Detection of Ampc and Extended-Spectrum Beta-Lactamases in Clinical Isolates of *Pseudomonas Aeruginosa* from Patients with Cystic Fibrosis

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ABSTRACT

Background and Objectives: *Pseudomonas aeruginosa* is the most frequent opportunistic pathogen isolated from the sputum of patients with cystic fibrosis (CF). Resistance to β -lactam antibiotics may arise from over expression of the naturally occurring AmpC cephalosporinases or acquired extended-spectrum β-lactamases (ESBL). The aim of this study was to determine the antibiotic resistance profiles as well as the prevalence of ESBL and AmpC production in clinical isolates of *P. aeruginosa* from CF patients in Tehran.

Methods: Antibiotic resistance of 50 non-duplicate *P. aeruginosa* isolates was determined by the disc diffusion method. AmpC β-lactamase production was detected by the antagonism disc test and ESBL production was detected by the phenotypic confirmatory test. The presence of ESBL and AmpC genes was assessed by PCR, followed by sequencing the PCR products.

Results: The antibiotic resistance rates were as follows: 22% to ceftriaxone, 20% to cefotaxime, 10% to imipenem, 8% to carbenicillin and 6% to ticarcillin, 4% each to cefepime, tobramycin, amikacin and aztreonam and 2% to each piperacilin, meropenem and ceftazidime. AmpC production was observed in 47 isolates (94%) and ESBL production was observed in one isolate (2%). PCR results showed that all isolates carried the bla_{AmpC} β-lactamase gene. One multidrug-resistant isolate carried both bla_{TEM} and bla_{PER-1} genes.

Conclusion: The results showed that despite the low rate of antibiotic resistance in *P. aeruginosa* CF isolates,the presence of multiple β-lactamases even in one isolate is alarming and can complicate the already difficult treatment of chronic infections in the lungs of CF patients.

KEYWORDS: *Pseudomonsa aeruginosa*; Cystic Fibrosis; ESBL, AmpC, Beta-Lactamases.

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INTRODUCTION

Chronic *Pseudomonas aeruginosa* lung infection is the main cause of morbidity and mortality in cystic fibrosis (CF) patients. About 80% of adults with CF suffer from the chronic *P. aeruginosa* infection (1,2). In the lung of CF patients, *P. aeruginosa* exhibits several genotypic and phenotypic changes such as antibiotic resistance and mucoid conversion (3). *P. aeruginosa* uses a wide variety of different resistance mechanisms and is essentially resistant to all classes of commonly used antibiotics (4). Several mechanisms are responsible for resistance to β-lactam antibiotics in *P. aeruginosa* including over expression of chromosomally mediated AmpC cephalosporinases, acquisition of transferable β-lactamase genes such as extended-spectrum β-lactamases (ESBLs) and metallo-β-lactamases (MBLs), presence of efflux systems, and reduced membrane permeability (5,6). In *P. aeruginosa*, the decreased susceptibility to the extended-spectrum cephalosporins such as ceftazidime mostly results from over expression of the naturally occurring AmpC β-lactamases (6). ESBLs are capable of hydrolyzing penicillins, cephalosporins and aztreonam (except for cephamycins or carbapenems). These enzymes are inhibited by β-lactamase inhibitors such as clavulanic acid (7). Up to 32 different ESBLs have been detected in *P. aeruginosa* belonging to class A (TEM, SHV, CTX-M, PER, VEB, GES, BEL), and class D (OXA type) β-lactamases (8). We studied the antibiotic resistance profiles in clinical isolates of *P. aeruginosa* from CF patients, by phenotypic detection of AmpC and ESBL production and their comparison in the presence of AmpC, TEM, SHV, CTX-M, PER and VEB β-lactamase genes among these isolates.

MATERIAL AND METHODS

Fifty non-duplicate clinical isolates of *P. aeruginosa* were collected from CF patients at Mofid Children's Hospital in Tehran between July 2012 and February 2013. Twenty-nine (58%) isolates were obtained from males and 21 (42%) were obtained from female patients (age range, 6 months to 26 years). The mean age (± SD) was 92.52 (± 70.99) months with a median of 72 months. The isolates were identified by Gram staining and conventional microbiological tests including oxidase production, blue–green pigmentation on Mueller Hinton agar [MHA, (Liofilchem, Italy)], acid and gas production on triple sugar iron agar slants (BBL, USA), and growth at 42°C (9). The isolates were maintained in brain heart infusion broth (Oxoid, UK) containing 10% dimethyl sulfoxide at -20 ^{o}C until use. *P. aeruginosa* PAO1 containing bla_{AmpC} (kindly provided by Dr Abdi, Alzahra University, Iran), DNA from *K. pneumoniae* 7881 harboring bla_{TEM}, bla_{SHV} and bla_{CTX-M}, *P. aeruginosa* KOAS carrying bla_{PER-1} and *P. aeruginosa* 10.2 harboring bla_{VEB-1} (obtained from Pasteur Institute, Tehran, Iran) were used as controls for PCR experiment. *P. aeroginosa* ATCC 27853 was used as control for susceptibility tests. Antibiotic susceptibility testing of the isolates was carried out by the disc diffusion method according to the Clinical and Laboratory Standard Institute (CLSI) guidelines (10). The antibiotic discs (MAST Group LTD, Merseyside, UK) used in this study included: ceftazidime (30 µg), cefotaxime (30 µg), ceftriaxone (30 µg), cefepime (30 µg), cefoxitin (30 µg), imipenem (10 µg), meropenem (10 µg), carbenicillin (100 µg), ticarcillin (75 µg), piperacillin (100 µg), amikacin (30 µg), tobramycin (10 µg) and aztreonam (30 µg). Minimum inhibitory concentrations (MICs) for imipenem and ceftazidime were determined by the microdilution assay as recommended by the CLSI (10). Initial screening for AmpC β-lactamase production was performed by the disc antagonism test on the isolates showing a zone diameter of <18 mm to cefoxitin disc (11). Briefly, a lawn culture of each test isolate (0.5 Mc Farland) was prepared on a MHA plate, and then ceftazidime (30µg) and cefoxitin (30µg) discs were placed 20 mm (centre to centre) apart from each other followed by incubation at 37 ˚C for 18-20 hours. Inducible AmpC β-lactamase production was detected when blunting of ceftazidime inhibition zone occurred adjacent to the cefoxitin disc. ESBL production was detected by the conventional double disc synergy test using ceftazidime (30 µg) and cefotaxime discs (30 µg) with or without clavulanic acid (10 µg) as recommended by the CLSI (10). An increase of ≥ 5 mm in the inhibition zone of either cephalosporin in combination with clavulanic acid compared to the cephalosporin alone was interpreted as ESBL production. DNA extraction was performed by boiling method. Briefly, a

loopful of an overnight grown culture of each isolate was suspended in 500 µl of sterile double distilled water, boiled at 100 °C for 10 min and then centrifuged at 12000 g for 10 min. The supernatant was used as DNA template for PCR amplification tests. PCR amplifications were performed using specific primers for Ambler class A (bla_{TEM}, bla_{SHV}, bla_{CTX-M}, bla_{VEB-1}, bla_{PER-1}) and class C (bla_{AmpC}) β-lactamase genes (Table 1). PCR experiments were carried out in 25 µl volume reaction mixtures containing 10 pM of each primer, 200 µM dNTP, 1.4 mM $MgCl_2$, 1 µl of DNA template and 1 U of Taq polymerase in the reaction buffer provided by the manufacturer (CinnaGen, Tehran, Iran). PCR process was carried out in a thermocycler (Techne, UK) using the following program: 5 min denaturation at 95 °C, followed by 30 cycles at 95 °C for 1 min, 1 min at the annealing temperature (43-63°C for ESBLs and 62°C for AmpC), 1 min at 72 °C and final extension for 10 min at 72 °C. Correlation of β-lactamase phenotypes with their related genes was assessed using the chi-square test. The relationship between antibiotic resistance and β-lactamase gene carriage was analyzed by the nonparametric Mann-Whitney U test. All statistical analyses were carried out using SPSS software version 20. *P-value* of ≤0.05 was considered statistically significant.

RESULTS

Among the 50 CF isolates, 48 (96%) were resistant to cefoxitin, one was susceptible, and one showed intermediate resistance. Resistance rates to other antibiotics were as follows (Figure 1): ceftriaxone (22%), cefotaxime (20%), imipenem (10%), carbenicillin (8%), ticarcillin (6%), cefepime, amikacin, tobramycin and aztreonam (4%) and piperacilin, ceftazidime and meropenem (2%). Overall, the highest resistance rate was to ertapenem and the lowest was to piperacilin, ceftazidime and meropenem. One isolate (2%) was multidrug-resistant with MIC ≥16 µg/ml for imipenem and ≥128 µg/ml for ceftazidime.

Table1- Primers used for PCR detection of ESBLs and AmpC genes

β -lactamase	Primer	Sequence (5' to 3')	Product size (bp)	Reference
Class A	TEM-F	GAGTATTCAACATTTCCGTGTC	851	12
	TEM-R	TAATCAGTGAGGCACCTATCTC		
	SHV-F	AAGATCCACTATCGCCAGCAG	231	12
	SHV-R	ATTCAGTTCCGTTTCCCAGCGG		
	CTX-M-F	CGCTTTGCGATGTGCAG	550	13
	CTX-M-R	ACCGCGATATCGTTGGT		
	PER-1-F	ATGAATGTCATTATAAAAGC	920	12
	PER-1-R	AATTTGGGCTTAGGGCAGAA		
	VEB-1-F	CGACTTCCATTTCCCGATGC	643	12
	VEB-1-R	GGACTCTGCAACAAATACGC		
Class C	AmpC-F	ATGCAGCCAACGACAAAGG	1243	14
	AmpC-R	CGCCCTCGCGAGCGCGCTTC		

Figure 1-Antibiotic resistance profiles of the 50 *P. aeruginosa* CF isolates. CAZ, ceftazidime; CTX, cefotaxime; CRO, ceftriaxone; CPM, cefepime; IPM, imipenem; MEM, meropenem; PY, carbenicillin; TC, ticarcillin; PRL, piperacilin; AK, amikacin; TN, tobramycin; ATM, aztreonam.

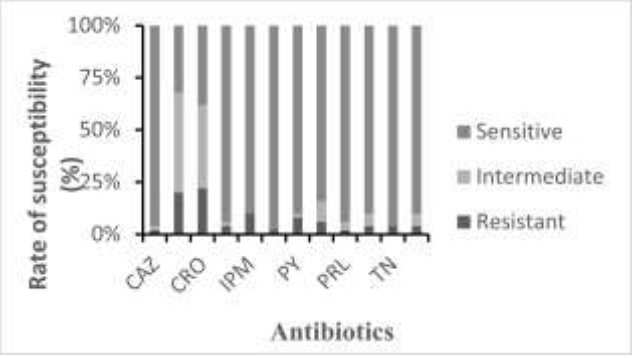
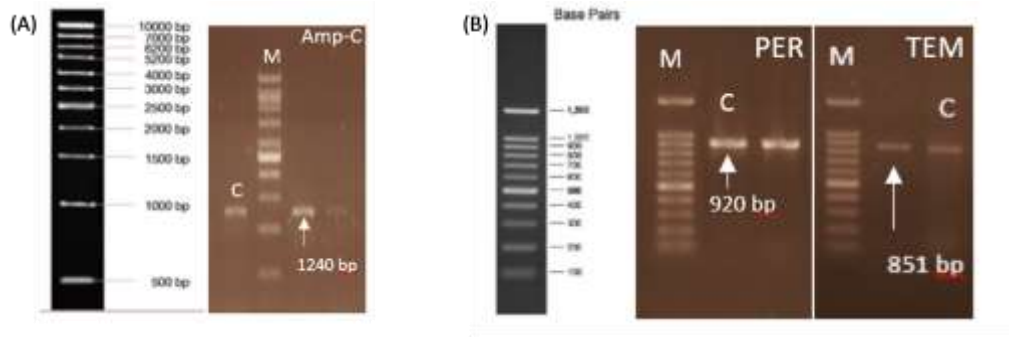


Figure 2- PCR amplification of β-lactamase genes in the *P. aeruginosa* CF isolates. (A): *bla*$_{AmpC}$ amplification product; M, 1 Kbp DNA ladder, C; positive control. (B): *bla*$_{PER}$ and *bla*$_{TEM}$ amplification products; M: 100 bp; C, positive control



AmpC production was observed in 47 isolates (94%) and ESBL phenotype was detected in one isolate (2%). PCR amplification results showed that all isolates carried the *bla*$_{AmpC}$ β-lactamase gene and the multidrug-resistant isolate carried *bla*$_{AmpC}$, *bla*$_{TEM}$ and *bla*$_{PER-1}$ genes (Figure 2). *bla*$_{SHV}$, *bla*$_{CTX-M}$ and *bla*$_{VEB}$ genes were not detected in any of the isolates. Sequence analysis of the PCR products showed the presence of a new allele for the *bla*$_{TEM}$ gene (www.lahey.org/studies/temtable.asp) (15). Overall, there was a good correlation between β-lactamase phenotype and its related gene carriage among the tested isolates ($p<0.05$).

DISCUSSION

β-lactam antibiotics are often inefficient in the treatment of *P. aeruginosa* lung infections. The main reasons for treatment failure are thought to be ESBL production, hyperproduction of chromosomally encoded AmpC β-lactamases and biofilm formation (1). The current CLSI guidelines have not provided a method for detection of AmpC β-lactamases. It has been suggested that cefoxitin-resistant isolates (showing a zone diameter of <18 mm to cefoxitin disc) are potential AmpC β-lactamase producers and the disc antagonism test should be used for screening AmpC β-lactamase production in these isolates (11,16).

In this study, all 50 CF isolates carried the *bla*$_{AmpC}$ gene, while 47 isolates (94%) were AmpC producers. These results show a good correlation between AmpC gene carriage and AmpC phenotype among the isolates. Of the three *bla*$_{AmpC}$ positive and AmpC negative isolates, one was susceptible to all tested antibiotics, two were resistant to cefoxitin, and one also showed intermediate resistance to cefotaxime. However, the susceptibility of these isolates to other 3rd generation cephalosporins was not investigated in the present study. The presence of genes does not necessarily mean that they are expressed, which could be influenced by the environment as well as other regulatory mechanisms. Limited number of studies on AmpC production by *P. aeruginosa* CF isolates are available. In a study in Africa, AmpC production was not detected in any of the 15 *P. aeruginosa* isolates from CF patients (17).

The rate of ESBL production in *P. aeruginosa* CF isolates varies worldwide. In a study in Iran, Vali et al. showed that 9.6% of 52 *P. aeruginosa* CF isolates carried *bla*$_{CTX-M}$ (18). Another study in Iran detected the *bla*$_{PER-1}$ gene in 10% of *P. aeruginosa* CF isolates (19). Mhlongo et al. reported the ESBL phenotype in all of their tested isolates (17). Other studies have reported the presence of *bla*$_{CTX-M}$ gene and coproduction of *PER-1* and *VIM-2* in one *P. aeruginosa* CF isolate (20, 21). We found ESBL production in one multidrug-resistant isolate, which also carried *bla*$_{AmpC}$, *bla*$_{TEM}$ and *bla*$_{PER-1}$ genes. Overall, the spread of different ESBL genes in *P. aeruginosa* CF isolates is increasing. The discrepancy between β-lactamase phenotype and gene carriage could be due to environmental conditions such as the presence of antibiotics that may allow the expression of β-lactamase genes in vivo but not in vitro.

CONCLUSION

The results showed that despite the low rate of antibiotic resistance in *P. aeruginosa* CF Isolates, the presence of β-lactamase genes could lead to their

expression under selective environmental pressures such as extensive use of antibiotics, resulting in treatment failure in these infections. In addition, resistance genes from even one multidrug-resistant isolate can spread among the susceptible strains and cause increased morbidity and mortality among the CF patients.

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CONFLICT OF INTEREST:

There are no conflicts of interest.

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