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Research Article

Molecular investigation of *Pseudomonas aeruginosa mexAB-oprM* efflux pump genes from clinical samples and their correlation with antibiotic resistance

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Abstract
Pseudomonas aeruginosa, one of the majority of common opportunistic infections, has become a public health concern, exhibiting intrinsic and acquired resistance to a wide range of antimicrobials. The present work aimed to study the correlation between the *P. aeruginosa* efflux pump mexAB-oprM genes and antibiotic resistance to different types of antibiotics. All 79 isolates were screened by Pseudomonas chromogenic agar, which was used as a selective medium for the isolation of *P. aeruginosa*. After incubation at 37°C for 24 hr, the results were confirmed by PCR using specific primer pairs for the 16S rDNA gene of Pseudomonas spp. and P. aeruginosa for identification of the isolates. MexABoprM genes were investigated by PCR. The antibiotic susceptibility test was accomplished according to CLSI-2021 using the disc diffusion method for 13 antibiotics. The results revealed that the antibiotic susceptibility of *P. aeruginosa* was highly resistant to ceftazidime (93.6%) and cefepime (77.2%). In comparison, high sensitivity for imipenem (77.2%) and meropenem (67%) was observed. The antibiotic resistance patterns revealed that 38% of isolates were MDR multidrug resistant and 41% were non-MDR, and the mexA(65\79), mexB(49\79) and oprM (37\79) genes were distributed as mexA 83.5%, mexB 63.29% and oprM .481%, respectively. The present study concluded that mexABoprM may be highly associated with resistance to ceftazidime and cefepime and moderately associated with piperacillin, gentamicin and tobramycin.

Keywords: Antibiotic resistance**,** Efflux pump, *mexABoprM*, *Pseudomonas aeruginosa*

INTRODUCTION

Pseudomonas aeruginosa is a gram-negative bacterium that is aerobic. It is widespread in nature and can adapt to a variety of situations; within hospitals, it may be isolated from practically any source (Moosavi *et al*., 2020). It is a significant source of infection in both the community and in hospitals. When compared to other bacterial pathogens, infections with this bacterium have been associated with a greater rate of morbidity and mortality (Sedighi *et al*., 2015). *P. aeruginosa* infections are difficult to handle due to high intrinsic resistance to a vast domain of medicines (multidrug resistance) and a significant likelihood of resistance emergence during treatment (Livermore *et al*., 2012). Because of the low permeability of its outer membrane and the development of multiple efflux pumps with broad substrate specificity, *P. aeruginosa* is innately resistant to a variety of drug treatments (Livermore 2001). It can use plasmids, transposons, and bacteriophages to gain more resistance genes from other organisms (Lambert 2002). One of the highly distributed chromosomally encoded traits of resistance is efflux pumps. *On the other hand, P. aeruginosa* is a species characterized by low outer membrane permeability (Nikaido *et al*., 1991; Sugawara *et al*., 2006), mainly because of the presence of its closed channels porin OprF (Plésiat and Nikaido 1992; Sugawara *et al*., 2010). Additionally, P. aeruginosa and Escherichia coli share the same similarities in the existing low-permeability lipid bilayer, which leads to easy drug passage through the outer membrane of mutant *P. aeruginosa* with deficiency in outer membrane and efflux pump activity (Zimmermann, 1980; Preheim *et al*., 1982). Multidrug-

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resistant bacteria (MDR) have become a major public health threat worldwide (Lambert 2002). One of the common antibiotic resistance mechanisms is efflux pumps. These pumps act in reducing the antibacterial efficacy of the antibiotics by extruding them out of the cytoplasm, leading to minimization of the drug intracellular concentration (Munita and Arias, 2016). The MexAB-OprM system is one of the familiar multidrug pumps in *P. aeruginosa* that has a broad-ranging substrate profile. This system involves antibiotics, including β-lactams, quinolones, chloramphenicol, macrolides, sulfonamides novobiocin, trimethoprim, tetracyclines, cerulenin, pacidamycin, and thiolactomycin, and extends to nonantibiotics, such as dyes, detergents, triclosan, organic solvents, and tea tree oil (Pesingi *et al*., 2019). The MexA, MexB, and OprM subunits of the P. aeruginosa mexAB-OprM efflux pump were thought to operate as the membrane fusion protein, the transporter's body, and the outer membrane channel protein, respectively. MexB and OprM are linked by the MexA subunit, demonstrating that MexA is a membrane bridge protein (Glavier *et a*l., 2020). The MexB subunit is essential to the pump function, which traverses the cytoplasmic membrane 12 times, chooses antibiotics for export and is thought to move substrates using the proton gradient's energy (Pesingi *et al*., 2019). The OprM component is a lipoprotein that is tethered to the outer membrane and is thought to play a role in the final step of antibiotic extrusion, allowing the antibiotic to pass through the outer membrane and over the whole protein moiety (Smithers *et a*l., 2021). In the present work, we studied the correlation between the P. aeruginosa efflux pump mexAB-oprM genes and antibiotic resistance to different types of antibiotics.

MATERIALS AND METHODS

Collection and identification of samples

A total of 127 specimens were collected from different clinical sites from December 2020 to April 2021. Among them, only 79 isolates belonged to Pseudomonas aeruginosa recovered from 7 types of specimens (CSF, vaginal swab, blood stream infection, ear swab, wound burn swab, Broncoalveolar lavage and midstream urine). Isolation was performed from private and governmental hospitals. All 79 isolates were screened by Pseudomonas chromogenic agar, which was used as a selective medium for the isolation of P. aeruginosa, after incubation at 37°C for 24 hr and confirmed by PCR using specific primer pairs for the 16S rDNA gene of Pseudomonas spp. and *P. aeruginosa* (Table 1).

DNA extraction and PCR technique:

The Favorgen Genomic DNA Extraction Kit was used to isolate genomic DNA from different sources, including Gram-negative bacteria. Using specified primer pairs, conventional PCR was performed to amplify the target (Table 1). The PCR conditions are clarified in Table 2 for a mixture of 20 µl consisting of 5 µl of Maxime PCR Premix kit (i-Taq) (Intronbio/Korea), 1 µl of forwards primer (10 pmol/µl), 1 µl of reverse primer (10 pmol/µl), (2 µl) of target DNA, and 13 µl of nuclease-free water.

Antibacterial susceptibility test

The Disc-diffusion method was used to assess the *in vitr*o susceptibility of *P. aeruginosa* isolates to 13 drugs (Clinical and Laboratory Standards Institute, 2021). Isolates were activated for 18 hours at 37°C in brain heart infusion broth and then adjusted to 0.5 McFarland's standard (1.5108 CFU/mL) and distributed on Mueller Hinton agar with a cotton swab. 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.

Investigation of mexab-oprm efflux genes by PCR assay

Conventional PCR was used to amplify target DNA. PCR typically consisted of three consecutive steps (denaturation, annealing, and elongation) of repeated cycles to obtain the PCR product (amplicon). The PCR thermal cycling conditions are listed in Table 2. The size of the PCR products (5 µl) was analysed in a 1.5% (w/v) agarose gel by electrophoresis using 1× TBE buffer and visualized by staining with simply safe dye. Product size was determined by comparison with the Gene Ruler 100 bp DNA ladder. The conventional PCR technique was accomplished for three genes involving the genes mexA, mexB, and oprM.

RESULTS

In the present study, including the use of 13 antibiotics for resistance testing depending on CLSI, 93.6% and 77.2% represented the resistance values of *P. aeruginosa* isolates towards ceftazidime (CAZ) and cefepime (FEP), respectively, while just 68% represented piperacillin (PRL), and 62% represented gentamycin (CN). Ciprofloxacin (CIP) was detected in just 44% of patients. On the other hand, 60% was recorded for tobramycin (TOB), 48% for aztreonam (ATM), 56% for amikacin (AK), 52% for ofloxacine (OFX) and levofloxacin (LEV), 50% for netilmicine, 20% for imipenem (IPM) and 44% for meropenem (MEM) (Fig. 5). The pathogenic bacterium *P. aeruginosa* is resistant to many types of drugs, including aminoglycosides, quinolones, and β-lactams. The feature of resistance may be innate resistance caused by different reasons, such as low permeability of the outer membrane or by the overexpression of pumps and the production of enzymes that

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 mexAB-oprM efflux pump genes

Efflux pump	Genes	Sequence	Product (bp)	Annealing temp. (°C)
RND	mexA	GACGGTGACCCTGAATACCG CGACGGAAACCTCGGAGAAT	620	60.3
	mexB	GTCTACCCGTACGACACCAC GGTGGAAAGGAACATCCGGT	600	60.3
	oprM	GGTAGCCCAGGACCAGAATG GAGCTGGTAGTACTCGTCGC	520	62.5

inactivate drugs. The other type of acquired resistance was caused by either horizontal transfer of genes or the change that happened by mutations, and the third type of adaptive resistance included the production of a layer of biofilm that acts as a diffusion barrier to reduce antibiotic entrance inside bacteria (Mulcahy *et al*., 2010; Breidenstein *et al*., 2011). The present results revealed high percentages of resistance to the beta-lactams Ceftazidime, Cefepime, and Piperacillin. Beta lactamases, considered an innate resistance mechanism, lead to disabling beta lactam activity (Tannous *et al*., 2020; Al Muqati *et al*., 2021). The results of the PCR assay for mexAB-oprM genes revealed that most isolates have these efflux pump genes. As concluded, the mexA gene exhibited a high rate of expression, with 83.54%, followed by 63.29% for mexB, which was the second most highly expressed, and 48.1% for the oprM gene, as shown in Figs. 1-4. Fig. 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. Fig. 2 explains the detection of the *mexA* gene *amplicon (620 bp) in isolates from 1-20 at a voltage of 110 volts for 50 min.* Fig. 3 explains the detection of the *mexB* gene *amplicon (600 bp)* in isolates from 1-20 at a voltage of 110 volts for 50 min. 4 explains the detection of the *oprM* gene amplicon (520 bp) in isolates 1-20 at a voltage of 110 volts for 50 min.

DISCUSSION

P. aeruginosa is still one of the most frequent nosocomial diseases, and it has gained resistance to a number of antibiotics. The researchers discovered that the drugs studied had a significant prevalence of resistance. These findings revealed that *P. aeruginosa* exhibited substantial antimicrobial resistance, which could be linked to the inappropriate use of antibiotics in this situation. A rise in multidrug resistance among *P. aeruginosa* isolates has been reported in several studies in recent years. A long hospital stay and heavy antibiotic treatment could be to blame for the high frequency. MDR in *P. aeruginosa* can be caused by a number of methods, including the formation of multidrug efflux systems, the production of enzymes, or the loss of outer membrane protein (porin) and target mutations (Zahra *et al*., 2011). Horan and his colleagues proved that the interplay between two Pumps for Pseudomonas aeruginosa efflux, MexAB-OprM and MexEF-OprN, is implicated in the development of antibiotic resistance, and their findings support the effect of efflux pumps on the change in clinical strain classification from susceptible to intermediate/resistant1 in the absence of alternative mechanisms (Horna *et al*., 2018)

The present results showed that the isolates with a prevalence of *mexAB-oprM* genes showed resistance to antibiotics, particularly piperacillin (65.8%) (Fig. 5). This result is compatible with the study that reported rates of 37.0% and 27.3%, respectively, representing piperacillin resistance of *P. aeruginosa* isolates from the bloodstream of ICU and non-ICU patients (Vitkauskienė *et al*., 2010), although this result is far from the reported 85.4% pipracillin resistance rate of *P. aeruginosa isolates* (Ghanbarzadeh *et al*., 2015), and resistance to cephems was 93% for ceftazidime and 77% for cefepime (Fig. 6). This result was close to the study, which reported a resistance rate of *P. aeruginosa* Ceftazidime of 73.6% (Othman *et al*., 2014).

The resistance shown at high levels to antibiotics of beta lactams involves ceftazidime (CAZ), cefepime (FEP), and piperacillin (PRL) due to the activity of beta lactamase enzymes, which represent one of the intrinsic mechanisms leading to bacterial resistance (Hussein *et al*., 2018). Mechanisms that show resistance, including β-lactams, fluoroquinolones, and aminoglycosides, greatly reduce the clinical efficacy of these agents (Perletti *et al*., 2010).

Resistance to aztreonam (39.2%) (Figure 5). A close result (48%) was previously documented by research (Kateete et al., 201 (7and (54.4%) by the study

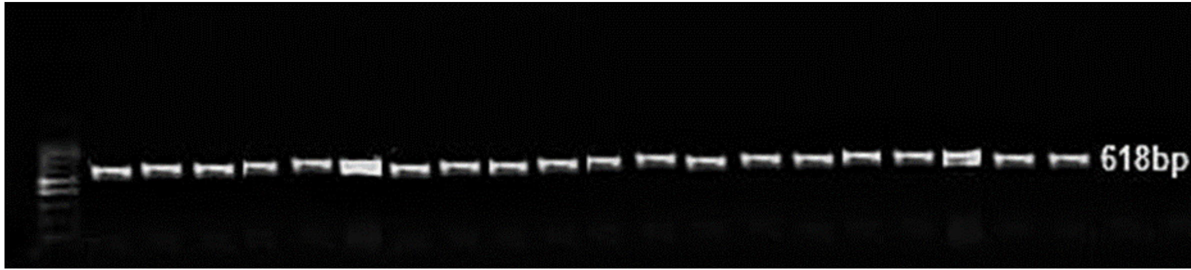

Fig. 1. *1.5% Agarose gel electrophoresis of 16S rDNA Pseudomonas spp. amplicon (618 bp). M represents the 100 bp DNA ladder, lanes 1-20 represent the isolates, TBE 1x, at a voltage of 110 volts for 50 min.*

Fig. 2. *1.5% Agarose gel electrophoresis of the mexA gene amplicon (620 bp). M represents the 100 bp DNA ladder, lanes 1-20 represent the isolates, TBE 1x, at a voltage of 110 volts for 50 min.*

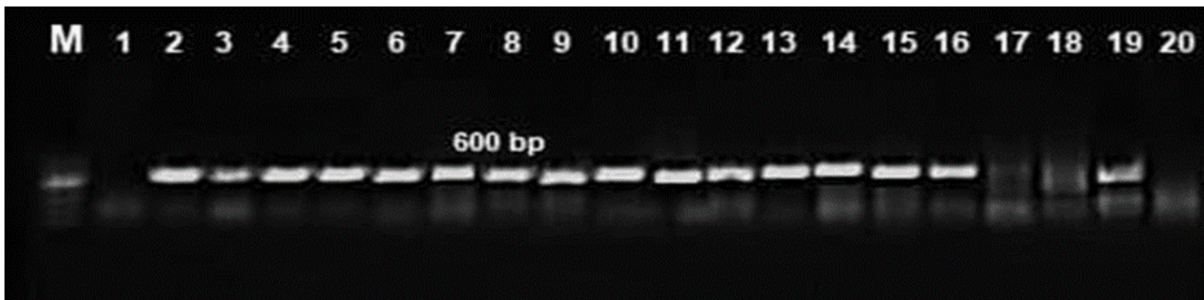

Fig. 3. *1.5% Agarose gel electrophoresis of the mexB gene amplicon (600 bp). M represents the 100 bp DNA ladder, lanes 1-20 represent the isolates, TBE 1x, at a voltage of 110 volts for 50 min.*

Fig. 4. *1.5% Agarose gel electrophoresis of the oprM gene amplicon (520 bp). M represents the 100 bp DNA ladder, lanes 1-20 represent the isolates, TBE 1x, at a voltage of 110 volts for 50 min.*

(Hussein *et al*., 2018) but disagrees with (81.8%), which was documented by another study (Ghanbarzadeh *et al*., 2015). The hydrolytic enzyme -lactamase is encoded by an inducible gene in the bacteria *P. aeruginosa*. This enzyme can break the amide bond of a -lactam ring, resulting in the inactivation of -lactam medicines, which is the cause of aztreonam, piperacillin, and ceftazidime resistance. (Pang *et al*., 2019). Resistance to carbapenems was 13.92% for imipenem and 25.3% for meropenem (Figure 8). The imipenem result was close to the study (Savari *et al*., 2016), which reported a rate resistance (22%), but different from the study (Fazeli *et al*., 2017) (98.7%), and far from the study (Coetzee *et al*., 2013), which reported an extremely higher rate (93.4%).

Carbapenem antibiotics (Imipenem, Meropenem) are lactams that are applied to deal with *P. aeruginosa* infections. Carbapenemase enzymes have been found in these bacterial strains, similar to those seen in Enterobacteriaceae, and are responsible for bacterial resistance. Furthermore, the porin OprD is known to aid in the internalization of imipenem and, to a lesser ex-

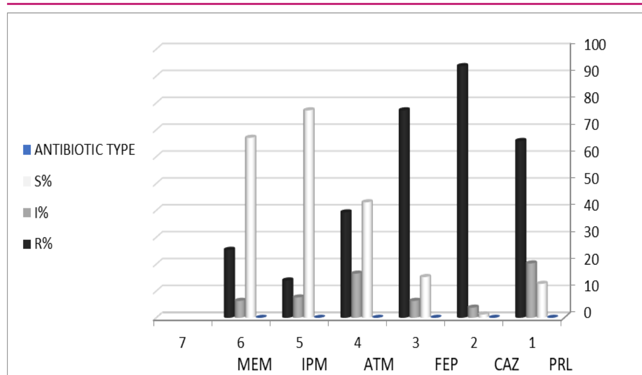

Fig. 5. *Antibiotic resistance percentage of P. aeruginosa to piperacillin (PRL), ceftazidime (CAZ), cefepime (FEP), aztreonam (ATM), imipenem (IPM) and meropenem (MEM).*

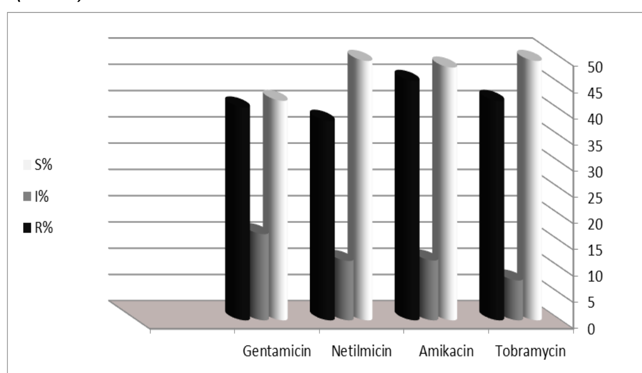

Fig. 6. *Antibiotic resistance patterns % of P. aeruginosa*

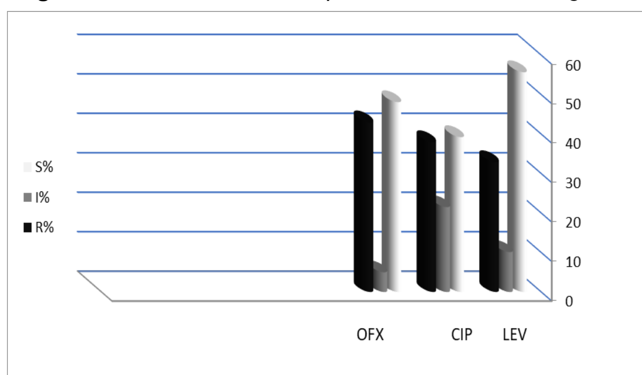

Fig. 7. *Antibiotic resistance patterns % of P. aeruginosa for fluoroquinolones (levofloxacin (LEV), ciprofloxacin (CIP), ofloxacin (OFX)*

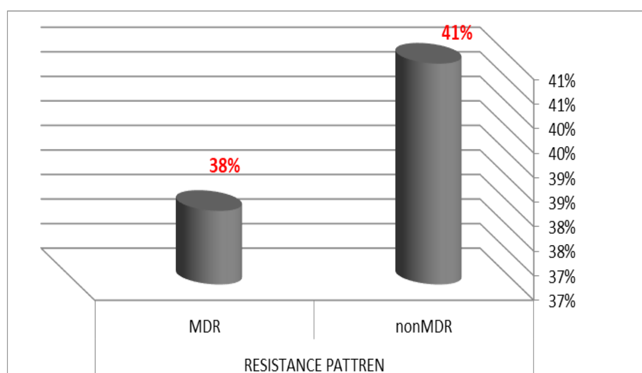

Fig. 8. *MDR distribution among p. aeruginosa isolates*

tent, meropenem but not other lactams. Imipenem susceptibility is lowered due to changes in OprD structure and expression. Modification of OprD is frequently associated with upregulation of efflux mechanisms, resulting in a high level of resistance to antibiotics other than imipenem, such as quinolones and aminoglycosides (Bassetti *et al*., 2018). Aminoglycosides resistance, gentamicin (40.5%), tobramycin (41.7%), amikacin (45.5%), and netilmicin (37.9%). Figure (6). The gentamicin resistance rate recorded in this study was close to that documented by the study (Vitkauskienė *et al*., 2010), which reported (37%), and incompatible with the study (Fazeli *et al*., 2017), which recorded the rate (91.2%).

The tobramycin resistance rate was 41.7%. This result is incompatible with (15.9 and 3.3%) by the study (Al-Derzi, 2012; Coetzee *et al*., 2013), respectively, and far away from study (Aljanaby and Aljanaby, 2018), which reported a rate of (78.8%). The amikacin results demonstrated a resistance rate of 45.5%. This result was close to those of studies (26%, 30%) (Aljanaby and Aljanaby, 2018; Juhi *et al*., 2009). On the other hand, it is incompatible with the findings of research with rete (77.4%) (Pang *et al*.2019) and (82%) other studies (Ghanbarzadeh *et al*., 2015). Aminoglycosides, quinolones, and beta-lactams are among the antibiotics that are resistant to *P. aeruginosa*. The pattern of resistance can be innate (low outer membrane permeability, coding for pumps, and the product of inactivating enzymes), acquired (horizontal transport of resistance genes or mutational changes), or adaptive (production of a biofilm layer that acts as a diffusion barrier to prevent drugs from reaching the cells) (Coetzee *et al*., 2013).

Resistance to flouroquinolones was 37.9%, 32.91%, and 43% to ciprofloxacin, levofloxacin, and ofloxacin, respectively (Figure 7). For ciprofloxacin, this result is compatible with the data reported in a previous study (23.9%) of isolates that were resistant to ciprofloxacin (Coetzee *et al*., 2013) but disagrees with another study in which the results showed 61.3% resistance (Othman *et al*., 2014). For Levofloxacin (32.9%), this rate was close to the results of the study with 30.6% and 36.1%, respectively (Al-Derzi, 2012; Lila *et al*., 2017), but disagrees with the study of 60.19% (Bassetti *et al*., 2018). Flouroquinolone drugs such as ciprofloxacin and levofloxacin interfere with the replication of DNA (33).

The results revealed that 38% of isolates were MDR and 41% were non-MDR (figure 8). This result was near (32%) MDR by (Rehman *et al*., 2019; Mirzaei *et al*., 2020) and disagrees with (69%) MDR by (Pérez *et al*., 2019), and the MDR rate in clinical isolates was 30 (51.7%) (44). Multiple drug resistance by *P. aeruginosa* has caused an increase in the mortality rate in hospitals from 25% to 60% (45).

Genotyping investigation using PCR surveying for mex-

AB-oprM efflux genes was performed and showed that 37 isolates from a total of 79 had all three genes of this pump, and most of these strains appeared to have multidrug resistance to different classes of antibiotics. Similar results were obtained from a study in Iran (Arabestani *et al*., 2015). Both studies proved the impact of these efflux pump genes on increasing resistance to antibiotics and found that the high rate of resistance to beta lactams (Ceftazidime and cefepme) may confer and/or develop resistance among different classes of antibiotics. According to a previous study (Yoneyama *et al*., 1997), the presence of the three proteins involved in this efflux is critical for pump function. In our study, several strains showed a greater increase in the mexA gene than in the mexB and oprM genes, leading to disruption of pump function, and the strain appeared to be susceptible to antibiotics due to their incomplete efflux parts, which agrees with a study in 2011 that concluded that efflux pumps correlated with multidrug resistance (Arabestani *et al*., 2015).

Conclusion

The present study observed an association between multidrug resistance and the efflux pump mexAB-oprM. In other words, the efflux pump may confer and/or develop resistance to various antibiotic classes. In all MDR isolated strains, Efflux mexAB genes were detected. It could be assumed that some of these multidrug cross-resistances among *P. aeruginosa* are caused by overexpression of the multidrug efflux pump *P. aeruginosa*, which has many mechanisms to avoid toxic substances. The pump mexAB-oprM is considered the most significant among efflux pumps. A strain that lacks MexAB-OprM showed lower levels of susceptibility in reducing the susceptibility towards different antibiotics, such as ceftazidime, cefepime, and piperacillin.

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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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