each test well. The wells for the positive control contained 5×10^5 CFU mL^{-1} and negative control well contained 100 µL of media alone. The microtitre plates were incubated overnight at 37 °C. MIC values were visually determined and interpreted as the lowest concentration of CCCP displaying no visible growth. The experiment was performed in triplicate.

Efflux pump inhibition assay

The efflux pump inhibition assay and determination of the MICs of CIP, CFM and TET were carried out simultaneously. To reduce the possibility of toxicity being responsible for the change in MIC of CIP, TET, and CFM resistance, a sub-MIC concentration of CCCP (32 µg mL^{-1}) was used. The MICs of CIP, TET, and CFM were tested both in the absence and presence of CCCP to evaluate the activity of the efflux-mediated resistance. A stock solution of antibiotics, CIP, TET, and CFM was prepared in sterile water to a final concentration of 20 mg mL^{-1}. The bacterial suspension (1×10^6 CFU mL^{-1}) was prepared as discussed earlier and subsequently added to each well. Antibiotic solution in a final volume of 100 µL of CIP, TET, and CFM (2048 µg mL^{-1}) was prepared in Muller Hinton broth and was added to the first row. For obtaining 5×10^5 CFU mL^{-1}

in each test well, a two-fold serial dilution was performed. The concentration of each antibiotic ranged from 1024 to 0.25 μg mL^{-1}. The tests containing antibiotics with CCCP were also performed simultaneously. A well containing 32 μg mL^{-1} of CCCP in 100 μL bacterial suspension (5×10^5 CFU mL^{-1}) served as a positive growth control. The experiment was performed in triplicate.

Results

In the present investigation, an attempt was made to determine the antibiotic sensitivity pattern and to detect the presence of efflux pump encoding genes in the clinical strain of *P. aeruginosa*, GC14. The antibiotic susceptibility test showed that GC14 is resistant to tetracycline, cefixime, ciprofloxacin, erythromycin, meropenem, azithromycin, doxycycline, aztreonam, co-trimoxazole and gentamycin and thus it revealed that the strain was multidrug-resistant.

A PCR based amplification strategy was adopted to detect the presence of efflux-associated genes in GC14, using the strain ATCC 27853 as a positive control. In the present investigation, the primers were designed in such a way to amplify the transporter component genes (*mexB, mexD* and *mexY*) of the efflux pumps. The results of PCR targeting *mexB, mexD* and *mexY* gene revealed the presence of 262, 180 and 165 bp amplicons respectively, indicating the presence of *MexAB-OprM, MexCD-OprJ*, and *MexXY-OprM* efflux pump-encoding operons in *P. aeruginosa* (Fig. 1).

We employed CCCP, an efflux pump inhibitor, to further evaluate the assumption that the efflux pumps are responsible for MDR. The oxidative phosphorylation of CCCP inhibits ATP synthesis and promotes membrane permeability in *P. aeruginosa* (Fanélus, Desrosiers 2013). The results showed that CCCP reduced the MIC of CFM and CIP by four- and 16-fold, respectively; however, no change in MIC was observed with TET (Table 2).

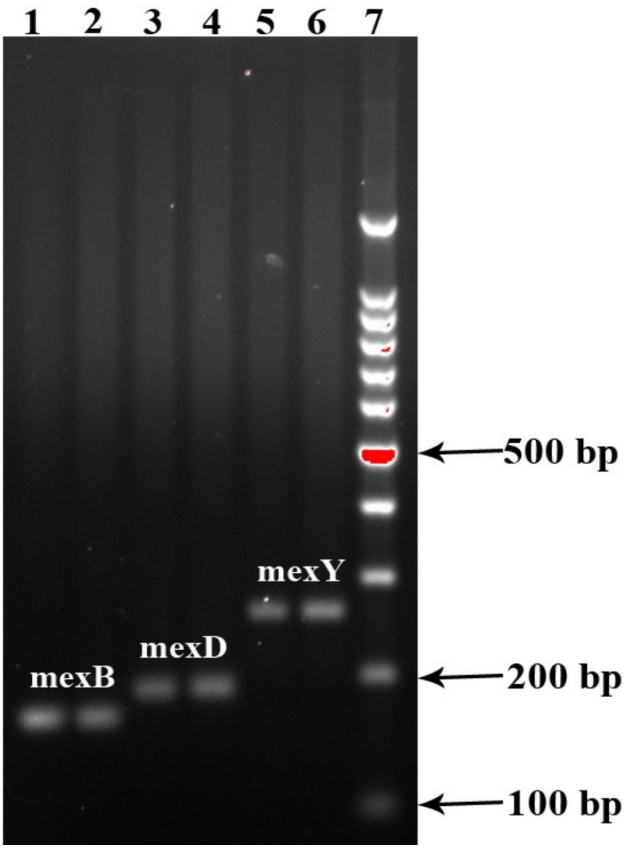

Fig. 1. Amplification of efflux pump genes: agarose gel showing PCR product of *mexB* (165 bp), *mexD* (180 bp), *mexY* (262 bp) genes. Lanes 1, 3 and 5: *P. aeruginosa* ATCC 27853, positive control); lanes 2, 4 and 6: *P. aeruginosa* GC14; lane 7, 100 bp ladder SRL).

Discussion

P. aeruginosa is an opportunistic pathogen with high intrinsic resistance to most antibiotics (Langendonk et al. 2021). The increasing trend of antibiotic resistance among *P. aeruginosa* indicates possible overuse and indiscriminate use of antibiotics. Understanding the resistance mechanisms and developing alternative therapy for MDR isolates is crucial to formulate control measures. The efflux pump mechanism is recognized as one of the major components of resistance to different classes of antibiotics in *P. aeruginosa*. Efflux pumps are clinically relevant as they can render the infections untreatable (Piddock 2006). Studies have shown the resistance of *P. aeruginosa* to a wide range of antibiotics like aminoglycosides, quinolones and β-lactams (Pang et al. 2019). Zhao et al (1998) confirmed that the efflux systems encoded by the *mexAB-oprM* operon extrude antibiotics like tetracycline, chloramphenicol, quinolones, novobiocin, macrolides, trimethoprim, β-lactams and β-lactamase inhibitors. CIP is the common substrate for many efflux pumps, and studies have shown that MIC of CIP is reduced when combined with CCCP (Xu et al. 2014). In this study, we used PCR to

Table 2. Effect of CCCP on the MIC of tetracyclin, cefixime and ciprofloxacin for *P. aeruginosa* GC14

Antibiotics	MIC of antibiotics alone (μg)	MIC of antibiotics in the presence of CCCP (μg)	MIC fold change
Tetracyclin	64	64	No change
Cefixime	256	64	4
Ciprofloxacin	1	0.0625	16

amplify the efflux pump-associated genes and CCCP-based efflux assays to confirm the efflux-mediated drug resistance in GC14. Our findings revealed that GC14 is resistant to multiple antibiotics. Our findings corroborate the previous reports of efflux-based antibiotic resistance in *P. aeruginosa* (Zhao et al. 1998; Suresh et al. 2016).

Considering the results obtained in this study and previous reports, it can be assumed that the aforementioned efflux pumps are responsible for the extrusion of CIP and CFM. Previous studies have shown that overexpression of *MexAB-OprM* in *P. aeruginosa* increases its resistance to various antibiotics, including TET (Siriyong et al. 2017). However, the present investigation revealed that the CCCP have no prominent role in the reduction of MIC of TET. Efflux pump inhibitors are a promising and alternative class of drugs to combat drug resistance. Although there are no reports on the mechanism of MDR in *P. aeruginosa* from South Kerala, the preliminary studies carried out in GC14 shed light on efflux-mediated resistance. Despite the fact that several EPIs have been proven at the experimental level in recent years, none have been approved by the FDA and used clinically. In this context, *P. aeruginosa*, GC14, exhibiting efflux-based drug resistance, may be employed to screen EPIs in future.

Conclusions

The results of the present study demonstrated that *P. aeruginosa* GC14 is resistant to multiple drugs and possesses *mexB, mexD* and *mexY* genes, and shows efflux-mediated resistance to CIP and CFM. Even though this study was carried out for a single strain, the results confirmed efflux-mediated drug resistance, and the identified strain GC14 could be used to screen efflux pump imhibitors in future investigations. *P. aeruginosa* infections are becoming more prevalent worldwide and impose a significant treatment challenge. Therefore, the present study necessitates continuous surveillance and regular monitoring of the emergence of multidrug-resistant *P. aeruginosa* isolates.

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