Antibiotic resistance patterns of efflux pumps MexAB-Opr M in pathogenic *Pseudomonas aeruginosa* isolates

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Research note

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Abstract

Objectives

Pseudomonas aeruginosa is one of the most important causes of Hospital infection especially in burn victims. The current study aimed to determine antibiotic resistance of the efflux Pumps MexAB-Opr M. In the present study, 115 samples of urine, blood, sputum, and ICU were collected from the reconstructive section of the patients. The drug susceptibility patterns were determined by disk diffusion method. Phenotypic activity of the efflux pump from the E-test was evaluated, in the presence and without the presence of efflux pump inhibitor. The *MexAB* gene was analyzed by PCR reaction.

Results

The resistant isolated was shown to be Ciprofloxacin 33.91%, Nurfloxacin 38.26%, Gentamicin 71.7%, Nalidixic acid 95.95%, Ceftazidim 38.46%, Emipenem 24.34%, Meropenem 26.36%, and Cefotaxim 40.86%. The highest and lowest resistance rates were Co-trimoxazole and Piperacilin, respectively. The findings of PCR reaction among 115 *P. aeruginosa* isolates indicated that 62.62% was MexAB gene. The results of MIC with E-test revealed that the role of efflux pumps in antibiotic resistance was 19 isolated. Due to the importance of antibiotic resistance to investigate other efflux pumps, comparison of efflux pump involvement in antibiotic resistance, and relationship between efflux pumps MexAB-Opr M are highly required and suggested.

Introduction

Pseudomonas aeruginosa is a pathogenic, Gram-negative, Opportunist, movable and mandatory aerobic bacteria. Occasionally, the formation of lethal infections in their hosts, can be a most important factors causing secondary Hospital-acquired infections in patients with burns and ICU (1, 2). *Pseudomonas* infections are commonly reported associated to infections, urinary tract infections and pulmonary diseases such as cystic fibrosis (3). One of the major problems in the treatment and prevention of infections caused by *P. aeruginosa* is antibiotic resistance. This bacteria has a direct relationship with increasing antibiotic resistance. Almost 30% of Hospital infections are related to antibiotic resistance of this bacteria. *Pseudomonas* antimicrobial resistance depends on several factors, such as the permeability of the outer membrane, the presence of induction AmpC, chromosomal β -lactamases, and the action of multidrug-based efflux systems. The most important causes of antibiotic resistance are pumps such as MexAB and OprM (4). Other Mex systems are less important. The role of the inherent aspect of pumps in the development of multi- drugs resistance is through the pumping of drug compounds into the outside of the cell. Decreasing the permeability of the membrane and activation of efflux system result in enhances efflux system of type resistance nodulation-cell division and also, increases drug resistance. Over the past several decades, the basic problem of drug resistance in many human pathogens is the inappropriate and excessive use of antimicrobial compounds (4, 5). Generally,

several bacterial multi-drug efflux pumps are exist. These groups include Resistance Nodulation Division (RND), ATP Binding Cassette (ABC), Major Facilitator Superfamily (MFS), Small Multidrug Resistance (SMR), and Multidrug and Toxic Compound Extrusion (MATE). Efflux pumps are clinically effective in relation to RND or MFS groups, which, by releasing energy and proton stimulus, play an important role in exude of antibiotics from the cell (6–8). Today, the biggest concern in the areas of care and burn is Hospital burns in patients with burns, which leads to death in more than 50% of patients. *P. aeruginosa* is the most important cause of Hospital infections in patients (9). This study was conducted to determine antibiotic resistance efflux pumps Mex AB-OprM using phenotypic and genotypic methods.

Methods

Study design

In cross-sectional descriptive study, from 115 *P. aeruginosa* isolates in patients of referred to Zare Hospital of Sari between 2016–2017, samples of wounds, urine, blood, sputum, ICU, and repair section were collected under sterile conditions. Afterward, they cultured in nutrient broth, and on Mac Conkey agar and Blood agar. Then, were incubated for 4 h at humidified 5% CO₂ and 37°C. Also, some biochemical tests such as oxidase, catalase, OF, SIM, MRVP, TSI, citrate tests, production of pigment, and smell were done (10). Bacterial samples in order to perform the test graduate in medium Tryptic soy broth containing 20% glycerol incubated at a temperature 20°C were kept.

Disk Diffusion method

The resistance patterns of the strains against the following antibiotics were determined by the Kirby bauer disk diffusion method as per the Clinical and Laboratory Standard Institute (CLSI) guidelines (11): Ciprofloxacin (5 µg), Nurfloxacin (10 µg), Gentamicin (10 µg), Nalidixic acid (30 µg), Ceftazidim (30 µg), Imipenem (10 µg), Meropenem (10 µg), Piperacilin (100 µg), Ofloxacin (5 µg), Cefotaxim (30 µg), Co-trimoxazole (1/25, 23/75 µg), and Tobramycin (10 µg) (MAST English). In brief, standard bacterial suspension was prepared by growth of bacteria on Muller Hinton agar (Merck, Germany) with subsequent adjustment equal to 0.5 McFarland inoculated on Muller Hinton agar and then, antibiotic discs were placed on the plate at identical distances. Afterward, plates were incubated for 18–24 hours at 37°C. The diameter of the zone of growth inhibition was measured and compared to standard values (sensitive, intermediate, and resistant).

Phenotypic method

For determination of minimal inhibitory concentrations (MIC) with E-test, antibiotics Levofloxacin (Himedia), and inhibitor Phenyl-Arginine-β-Naphthylamide (Sigma, Switzerland) were used in accordance with CLSI guidelines (12). Briefly, inhibitor Phenyl-Arginine-β-Naphthylamide on Muller Hinton agar added, and then, suspension of the microbial equal to 0.5 McFarland Muller Hinton agar applied. Bars E-test Levofloxacinin on Muller Hinton agar plate were put for 24hours at 37°C under sterile conditions. After the elapse of time desired, MIC readings and results were reported based on the standard table according

to CLSI. The difference between numbers of resistant strains was considered, in the presence, and without the presence of Paβ Nof, based on the following formula as resistance of efflux pumps. $A = B - C$

A, numbers of resistant strains to known efflux pumps. B, numbers of resistant strains without the presence of PAβN. C, numbers of resistant strains in presence PAβN.

PCR of MexA-B genes

DNA extraction was performed using simple boiling method of bacterial colonies which were identified as *P. aeruginosa* by phenotypic criteria (13). PCR primers were listed in Table 1(14, 15). These primers amplified a fragment of 293 base pairs (bp) and 280 bp target genes. PCR reaction was done with volume 20 µl including: 2 µl DNA template, 10 µl PCR buffer, 7 µl distilled water, and 1 µl primers. The amplification program consisted initial denaturation at 94 °C for three minutes, followed by 35 cycles of denaturation at 94°C for 30 seconds, annealing at 58°C for 30 seconds for the Mex A gene, and 58°C for 30 seconds for the Mex B gene, extension at 72°C for 30 seconds, and a final extension at 72°C for 7 minutes. The PCR products were analyzed using agarose gel electrophoresis on 1.5% agarose containing ethidium bromide. DNA green viewer, and DNA ladder 100 bp were used at the size marker. The findings were recorded using gel documentation system.

Table 1
Primer sequences mexA,

Primers	Sequence 5'-3'	Product length
MexA	CGACCAGGCCGTCAGCAAGCAGC(Forward) GGAGACCTTCGCCGCGTTGTCGC(Reverse)	293 bp
MexB	TGTCGAAGTTTTTCATTGATG(Forward) AAGGTCACGGTGATGGT(Reverse)	280 bp

Statistical analysis

The SPSS software (SPSS18) was used for data analysis.

Results

In the current study, from 115 *P. aeruginosa* isolates, 39 female patients and 76 male patients were merged. More patients suffered from burn of the thermal in age category (20–40) 82 people, age category (40–60) 23 people, and age category (60–90)10 people attended. Also, in this study, 53 (%46.10) wound, 26 (%22.60) urine, 18 (%15.65) blood, 10 (%8.70) sputum, 5 (%4.35) ICU, and 3 (%2.60) department of reconstructive were seen. Resistant isolates of *P. aeruginosa* in activity efflux pumps compared to antibiotics of Ciprofloxacin (%33.91), Norfloxacin (%38.26), Gentamicin (%71.30), Nalidixic acid (%95.65), Ceftazidim (%38.26), Emipenem (%24.34), Meropenem (%26.95), Piperacilin (%19.13), Ofloxacin (%62.60), Cefotaxime (%40.86), Co-trimoxazole (%96.52), and Tobramycin (%52.18) Table 2.

Table 2
Antimicrobial Susceptibility Patterns of isolates
Paeruginosa (N = 115) from wound, urine, blood, sputum,
ICU, by the Means of the Disk Diffusion Method.

Antibiotic	Resistant	Sensitive	Intermediate
Ciprofloxacin	33.91	62.61	3.48
Norfloxacin	38.26	60	1.74
Gentamicin	71.30	24.35	4.35
Nalidixic acid	95.65	4.35	0
Ceftazidim	38.26	59.14	2.60
Emipenem	24.35	75.65	0
Meropenem	26.95	73.05	0
Cefotaxim	40.87	56.53	2.60
Piperacilin	19.14	80.86	0
Co-trimoxazole	97.40	0	2.60
Ofloxacin	62.60	32.18	5.22
Tobramycin	52.18	43.47	4.35

PCR products in MexA and MexB genes to arrange 293, 280 bp. Molecular review activity efflux pump represents presence of MexA and MexB genes to arrange in 0 (%0), 72 (%62/70) was isolated (Fig. 1). Our findings showed that dilution reduce results in resistance *P. aeruginosa* isolates to Levofloxacin (Table 3).

Table 3
Dilution reduce results in resistant isolates *P.aeruginosa* to Levofloxacin

Dilution reduce MIC	MIC-PAβN	MIC-NON PAβN	Resistant bacteria
More than 4 times	0.64	12	Sample 1
3 times	4	32	Sample2
More than 5 times	0.43	16	Sample 3
More than 4 times	0.47	12	Sample 4
1 times	4	8	Sample 5
More than 5 times	0.64	24	Sample 6
More than 4 times	0.47	12	Sample 7
More than 4 times	0.67	12	Sample 8
More than 4 times	0.49	8	Sample 9
1 times	4	8	Sample 10
More than 3 times	0.64	8	Sample 11
More than 5 times	0.43	16	Sample 12
More than 2 times	4	24	Sample 13
3 times	4	32	Sample 14
More than 4 times	0.47	8	Sample 15
3 times	2	16	Sample 16
More than 2 times	4	24	Sample 17
More than 2 times	2	12	Sample 18
More than 3 times	0.67	8	Sample 19

Discussion

This study revealed that resistance to Piperacilin was 19.13%, while our study in 2010 in Tehran was 170 *P. aeruginosa* isolated directly from sample ulcer patients performed. Resistance to Piperacilin 67% was reported (16). In a study conducted by Sonnet in 2012, indicated that inhibitor PAβN reduction MIC antibiotics Fluorquinolones in *P. aeruginosa* is the impact (17). In this study dilution reduce most MIC antibiotic Levofloxacin in range more than four times was seen. Several studies in field the effect efflux pump inhibitors and the impact on increase MIC is done antibiotics difference. Pannek in 2007 in Germany, Peleg in 2007 in America, Vecchione in 2009 in America, and Li in 2011 in China on increase property anti-microbial antibiotics difference of the inhibitors efflux pump have been emphasis (18–21).

So far, further studies on antibiotics resistance of *P. aeruginosa* isolates are done from clinical samples. In a review study conducted by AL Marjani in 2015 in Iraq on the role efflux pump *P. aeruginosa* isolates in creation antibiotic resistance, Norfloxacin (%14.8), Ciprofloxacin (%7.4), and Levofloxacin (%11.1) was reported (22). In this study resistance to Nurfloxacin (%38.26) and Ciprofloxacin (%33.91). In study of Salehi in 2014 on Quinolone resistance with efflux pumps MexAB-OprM, antibiotics resistance been Piperacilin (%44.9) and Imepenem (%40.91) (23). In this study, resistance to Piperacilin (%19.13) and Imepenem (%24.4). In our study, resistance to Meropenem, Ceftazidim, and Ofloxacin to arrange (%26.95), (%38.26), (%62.60) has been reported, respectively. In study of Khosravi in 2016 on the role of MexAB-OprM in antibiotics resistance of *P. aeruginosa* isolates from patients with burn and wound infections, resistance to Meropenem (%62), Ceftazidim (%68), and Ofloxacin (% 73.33) reported (24). In study of conducted by Rastegar Lari, frequency of *P. aeruginosa* resistance to Ciprofloxacin and Gentamicin isolated from burn centers of Tehran, Iran, was over 85% (25). Also, study by Brown and Izundu in 2004 in Jamaica revealed that resistance percentage to Nalidixic acid were %82.4 and resistance rates were 25.5% or lower for Gentamicin (26). In study of Deplano, 100% resistance to Meropenem, Imipenem, Gentamicin and Ciprofloxacin, 94% to other antibiotics were reported (27). A study by Yi showed that all isolates of *P. aeruginosa* which were resistant to Imipenem 86.6% and Meropenem 44.4% (28). In study of AL Grawi in 2012 in Iraq, all 53 *P. aeruginosa* isolates had MexAB-OprM gene (29). While in study of conducted by Rahmanibadi in 2007, only 17% of isolates had efflux pump MexAB-OprM gene. Najjarpeerayeh in 2011 in *P. aeruginosa* isolates didn't view efflux pump MexAB-OprM (30). Goli in 2016 evaluated the role of efflux pump MexAB-OprM overexpression in antibiotics resistance of *P. aeruginosa* clinical isolates, four-fold reduction in MIC with PAβN sixty-two present and 65% of isolates overexpressed MexB showed (12). In this study 72 (%62.70) isolates was seen efflux pump MexAB-OprM. The results of study expresses important communication in creation of antibiotics resistance *P. aeruginosa* can be established MexB gene. In this study, according to review phenotypic efflux pump signs a relationship between MexB in create is antibiotic resistance. Also, by comparing phenotypic and genotypic methods, we can use inhibitor PAβN amount of activity other efflux pumps put reviewed.

Limitations

The present study had some limitations. First, this was a single-center study; therefore, generalization of the results to other regions requires further investigations. Second, it was a retrospective study with a relatively small sample size. More studies with a larger sample size.

Abbreviations

ICU: intensive care unit, PCR: Polymerase chain reaction, ABC: ATP Binding Cassette, MFS: Major Facilitator Superfamily, SMR Small Multidrug Resistance and MATE :Multidrug and Toxic Compound Extrusion.

Declarations

Ethics approval and consent to participate

This study was in accordance with the declaration of Helsinki and an ethical permission was sought from the institutional Ethics Committee of Faculty of Medicine, Mazandaran University of Medical Science, Sari, Iran (Approval No. IR.MAZUMS.1395.S247). However, because we only used leftovers from clinical specimens, the local ethics committee waived the need for informed consent.

Consent for publication

There is no limit to the publication.

Competing interests

We declare that we have no conflict of interest.

Availability of data and materials

All data generated or analyzed during this study are included in this published article.

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Authors' contributions

Mohammad Ahanjan and Javad Rasouli: conceived and design of the study. Behnam hashemi and Hamed Afkhami: performed the statistical analysis and interpreted the data. Javad Fathi, Mahtab Hadadi: Mohammad Ahanjan: Supervised data collectors. Mansoor Khaledi, Reza valadan and Javad fathi: Drafting the article or revisiting it critically for important intellectual content. All authors read and approved the final manuscript.

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