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Research Article

Molecular detection of *mexXY-oprM, mexPQ-opmE* Efflux pumps in multi-drug resistant *Pseudomonas aeruginosa* isolates in patients referred to teaching hospitals in Babylon province, Iraq

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Abstract
One of the global health issues is antibiotic resistance in *Pseudomonas aeruginosa*, a causative agent of bacterial infections due to multidrug resistance (MDR), which may be mediated by efflux pumps' overexpression. The present study investigated the prevalence of *mexXY-oprM, mexPQ-opmE* genes as encoding agents of efflux pumps and the determination of antibiotic resistance rate in clinical isolates of *P. aeruginosa*. Different clinical specimens of infectious patients, such as wounds, urine, blood, discharge, and abscesses except for stool, were examined. Identification of the isolates was performed using *Pseudomonas chromogenic* agar. A selective medium for the isolation of *P. aeruginosa*, used to screen 79 isolates. The results were validated by *Polymerase chain reaction* (PCR) utilizing particular primer pairs for the 16S rDNA gene of Pseudomonas spp. for identification of the isolates after incubation at 37°C for 24 hours. According to Clinical and Laboratory Standards Institute (CLSI) (2021) recommendations, a microbial susceptibility test was performed using the Kirby–Bauer disk diffusion method. *P. aeruginosa* was extremely resistant to ceftazidime (93.6%) and cefepime (77.2 %). In contrast, imipenem (77.2%) and meropenem (67%) showed high sensitivity. Finally, *mexXY-oprM, mexPQ-opmE* genes were investigated by PCR technique. Molecular investigation revealed *mexX* 43%, *mexY* 51.89%, *oprM* .481%, *mexP* 36.70% *mexQ* 46.83% and *opmE* 51.89%. The present study concluded that *mexXY-oprM and mexPQ-opmE* may have a role in *P. aeruginosa* resistance to various antibiotics. Identifying resistant isolates and antibiotic monitoring programs is essential to prevent the spread of MDR isolates.

Keywords: Antibiotic resistance, Efflux pump, *mexXY-oprM, mexPQ-opmE, Psuedpmonas aeruginosa*

INTRODUCTION

Pseudomonas aeruginosa is an aerobic gram-negative rod. It can adapt to a range of circumstances and is widely distributed in nature (Micek *et al*., 2015). It can be isolated from practically any possible source within hospitals. It is a major source of infections acquired in the community and in hospitals. Diseases with this bacteria have been associated with a greater rate of morbidity and mortality when compared to other bacterial infections (Shortridge *et al*., 2019). In view of this, *P. aeruginosa* infections are resistant to the majority of antibiotics; this could be due to the bacteria's development of numerous mechanisms to counteract the agents' effects (Pang *et al*., 2019). One of the essential mechanisms is efflux pumps, which are responsible for multidrug resistance by extruding different antimicrobial agents (Soares *et al*., 2020). In *P. aeruginosa* efflux systems, the RND family is fully defined, which is clinically significant (Soares *et al*., 2020). This family's three members are the transporter, the linker, and the outer membrane pore (Meliani, 2020). In *P. aeruginosa*, there are 11 RND effluxes. Using efflux pump inhibitors to improve the clinical effectiveness of various antibiotics is a unique and promising strategy for treating multidrug-resistant bacteria (Scoffone *et al*., 2021; Zechini and Versace, 2009).

Higher levels of resistance may be attributed to efflux pump overexpression, which can become unstable when specific phenotypic resistance inducers or constitutive inducers for acquired resistance are revealed (Langendonk *et al*., 2021). The MexXY pump, which

confers intrinsic resistance to aminoglycosides, has been inducibly expressed in *P. aeruginosa* (Lau CH, Hughes and Poole, 2014). When MexXY is overexpressed via plasmid vectors, it generates aminoglycoside resistance in clinical isolates and fluoroquinolone resistance in *P. aeruginosa* (Singh *et al*., 2020). MexPQ -OpmE uses fluoroquinolone as a substrate. Even while MexPQ-OpmE was found to be quiet in *P. aeruginosa*, studies revealed that it can behave as a multidrug efflux pump. If there are mutations in their promoter regions or regulatory genes, or if there are suitable inducers, this pump can be developed. As a result of such mutations or inducers, *P. aeruginosa* could cause resistance to a number of antibiotics (Ranjitkar *et al*., 2019). The present work aimed to find out the correlation between the *P. aeruginosa* efflux pump *mexXY-oprM, mexPQ-opmE* genes and antibiotic resistance to different types of antibiotics.

MATERIALS AND METHODS

Collection and identification of samples

Between December 2020 and April 2021, 127 specimens were obtained from teaching hospitals in Babylon. Only 79 isolates of *P. aeruginosa* were found among 127 specimens, after culturing the specimens on a special medium for *P.aeruginosa* (*P. aeruginosa* chromogenic agar). Before that, the swabs were used with gel or liquid transport medium to collect the specimens from different categories of material (CSF, vaginal swab, bloodstream infection, ear swab, wound burn swab, broncoalveolar lavage, and midstream urine) from the patients admitted to the burns Department and the Intensive care unit, as well as the respiratory and internal consultations. Isolation was carried out at private and public hospitals (Marjan Teaching Hospital, Babylon Maternity and Children Hospital, Al Hilla Teaching Hospital, Al Salam Hospital and Al Hayat Hospital). The informed consent was obtained from all human adult participants or parents or legal guardians of minors. The incubation at 37°C for 24 hours, all isolates were screened on a selective medium for this bacteria isolation and validated by PCR using particular primer pairs for the 16S rDNA gene of Pseudomonas sp.

DNA extraction and PCR technique

Genomic DNA Extraction Kit was used to isolate total genomic DNA from cultured bacterial growth for the 79 isolates on Pseudomonas chromogenic agar and incubated overnight. Using specified primer pairs, conventional PCR was performed to amplify the target (Table 1). PCR condition is clarified in Table 2 for the mixture of 20µl consisting of 5 µl of Maxime PCR Premix kit (i-Taq) (Intronbio/Korea), 1µl of forwarding primer (10 (pmole/µl), 1µl of reverse primer (10 pmole/µl), (2 µl) of target DNA, and 13µl of nuclease-free water.

Phenotypic approach

Antibacterial susceptibility Test(disk diffusion method)

Isolates of *P. aeruginosa* were activated in brain heart infusion broth for 18 hours at 37°C, then adjusted to 0.5 McFarland's standard (1.5108 CFU/mL) and disseminated with a cotton swab on Mueller Hinton agar. Antibiotic discs were used with this test on MHA and carefully pressed down to establish complete contact with the bacteria-inoculated agar. The incubation lasted for 18–24 hours at 37°C, and the diameter of the inhibitory zone in mm was measured, followed by estimated phenotypic types according to their resistance to different classes of antibiotics.

Genotypic approach

Investigation of MexXY-OprM, mexPQ-opmE efflux's genes by PCR assay

The PCR assay was used to look into the *MexXY-OprM, mexPQ-opmE* efflux genes. Target DNA was amplified using conventional PCR. To generate the PCR product, PCR is normally made up of three sequential phases (denaturation, annealing, and elongation) of repeated cycles (amplicon). Table 2 lists the PCR thermal cycling settings. The size of the PCR products (5 l) was determined by electrophoresis in a 1.5 % (w/v) agarose gel using 1 TBE buffer and stained with Simply Safe Dye. The size of the product was assessed by comparing it to the Gene Ruler 100 bp DNA ladder. Genes, mexX, mexY, oprM, mexP, mexQ and opmE were tested using PCR technique, the last step ending with the visualization of the gel by UV transilluminator.

RESULTS AND DISCUSSION

The results of isolation of *P. aeruginosa* revealed its high percentage of 79 isolates distributed as UTI patients 35.4%, lower respiratory tract infection patients 29.1%, wounds and burn infection 18.9% while 8.8% for otitis media, 2.5% for bacteremia and 3.7% for bacterial vaginosis and 1.2% for meningitis (Table 3).

According to a study (Kamali *et al*., 2020) found that the most isolates of *P. aeruginosa* (36.25%) came from endotracheal secretions, followed by urine (32.5%), blood (13.75%), wound (10%), CSF (5%), and ear (5%). In a new study, the prevalence of *P. aeruginosa* infections in the bloodstream, urinary tract, and surgical site infections were found to be (8.9%), (8.3%), and (6.3%), respectively (Motbainor *et al*., 2020). According to a study (Kamali *et al*., 2020) that looked that among the percentage of *P. aeruginosa* isolates from various clinical collections, the most isolates (36.25%) came from endotracheal secretions, followed by urine (32.5%), blood (13.75%), wound (10%), CSF (5%), and

Table 1. Primer pair sequences and PCR conditions for the identification of *P. aeruginosa*

Primer	Sequence (5′ to 3′)	Product (bp)	Annealing temp. (°C)	Ref.
Ps.spp-F	GACGGGTGAGTAATGCCTA	618	56.0°C	Spilker *et al*., 2004
Ps.spp-R	CACTGGTGTTCCTTCCTATA			

Table 2. PCR conditions for *MexXY-OprM, mexPQ-opmE* efflux pump genes

Efflux pump Class	Genes	Sequence	Product (bp)	Annealing temp. (ºC)
RND	mexX	CATCAGCGAAC-GCGAGTACA TGTGGGTTGAC-CACCTTGAC	500	60.3
	mexY	CCGTACGGTG-TATGCGATGAG CTCGAGGTTGAAC-GAGGGAT	554	60.3
	oprM	GGTAGCCCAG-GACCAGAATG GAGCTGGTAG-TACTCGTCGC	520	62.5
	mexP	ACATCCAGGAC-GTTACGGTG CATAGGACTCGTC GGTGAGC	534	60.3
	mexQ	CTGGCTCTGGTGG TGTATGG GCAATGCCTCGAA CACATCG	492	60.3
	opmE	TGTATCCG-CAGGTCGAGGTA AGAGGTATCGTCG GTAGCCA	522	60.3

ear (5%). (2.5%).

P. aeruginosa is a prevalent infection in hospitals, particularly in intensive care units, due to its intrinsic resistance to numerous antibiotics and antiseptics, capacity to develop more resistance mechanisms to various classes of antibiotics, and ability to persist in damp settings. Endocarditis and septicemia, urinary tract infections, cystitis, pneumonia, and surgical wound infections are all life-threatening infections in the ICU (Diggle and Whiteley, 2020).

Fig. 7 showed that the isolates of *P.aeruginosa* with the highest resistance rates were ceftazidime (CAZ) and cefepime (FEP) with 93.6 % and 77.2 %, respectively, followed by tobramycin (TOB) at 60%, amikacin (AK) at 56%, ofloxacine (LEV) at 52%, netilmicine at 50%, imipenem (IPM) at 20%, and meropenem (MEM) at 44%. The result of the PCR assay for *MexXY-OprM, mexPQ-opmE* genes revealed that mexX (33/79) 43% was followed by the mexY (40/79) 51.89%, and the OprM (37/79) 48.1%, while the data for *mexPQ-opmE* was recorded as mexP (28/79) 36.70%, mexQ (36/79) 46.83%, and opmE (40/79) 51.89%.

The bacterium *P. aeruginosa* has become a widespread problem in the world correlated with drug resistance . These multidrug-resistant clinical isolates pose a serious health risk, with only a few treatment options available (Pachori *et al*., 2019). According to the present results, the obtained *P. aeruginosa* isolates exhibited high antibiotic resistance rates reaching to 93.6 % for ceftazidime (CAZ), 77.2% for cefepime (FEP) , 60% for tobramycin (TOB), 56% for amikacin (AK), 52% for ofloxacine (LEV), 50% for netilmicin, 20% for imipenem (IPM) and 44% for meropenem (MEM) which are commonly used to treat *P. aeruginosa* infections. Talebi-Taher*et al*. (2016) also found that all isolates were resistant to three or more tested medicines, including cefepime, ciprofloxacin, and gentamicin. Similarly, another study found that all *P. aeruginosa* isolates were highly resistant to ceftazidime (100%), cefotaxime (92%), ceftriaxone, and cefepime (74%) and that all *P. aeruginosa* isolates were classified as MDR. *P. aeruginosa* isolates were highly resistant to ceftazidime (100% for each), cefotaxime (92%), ceftriaxone and cefepime (74% for each) and all of these isolates were considered MDR (Abbas *et al*., 2018). The resistance rate for amikacin was found to be 45.5 %. This finding was comparable to the study correlated with amikacin (26 % and 30%, respectively) (Aljanaby

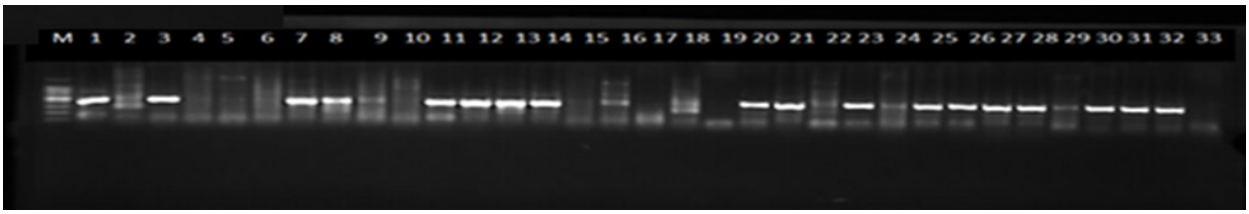

Fig. 1. *1.5% agarose gel electrophoresis of mexX gene amplicon (500 bp). M represent 100bp DNA ladder, lane 1-33 represent the isolates, TBE 1x, at Voltage 110volt for 50min*

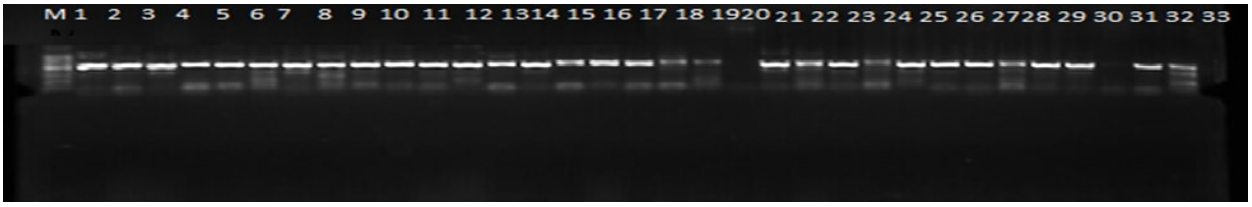

Fig. 2*: 1.5% agarose gel electrophoresis of mexY gene amplicon (554 bp). M represent 100bp DNA ladder, lane 1-33 represent the isolates, TBE 1x, at Voltage 110volt for 50min*

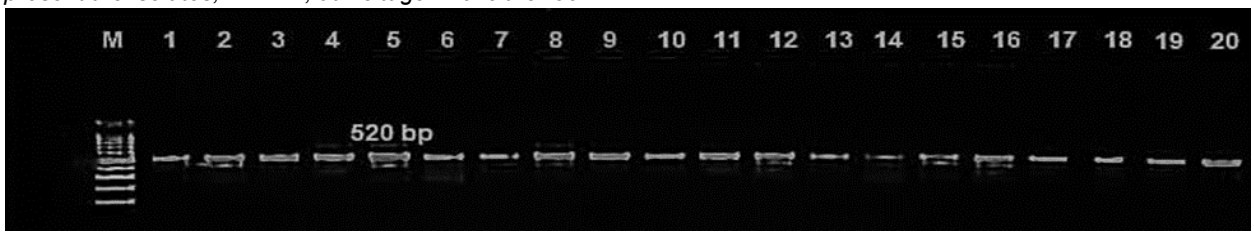

Fig. 3: *1.5% agarose gel electrophoresis of oprM gene amplicon (520 bp). M represent 100bp DNA ladder, lane 1-20 represent the isolates, TBE 1x, at Voltage 110volt for 50min*

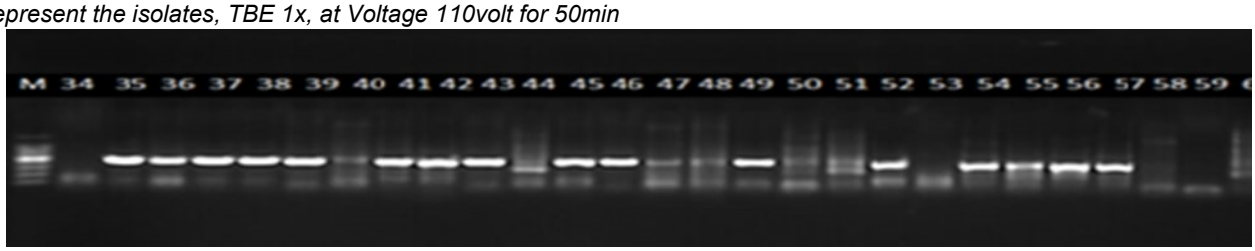

Fig. 4*: 1.5% agarose gel electrophoresis of mexP gene amplicon (534 bp). M represent 100bp DNA ladder, lane 34-60 represent the isolates, TBE 1x, at Voltage 110volt for 50min*

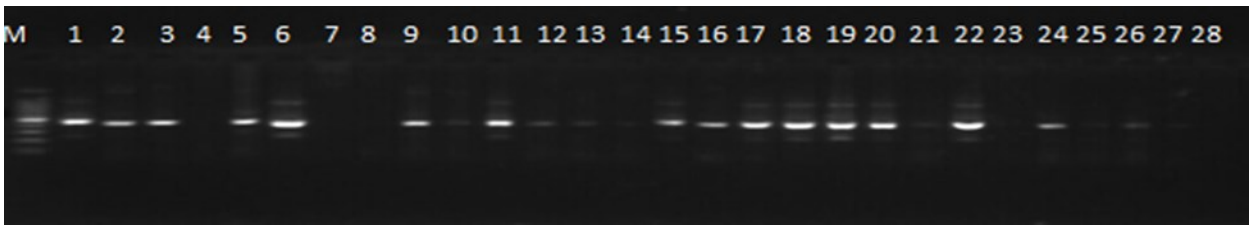

Fig. 5*: 1.5% agarose gel electrophoresis of mexQ gene amplicon (492 bp). M represent 100bp DNA ladder, lane 34-60 represent the isolates, TBE 1x, at Voltage 110volt for 50min*

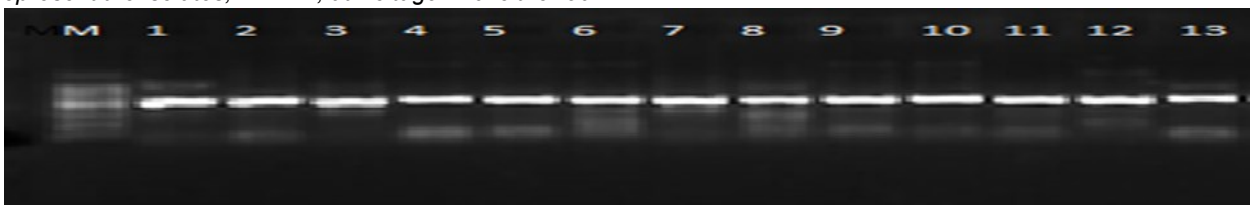

Fig. 6*: 1.5% agarose gel electrophoresis of opmE gene amplicon (522bp). M represent 100bp DNA ladder, lane 1-13 represent the isolates, TBE 1x, at Voltage 110volt for 50min*

and Aljanaby, 2018; Juhi *et al*., 2009).

High levels of resistance to beta-lactam antibiotics (ceftazidime (CAZ), cefepime (FEP), and piperacillin (PRL)) have been seen due to the activity of beta-lactamases. The resistance mechanisms such as beta-lactams, fluoroquinolones, and aminoglycosides have greatly reduced the therapeutic efficacy of these medications (Hussein *et al*., 2018). In present study, aztre-

Table 3. Distribution of *P. aeruginosa* isolates among diseases

Disease	Specimen	Bacterial Isolate No.	%
Urinary tract infections(UTIs)	midstream urine	28	35.4%
Respiratory tract infections (RTIs)	Broncoalveolar lavage	23	29.1%
Wound and burn infections	Wound burn swab	15	18.9%
Otitis Media	Ear swab	7	8.8%
Bacteremia	Blood stream	2	2.5%
Vaginosis	High vaginal swab	3	3.7%
Meningitis	CSF	1	1.2%

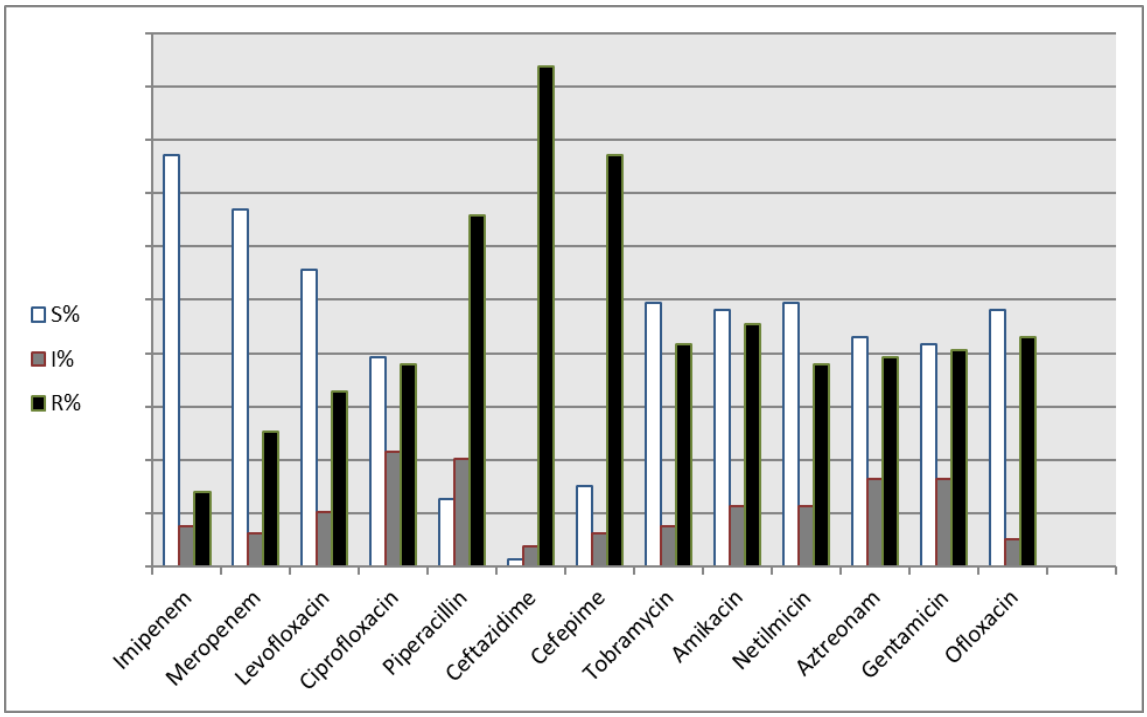

Fig. 7. *Antibiotic resistance percentage of P. aeruginosa to piperacillin (PRL), (ceftazidime (CAZ), cefepime (FEP),aztreonam (ATM), imipenem (IPM) and meropenem (MEM), levofloxacin (LEV), ciprofloxacin (CIP), ofloxacin (OFX), Tobramycin(TOB), Amikacin(AK), Netilmicin (NET),Gentamicin(GEN)*

onam resistance was 39.2 %. A comparable result (48%) was earlier documented by (Kateete *et al*., 2017), and another study (Hussein *et al*., 2018) reported a similar result (54.4%), while another study showed a different result that conflicts with the present results with 81.8% The percentage of resistance to aztreonam documented by other studies (Corehtash *et al*., 2015). A study from (Shaaban *et al*., 2019) and another study by El-Mahdy and El-Kannishy, 2019)reported that more than 70% of *P. aeruginosa* isolates were MDR isolates in the same field. Surprisingly, resistance to unrelated antibiotic classes was identified; most *P. aeruginosa* isolates had multidrug resistance to two or three of the antibiotic classes such as ceftazidime, cefepime, piperacillin, aztreonam, and levofloxacin. The results of the PCR assay for efflux pump genes *MexXY-OprM, mexPQ-opmE* revealed as concluded, *mexX* 43%, *mexY* 51.89%, *oprM* 48**,%1.** *mexP* 36.70% *mexQ* 46.83% and *opmE* 51.89%., as shown in Fig. 1-6. Also, Table 1 clarifies the identification of isolates using a *P. aeruginosa*-specific primer pair (16S rDNA Pseudomonas spp. amplicon (618 bp)) with 1.5% agarose gel electrophoresis at a voltage of 110 volts for 50 min. and Table 2 explains the detection of *MexXY-OprM, mexPQ-opmE* genes in isolates at a voltage of 110 volts for 50 min.

The Multi Drug Resistance (MDR) phenotype of *P. aeruginosa* is a key source of concern. In addition to traditional drug resistance mechanisms, *P. aeruginosa* can develop resistance to antibiotics as the infection progresses (Scoffone *et al*., 2021). Efflux pumps in the Resistance-Nodulation-cell Division (RND) family are able to translocate various compounds (including antibiotics) out of the bacterial cell in an atypical manner,

boosting bacteria's resistance to a wide range of therapies (Nikaido, 2018). Efflux pump genes MexXY-OprM contribute to intrinsic resistance to aminoglycosides, cefepime, tetracyclines, and erythromycin (Hocquet *et al*., 2006).

It is possible that overexpression of multidrug efflux pumps causes some of these multiple cross-resistances in *P. aeruginosa*, and that each efflux pump expels numerous antibiotic classes. By stimulating the upregulation of efflux pumps and selecting mutants with multidrug cross-resistance, inappropriate antibiotic use could eventually lead to resistance to other types of antibiotics.

Although the present study used a combination of phenotypic by antibiotic susceptibility test (AST) and genotypic approaches by conventional PCR techniques to diagnose resistance mediated by the two MexXY-OprM and MexPQ-OpmE efflux pumps in *P. aeruginosa*, phenotypic data interpretation in clinical strains remained difficult owing to the co-expression of resistance mechanisms other than efflux. Dalmolin and his co-workers determined that phenotypic methods developed and the high resistance of *P. aeruginosa* can be attributed to several mechanisms, including efflux pumps, reduced activity of outer membrane porins, and the production of B-lactamases (Dalmolin *et al*., 2017).

Conclusion

The present study concluded that efflux pump genes (*MexXY-OprM, mexPQ-opmE*) action resulted in multidrug resistance in *P. aeruginosa* isolated from clinical samples from patients suffering from drug resistance, especially towards ceftazidime, cefepime, piperacillin, aztreonam, and levofloxacin and that may lead to death. More research into efflux pumps appears to be a promising strategy for enhancing the clinical efficacy of antibiotics that are substrates for these pumps. This knowledge could be useful for rationalizing antibiotic selection at the individual patient level and developing antibiotic policies at the hospital level based on epidemiological surveys demonstrating the most common resistance mechanisms.

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Ethical approval

Informed consent was obtained from all human adult participants or parents or legal guardians of minors.

Conflict of interest

The authors declare that they have no conflict of interest.

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