Molecular Characterization of Efflux Pumps in Beta-lactam Resistant *Pseudomonas aeruginosa*

Running title: Beta-lactam-resistant *Pseudomonas aeruginosa*

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Abstract

Background: *Pseudomonas aeruginosa* is a common causative agent of hospital-acquired infections and exhibits resistance to many antibiotics, including beta-lactams. One of the mechanisms of resistance to beta-lactams is the MexAB-OprM efflux pump. This study investigates the genetic pattern of resistant *P.aeruginosa* strains concerning the presence of gene encoding the MexAB-OprM efflux pump.

Methods: This descriptive-analytical study was conducted between 2022 and 2023 in Isfahan, and 110 strains of *P.aeruginosa* isolated from various clinical samples were identified. Antibiotic susceptibility testing of the isolates was conducted using the disk diffusion method, and strains producing extended-spectrum beta-lactamases (ESBL) were identified by the double disk diffusion. The gene encoding the MexAB-OprM efflux pump in these strains was investigated by polymerase chain reaction (PCR).

Results: A significant proportion of the 101 *P.aeruginosa* isolates originated from the emergency department and ICU-2, highlighting the clinical significance of this pathogen in these settings Meropenem demonstrated a high resistance rate (74%), while gentamicin exhibited lower resistance (33.33%). Resistance rates to amikacin, levofloxin, cefepime, ceftazidime, tazocin, ciprofloxacin, and ceftriaxone were 40.4%, 68%, 65.34%, 66.33%, 57.42%, 71.42%, and 50%, respectively. The prevalence of extended-spectrum beta-lactamases (ESBLs) was 29.7%, and the MexAB-OprM efflux pump gene was identified in 80% of ESBL-producing strains, suggesting a potential role in multidrug resistance.

Conclusion: Our findings reveal a strong association between the presence of the MexAB-OprM efflux pump and extended-spectrum beta-lactamase production in *P.aeruginosa*. This observation suggests that the MexAB-OprM efflux pump plays a pivotal role in the development of multidrug resistance in this pathogen. Future studies should focus on elucidating the molecular mechanisms underlying the regulation and function of this efflux system to inform the design of novel antimicrobial agents and combination therapies.

Keywords: MexAB-OprM Efflux Pump, *Pseudomonas aeruginosa*, Extended-Spectrum Beta-Lactamase

Introduction

Pseudomonas aeruginosa (P.aeruginosa) is a Gram-negative, opportunistic bacterium responsible for a wide range of human infections, including endocarditis, meningitis, sepsis, and chronic pulmonary infections in cystic fibrosis patients. This bacterium is a major causative agent of hospital-acquired infections and a significant contributor to mortality rates in immunocompromised individuals, such as leukemia patients, burn victims, and those with cystic fibrosis (1). Accounting for 21% of nosocomial infections, this bacterium is a significant pathogen, particularly in critical care settings. The use of medical devices, such as catheters, combined with the widespread use of broad-spectrum antibiotics among critically ill patients, contributes to increased susceptibility to infection (2). The increasing prevalence of antibiotic resistance in healthcare settings, notably intensive care units, has created a critical public health problem, complicating the management of bacterial infections (3). The increased consumption of beta-lactam antibiotics has resulted in elevated resistance rates, especially among cephalosporins. Bacteria produce beta-lactamases, a class of hydrolytic enzymes, as a defense mechanism. These enzymes hydrolyze the beta-lactam ring of antibiotics, rendering them inactive and compromising treatment outcomes (4). The global spread of antibiotic resistance among *P.aeruginosa* strains has severe clinical consequences. Resistance to beta-lactams in *P. aeruginosa* is multifactorial, involving alterations in porin expression, the production of beta-lactamases, and overexpression of efflux pumps, which actively expel antibiotics from the bacterial cell (5). Among these, efflux pumps are the most significant contributors to antibiotic resistance (6) and play a crucial role in expelling toxic compounds from the cell. Efflux pumps are classified into five families: The Major Facilitator Superfamily (MFS), ATP-binding Cassette (ABC) transporters, Multidrug and Toxic Compound Extrusion (MATE) family, Resistance-Nodulation-Division (RND) family, and Small Multidrug Resistance (SMR) family. Specifically, proteins belonging to the RND efflux pump family play a key role in antibiotic resistance in *P.aeruginosa*.

The MexAB-OprM efflux pump is a major efflux pump in *P.aeruginosa*, widely found in isolates of this bacterium, and plays a significant role in the intrinsic resistance of this bacterium to certain antibiotics. Therefore, the overexpression of the MexAB-OprM efflux pump plays a significant role in the antibiotic resistance of *P.aeruginosa* isolates to beta-lactam antibiotics (7). The principal purpose of this research was to ascertain the presence of MexAB-OprM efflux pump genes in clinical isolates of *P.aeruginosa* that produce extended-spectrum beta-lactamases.

Methods

This descriptive cross-sectional study involved the isolation and identification of 101 *P.aeruginosa* strains collected between 2022 and 2023 from educational hospitals in Isfahan. Following culture on blood agar and MacConkey agar media (Merck, Germany), the isolates were transferred to the research laboratory of Islamic Azad University, Falavarjan, and incubated at 37°C for 24 hours. *P.aeruginosa* strains were subsequently identified using a battery of microbiological and biochemical tests based on the Clinical and Laboratory Standards Institute (CLSI 2020) guidelines. These tests included Gram staining, catalase and oxidase tests, pyocyanin pigment production on Mueller-Hinton agar, TSI, OF, IMViC, and growth at 42°C.

Antibiotic resistance of the isolates was assessed using the standard disk diffusion method according to the Clinical and Laboratory Standards Institute (CLSI 2020) guidelines. Disks were purchased from Padtan Teb company and included amikacin (20 µg), meropenem (10 µg), levofloxacin (20 µg), ciprofloxacin (30 µg), ceftazidime (30 µg), tazocin (0.5 µg), gentamicin (30

µg), cefepime (30 µg), and ceftriaxone (30 µg). *P.aeruginosa* strain ATCC 27853 was used as a positive control.

Rosco's ceftazidime-clavulanic acid (10 µg/30 µg) double disk diffusion was utilized for phenotypic confirmation. In this method, the inhibition zone diameter of the ceftazidime disk was compared with the inhibition zone diameter of the ceftazidime/clavulanic acid disk, and the inhibition zone diameter of the cefotaxime disk was compared with the inhibition zone diameter of the cefotaxime/clavulanic acid disk. The presence of ESBL in the investigated strain was indicated by an increase of 5 mm or more in the inhibition zone diameter when the antibiotic was used alone (8).

DNA extraction was performed using the boiling method. Polymerase chain reaction (PCR) and specific primers were used to amplify the gene encoding the MexAB-OprM efflux pump. The forward and reverse primers used in this study to amplify the *mexA* gene were 5′-ACAACGCGGCGAAGGTCTCC-3′ and 5′-AGCAGCTCGTTGTTCGGGTTG-3′, respectively. A commercial PCR master mix (Sinaclon, Iran) was employed for the amplification reaction, supplemented with 1 µL of DNA template, 1 µL of each primer, and sterile distilled water to attain a final volume of 20 µL. The PCR was conducted on a thermocycler using 35 cycles with the following conditions: an initial denaturation step at 94°C for 5 minutes, followed by 35 cycles of denaturation at 94°C for 5 seconds, primer annealing at 54°C for 35 seconds, and extension at 72°C for 30 seconds. A final extension step was performed at 72°C for 5 minutes. Electrophoresis on a 1.5% agarose gel was carried out to visualize the PCR product, with a 100 bp DNA ladder used for molecular weight estimation.

After collecting the data, statistical methods were used to describe and analyze them. The collected data was analyzed using SPSS version 2025. The results were presented in two sections: descriptive statistics and inferential statistics. Descriptive statistics included quantitative and qualitative variables. Quantitative variables were summarized using mean and standard deviation. Qualitative variables were presented as frequencies and relative frequencies. In the inferential analysis, the relationships between qualitative variables were assessed using the Likelihood Ratio Test. Statistical significance was determined at the 0.05 level.

Results

The clinical setting of infection with *P.aeruginosa* was predominantly in males (60.4%) compared to females (39.6%) among the 101 isolates. The emergency department and ICU 2 were the most common sources of isolates, accounting for 16.8% of all cases (Figure 1).

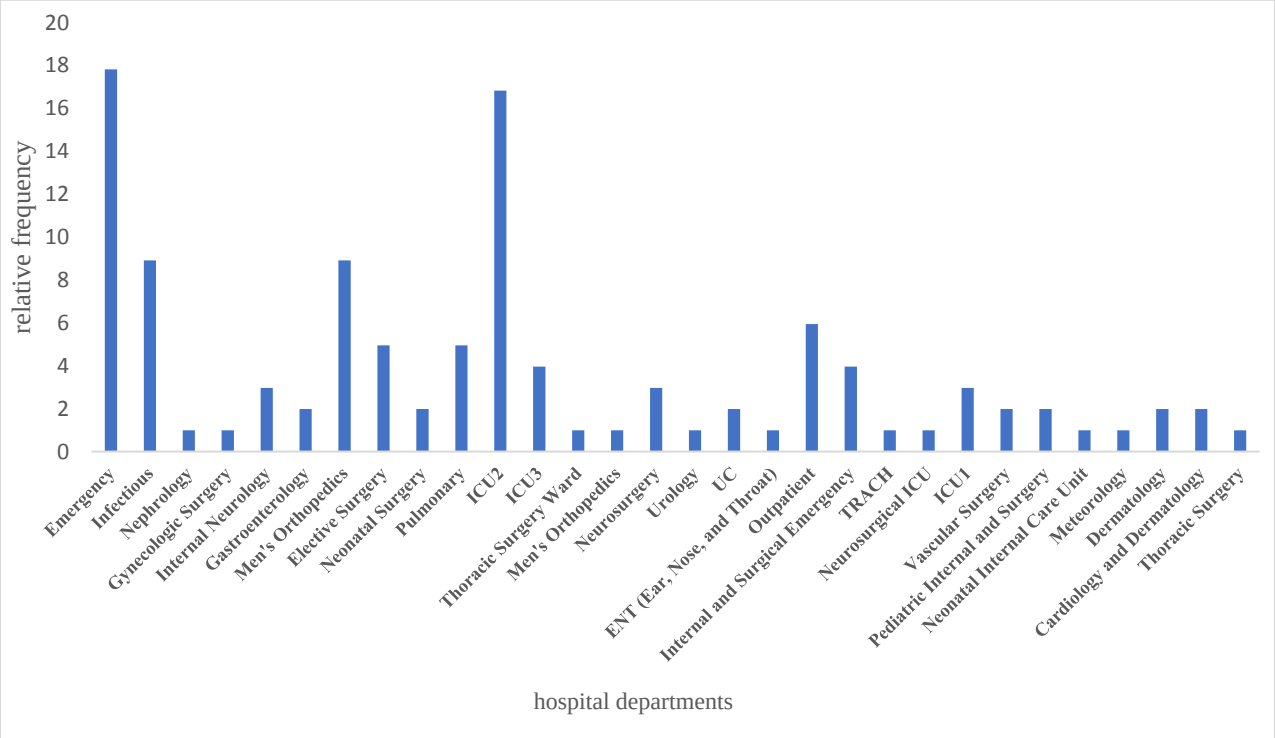

Figure 1: Distribution of total isolates by hospital departments

Antibiotic susceptibility testing revealed that meropenem demonstrated the highest resistance rate (74%), whereas gentamicin exhibited the lowest resistance rate (33.33%) among the strains investigated (Table 1).

Table 1: Antibiotic susceptibility testing of *P.aeruginosa*

Antibiotics	Resistant isolates (Percentage)	Intermediate isolates (Percentage)	Sensitive isolates (Percentage)
Amikacin	40.40	4.04	55.55
Meropenem	74	1	25
Levofloxacin	68	4	28
Ceftazidime	66.33	1.98	31.68
Tazocin	57.42	13.86	28.71
Gentamicin	33.33	0	66.66
Ceftriaxone	50	0	50
Cefepime	65.34	2.97	31.68
Ciprofloxacin	71.42	5.10	23.46

The prevalence of ESBL production among the 101 strains was 29.7% notably, 80% of the ESBL-positive strains possessed the *MexA* gene. The emergency department contributed 40%, the surgical ward 26.6% and the infectious disease ward 33.3% to the total number of ESBL-producing isolates. Urinary tract infections accounted for 23.33% of the ESBL-positive samples. Statistical analysis confirmed a significant correlation between ESBL production and the presence of the *MexA* gene ($P < 0.05$), suggesting a potential role for *MexA* in ESBL expression.

Discussion

P.aeruginosa is a significant pathogen in nosocomial infections with a high level of antibiotic resistance. This resistance arises from inherent mechanisms such as low permeability, efflux systems, and acquisition of resistance genes through plasmids, transposons, integrons, and biofilm production. Efflux pumps can extrude a wide range of antimicrobial compounds without any structural or functional similarities between them (9; 10). The antibiotics used to treat infections caused by this bacterium fall into the category of these toxic compounds, and their expulsion from the bacterial cell significantly reduces their therapeutic efficacy (11).

In this study, the antibiotic resistance pattern was determined using the disk diffusion and combined disk diffusion methods. The presence of the *mexA* gene was then investigated using PCR. According to the results, the highest resistance was observed against meropenem, ciprofloxacin, ceftazidime, and cefepime, while the lowest resistance was against gentamicin and amikacin. Beta-lactamase testing showed that 29.7% of the strains produced this enzyme. PCR results indicated the presence of the *mexA* gene in 80% of clinical isolates. Statistically, there was a significant association between the presence of the *mexA* gene and antibiotic resistance.

The prevalence of these microorganisms was determined to be 61 samples (60.39%) in males and 40 samples (39.61%) in females. Consistent with a previous study by Munde et al. (2011), our findings suggest a higher susceptibility to *Pseudomonas* infections among males compared to females (12). Additionally, the majority of samples were from the emergency department (16.8%) and ICU2 (16.8%). The prevalence of antimicrobial resistance is probably increasing due to the widespread use of antibiotics in the hospital environment. This can selectively increase the pressure on the hospital microbiota and in turn increase the emergence and spread of resistant bacteria. Therefore, it affects the morbidity and mortality of patients. ICUs are major sources of dissemination of multidrug-resistant organisms, where the selection pressure for the emergence of resistance is highest. *P.aeruginosa* grows significantly in hospital environments and its establishment is directly related to the length of hospital stay (13). These bacteria often lead to outbreaks of nosocomial infections, especially in ICU patients and people with weakened immune systems (14).

These results are consistent with the findings of this research. The present study showed that meropenem had the highest resistance rate (74%) and gentamicin had the lowest resistance rate (33.33%). Although meropenem has been a mainstay in the treatment of *P.aeruginosa* infections, the emergence of resistance to this antibiotic necessitates the exploration of new therapeutic approaches. In a study by Mihani et al., (2007) on 100 clinical isolates of *P.aeruginosa* in Ahvaz, resistance to imipenem and meropenem was 41% and 23%, respectively (15). In a study by Aminzadeh and colleagues in 2012 on isolates of *P.aeruginosa* from the ICU, the highest and lowest resistance rates were 87% to ceftazidime and 6.5% to imipenem, respectively. In the present study, the highest and lowest resistance rates were to meropenem and gentamicin, respectively. The differences in results among these studies indicate that the antibiotic resistance pattern of the bacteria has changed. This discrepancy could be due to different antibiotic consumption patterns,

geographical regions, resistance patterns in different areas, and the excessive use of antibiotics in our country (16).

The prevalence of beta-lactamase production in *P.aeruginosa* strains was reported to be 41% by Shakibaei et al. (1999) and 49% by Bharty et al. (2024), both of which were higher than our observed prevalence (17, 18).

Comparing these results shows that the levels of beta-lactamase enzymes vary among isolated strains from different countries and even from different hospitals within the same country. This variation depends on the infection control system and the treatment methods of patients in those hospitals. In the present study, 80% of the beta-lactamase-producing strains had the MexAB-OprM efflux pump on their chromosome. Rahmani-Badi et al.**,** (2007) observed the MexAB-OprM efflux pump in only 17% of the studied strains (18). In contrast, Najjarpirayeh et al. (2010) did not observe the MexAB-OprM efflux pump in any of the 33 MDR strains of *P.aeruginosa*. The results of these studies do not align with our findings, which may indicate an increased prevalence of MexAB-OprM efflux pump genes among hospital strains in recent years (19).

Conclusion

Investigating the underlying causes of antibiotic resistance among common hospital pathogens is paramount. A comprehensive understanding of these mechanisms is essential for selecting appropriate medications, modifying treatment protocols, and managing the judicious use of broad-spectrum antibiotics to mitigate the spread of resistance and prevent hospital-acquired infections. The findings of this study demonstrate a statistically significant association between the presence of the *mexA* gene, the MexAB-OprM efflux pump, and antibiotic resistance in *P.aeruginosa*. Given the critical nature of antibiotic resistance, further research on other efflux pumps, comparative analyses of antibiotic resistance patterns, and the correlation between efflux pump expression and the clinical origin of strains is warranted.

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Ethical statement

This study was approved by the Ethics Committee of the Falavarjan Branch, Islamic Azad University (Ethical approval code: IR.IAU.FALA.REC.1400.029).

Conflicts of interest

There is no conflict of interest.

Author contributions

All the authors have made substantial, direct, and intellectual contributions to the work and read and approved the final manuscript.

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