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Relation between Resistance to Antipseudomonal β-Lactams and *ampC* and *mexC* Genes of *Pseudomonas aeruginosa*

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ABSTRACT

***Background*:** In order to select a better antibiotic choice for treatment of *Pseudomonas aeruginosa* infections, this study was conducted to determine the frequency of resistance to some antipseudomonal β-lactams in *P. aeruginosa* isolates from patients in Tehran, Iran. In addition, the relation between presence of genes known to be responsible for resistance to β-lactams (*ampC*, *mexC1,2*, and *mexC3,4* genes) and resistance phenotype among *P. aeroginosa* isolates was evaluated.

***Methods*:** *P. aeruginosa* strains were isolated and identified by routine methods and PCR for *oprL* gene. Disk diffusion method was employed to determine the antimicrobial susceptibility pattern according to CLSI recommendations. PCR was used to detect the resistance genes.

***Results*:** Among 100 isolates of *P. aeruginosa*, 82% had *ampC*, 86% *mexC1,2* and 89% *mexC3,4* genes and combinations of these genes were seen in most of isolates and only 3% of isolates had none of these genes. Resistance to mezlocillin, cefepime, ceftazidime and piperacillin/ tazobactam was seen in 46%, 41%, 36% and 29% of isolates, respectively. Significant relation (*P* value ≤0.05 by Chi-square or Fisher Exact test) was observed between the presence of *ampC* gene and resistance to all the studied β-lactams in this study. No relation was observed for *mexC* genes, although many of isolates containing these two genes were phenotypically resistant.

***Discussion*:** This study had shown for the first time, the presence of *ampC* and *mexC* genes in significant percent of clinical isolates of *P. aeruginosa* in Tehran, Iran, and relation between presence of *ampC* gene and resistance to β-lactams.

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Introduction

Pseudomonas aeruginosa is a member of skin normal flora in humans but has emerged as a major nosocomial pathogen in immune-compromised patients as a result of burns or other severe trauma, and underlying diseases including cancer, diabetes and cystic fibrosis (1).

This bacterium is associated with different kinds of infections such as otitis externa, burn wounds, urinary tract infections, ventilator associated pneumonia and septicemia (2). It is responsible for about 10% of nosocomial infections and is considered as a major cause of mortality and morbidity in these patients (3).

P. aeruginosa has different mechanisms of resistance against antimicrobial agents; therefore, it is an important problem in clinical centers (1). It uses special outer membrane porins to restrict the uptake of antibiotics and the secondary resistance mechanisms such as energy-dependent multidrug efflux and chromosomally producing β-lactamase (4). A major component of bacterial resistance to many classes of antibiotics is expelling them out of bacteria cells that occur due to the activity of membrane transporter proteins called drug efflux pumps (5). Extrusion of antibiotics and restricted uptake through porins of outer membrane can cause a decrease in intracellular concentration of antibiotics (6). There are many genes that encode putative efflux pumps. One of the important families of chromosomally encoded bacterial efflux pumps is the resistance nodulation division (RND) family. This kind of pumps has three components: a membrane fusion protein that is associated with the cytoplasmic membrane, a periplasmic accessory protein (such as MexA, MexC, MexE and MexX), and an outer membrane protein (OMP) (such as OprM, OprJ and OprN) (7-11). As a result of synergy between outer membrane impermeability and chromosomally encoded efflux pumps, *P. aeruginosa* shows a remarkable intrinsic resistance to various antibiotic families (9). This antibiotic resistance is mediated by several resistance genes using multiple mechanisms resulting in making the treatment of many Pseudomonal infections more complicated.

The existence of three resistance genes (*ampC*, *mexC1,2* and *mexC3,4*) are related to resistance to antipseudomonal β-lactams in clinical isolates of *P. aeruginosa* (12). Due to the absence of information about distribution of these genes in Iran, this study was performed to determine the frequency of these genes among *P. aeruginosa* isolated from patients in Tehran, Iran. This study has shown for the first time, the presence of *ampC* and *mexC* genes in significant percent of clinical isolates of *P. aeruginosa* in Tehran, Iran, and relation between presence of *ampC* gene and resistance to β-lactams.

Materials and Methods

Bacterial isolates

P. aeruginosa isolated from patients in three hospital laboratories (Pars and Milad hospitals and Motahari Burn Center) in Tehran, Iran, in 2013 were collected and 100 of them selected randomly and used in this study. Identification of isolates as *P. aeruginosa* was done based on general phenotypic methods including colony pigmentation, Gram staining, oxidase test, oxidative/fermentative (OF) test for carbohydrate utilization, growth at 42˚C and growth on cetrimide agar (13).

PCR for detection of genes

Genomic DNA was extracted based on the Ozer *et al.* method (12) with some modifications. The isolates were screened for presence of resistance genes *ampC*, *mexC1,2* and *mexC3,4* genes according to PCR method of Ozer *et al.* (12). Molecular identification of *P. aeruginosa* was performed with PCR using *oprL* gene primers (14). Primers used in this study are shown in Table 1.

We performed Duplex PCR assay for the detection of studied genes in a thermal cycler (Techne, UK). This Duplex PCR reaction were carried out in a final volume of 25µl containing 12.5µl Master Mix (Amplicon Taq DNA Polymerase 2x Master Mix Red, ViraGene Company, Iran), 9.5µl DDW, 1µl of

Table 1
Primers used in this study

Gene	Forward and Reverse Primers Sequences (5′ to 3′)	Product length (bp)	Reference
ampC	CGGCTCGGTGAGCAAGACCTTC	218	(12)
	AGTCGCGGATCTGTGCCTGGTC		
$mexC_{1,2}$	ATCCGGCACCGCTGAAGGCTGCG	344	(12)
	CGGATCGAGCTGCTGGATGCGCG		
$mexC_{3,4}$	GTACCGGCGTCATGCAGGGTCC	164	(12)
	TTACTGTTGCGGCGCAGGTGACT		
oprL	ATGGAAATGCTGAAATTCGGC	504	(14)
	CTTCTTCAGCTCGACGCGACG		

each primers (0.5 µl Forward primer and 0.5µl Reverse primer) and 1µl DNA template. Master Mix1 contained *ampC* and *mexC1,2* primers, and Master Mix2 contained *mexC3,4* and *oprL* primers. Program of amplification process was as follows: Initial denaturation at 93˚C for 5 min, 30 cycles of initiation at 93˚C, annealing at 55˚C and extension at 72˚C; each 1 min; and final extension at 72˚C for 5 min. The PCR products and 100bp DNA ladder were visualized under gel documentation system (UVItec, UK) after electrophoresis on a 1% agarose gel and staining by Ethidium Bromide.

Antimicrobial susceptibility testing

Disk diffusion method was used for detection of antimicrobial susceptibility pattern in clinical isolates of *P. aeruginosa* according to the Clinical and Laboratory Standards Institute (CLSI) guidelines (15). The following antibiotic disks from MAST Group Ltd. (Merseyside, UK), were used: Mezlocillin (MEZ; 75µg), cefepime (CPM; 30µg), ceftazidime (CAZ; 30µg) and piperacillin/ tazobactam (PTZ; 100/10µg). Control strains used for piperacillin/ tazobactam was *E. coli* ATCC35218, and for other antibiotics was *P. aeruginosa* ATCC27853.

Data analysis

All collected data were analyzed and frequencies were computed by Statistical Package for Social Sciences version 20 (SSPS Inc, Chicago, IL, USA). The relation between antibiotic resistance and the presence of the resistance genes is determined by Chi-square or Fisher Exact test and *P* value ≤0.05 was considered statistically significant.

Results

From 100 collected *P. aeruginosa* isolates 67, 25 and 8 were isolated from patients in Pars Hospital, Milad Hospital, and Motahari Burn Center, respectively, which were isolated from sputum (50%), urine (35%), wound (13%), CSF (1%), and blood (1%). Characteristics of patients showed that 51 of them were male and 49 female, 62 were outpatient and 38 inpatients, and mean age was 52.57 ± 27.15 (12 cases had below 15, 20 cases 15-44, 23 cases 45-64 and 45 cases 65-94 years old.

In all isolates identified by phenotypically methods as *P. aeruginosa,* the *oprL* gene was also detected by PCR method. Altogether, *ampC*, *mexC1,2* and *mexC3,4* genes were detected in 82%, 86%, and 89% of isolates, respectively, and combination of genes were also seen in many of isolates and only three isolates had neither of these genes (table 2).

Antibiotic susceptibility of studied isolates had shown in Fig. 1. Resistance to mezlocillin, cefepime, ceftazidime and piperacillin/

Table 2
Frequency of studied genes in Iranian *P. aeruginosa* isolates

Gene (s)	Percent
ampC	82
$mexC_{1,2}$	86
$mexC_{3,4}$	89
ampC & $mexC_{1,2}$	75
ampC & $mexC_{3,4}$	75
$mexC_{1,2}$ & $mexC_{3,4}$	80
ampC & $mexC_{1,2}$ & $mexC_{3,4}$	70
Without *ampC* & $mexC_{1,2}$ & $mexC_{3,4}$	3

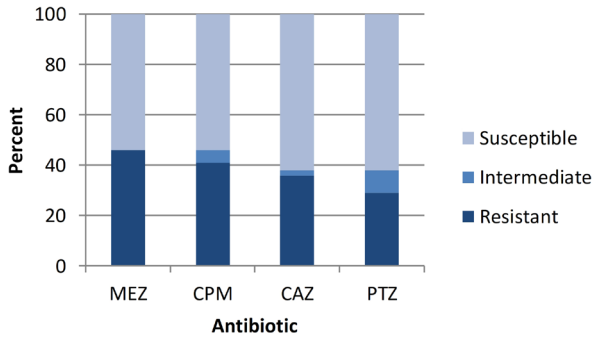

Fig. 1
Results of susceptibility test for Iranian *P. aeruginosa* isolates

tazobactam was seen in 46%, 41%, 36% and 29% of studied *P. aeruginosa* isolates, respectively. Relation between resistance to theses antipseudomonal β-lactams and the presence of *ampC*, *mexC1,2* and *mexC3,4* genes among studied *P. aeruginosa* isolates were also studied by statistical methods. Significant relation (*P* value ≤ 0.001) was shown between the resistance to each studied antibiotic and presence of *ampC* gene. This relation was not found for *mexC* genes, although a high number of resistant isolates had these genes.

Discussion

Because of the importance of *P. aeruginosa* in human infections, many studies are undertaken in the world about resistance to different antibiotics in clinical isolates of this bacterium. Many reports were published in Iran about frequency of resistance to different antibiotics in *P. aeruginosa* isolated from patients (16-23); although there is no report of resistance to mezlocillin. In this study we focused on determination of the frequency of resistance to four extended expectrum penicillins which are used in treatment of *P. aeruginosa* infections. Among clinical isolates of *P. aeruginosa* collected in three Tehran hospitals, resistance to ceftazidime was 36%, which was higher than Shahcheraghi *et al.* report (25%) and lower than other studies in Iran, which were 57.5% to 89.5% (17, 23-26). In our study, resistance to cefepime was 41% that was almost similar to a report with the result of 39%, but much less than other study which was 91.7% (17, 25). Furthermore we found that resistance to piperacillin/ tazobactam was 29% which showed similarity to study of Shahcheraghi *et al.* (28%), and a little higher than the result of Salimi *et al.* (19.1%), however, it was reported 87.2% resistance that was much higher than our study (17, 24, 26). The rates of resistance to ceftazidime, piperacillin/ tazobactam and mezlocillin in this study were 36%, 29%, and 46%, respectively, similar to a report (12) from Turkey, our neighbor country, which were reported respectively 30%, 24%, and 50%, but the resistance to cefepime in this study was higher than mentioned report (41% versus 18%). searching in studies of other countries, the rates of resistance to mezlocillin were reported 48% that shows higher rate in comparison with our findings (19, 20). Geographic differences in antimicrobial resistance were also shown in other studies, and some of the variables explaining these differences in population demographics, access to medical care and illicit drug use (27, 28).

P. aeruginosa use several genes to mediate resistance to β-lactam antibiotics including *ampC*, *mexC1,2* and *mexC3,4* genes (12, 29). The *ampC* gene encodes an inducible chromosomal

β-lactamase. The *mexC* genes are related to MexCD-OprJ efflux system and are belonging to the RND family (30, 31). In the genetic map of this bacterium, *ampC* is located beside the genes of the MexCD-OprJ efflux system (30). Besides, it is shown that this efflux system is an inducible pump, which expression could be induced after more using of some inducer antibiotics, pressure of antibiotics and following mutation in *mexR* gene that control the expression of genes belonging to MexCD-OprJ efflux system (32, 33). Only a few reports were published about presence of *ampC* gene in clinical isolates of *P. aeruginosa* in Iran, such as study of Aghazadeh *et al*. (34), and presence of *mexC* genes have not be done before this study. The studied genes (*ampC*, *mexC1,2* and *mexC3,4*) were detected in most of *P. aeruginosa* isolated from patients in Tehran, Iran. Unfortunately, we could not found any report about the rate of *mexC* genes in clinical isolates of *P. aeruginosa* in other countries for comparison, except the study of Ozer *et al.* in Turkey ,(12) which reported lower rates for these genes.

In this study ,relation between the resistance to mezlocillin ,cefepime ,ceftazidime and piperacillin /tazobactam and presence of *ampC* gene in *P. aeruginosa* isolated from patients in Iran were seen same to the Ozer *et al.* study in Turkey (12) but reported relation between the resistance to these antibiotics and presence of *mexC* genes was not shown in this study, although *mexC* genes were found in a high number of resistant isolates.

Conclusion

The study presented high frequency of resistance to mezlocillin, cefepime, ceftazidime, and piperacillin/ tazobactam in *P. aeruginosa* isolated from patient in Tehran, Iran, which could help in selection of the best antibiotic for empirical therapy in treatment of severe pseudomonal infections. Moreover, it was shown that most of these isolates had *ampC* and *mexC* genes and there was significant relation between resistant to used antipseudomonal β-lactams and presence of *ampC* gene.

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Conflict of interest

The authors declare that there is no conflict of interests.

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