Identification of Class-1 Integron and Various B-Lactamase Classes among Clinical Isolates of *Pseudomonas aeruginosa* at Children's Medical Center Hospital

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**ABSTRACT**

*Background:* *Pseudomonas aeruginosa* is one of the most important opportunistic pathogens responsible for various types of infections. Children suffer significant morbidity and mortality due to nosocomial infections. The aim of this study was to investigate the presence of Class-1 integron, *bla*<sub>BEL</sub>, *bla*<sub>PER</sub>, *bla*<sub>KPC</sub>, *bla*<sub>VIM</sub>, *bla*<sub>IMP</sub> and *bla*<sub>OXA-group-1</sub> genes among *P. aeruginosa* isolates at Children's Medical Center Hospital in Iran and to determine phenotypic evidence of ESBL and MBL production.

*Methods:* Antibiotic susceptibility tests were analyzed for 72 *P. aeruginosa* clinical isolates. Isolates were identified by using biochemical tests and confirmed by PCR assay for *oprL* gene. ESBL and MBL producer isolates were identified by phenotypic tests (double disc synergy tests). Detection of β-lactamase genes and class-1 integron were performed by PCR method.

*Results:* All of the isolates were susceptible to ceftazidime / clavulanate, meropenem, imipenem and ciprofloxacin. About 83.3% and 16.7% of isolates were resistant to ceftazidime and amikacin respectively. Approximately, 83.3% of isolates were considered as potential ESBL producers. None of the clinical isolates showed above β-lactamase genes. It seems that, the reason is the absence of class-1 integron in all of isolates. About 16.7% of strains were identified as multidrug resistant. Fortunately, all of the isolates were susceptible to meropenem and imipenem which are effective against ESBL producing strains.

*Conclusion:* The absences of class-1 integron decreases the probability of acquired β-lactamase especially MBL. Thus, absolute susceptibility to carbapenems and ciprofloxacin among *P. aeruginosa* isolates in pediatric hospital has important implications for empirical antimicrobial therapy. It seems that these properties help to decrease mortality of nosocomial infections within children.

Introduction

Children suffer from significant morbidity and mortality due to nosocomial infections. The consequences of these infections include prolongation of hospitalization, transfer to intensive care units, antibiotic therapy, placement or replacement of invasive devices and surgical procedures (1, 2).

Pediatric wards and hospitals are particularly suited for the transmission of infection. Infants and toddlers constitute a large proportion of the patients admitted. They frequently harbor infectious organisms and may transfer pathogens, especially respiratory and gastrointestinal microorganisms (1, 3). Young children are also susceptible to many infections because they have not yet developed sufficient immunity. Behavioral characteristics of young children, such as incontinence, inadequate hygiene, frequent mouthing of hands and objects, drooling and direct contact between children during play, facilitate the spread of infection. Basic care requires frequent hands-on contact from health care personnel and parents. Multibed rooms, shared toys and playrooms, and visiting siblings contribute to the risk of transmission (1, 2, 4). Transmission rates increase with understaffing and overcrowding (5, 6).

*Pseudomonas aeruginosa*, an aerobic Gram-negative organism and an opportunistic pathogen that is commonly discovered in soil, water, and plants, rarely causes illness in healthy people. However, *P. aeruginosa* sepsis often occurs in patients with burns, malignancies or immunodeficiency or in preterm infants. Most of these infections are nosocomially acquired (1). *P. aeruginosa* is a virulent organism that is susceptible to a limited number of antimicrobial agents including antipseudomonal penicillins and cephalosporins, carbapenems, fluoroquinolones and ciprofloxacin (7-10). The intrinsic resistance of *P. aeruginosa* to various antibiotics is generally due to its low outer membrane permeability, production of inducible chromosomal cephalosporinases (AmpC), and multidrug efflux pumps (8, 11). Despite recent improvements in therapy, *P. aeruginosa* bacteremia still remains fatal in children (12). In a large multi-center study of all age groups, *P. aeruginosa* bloodstream infection was associated with crude mortality rates of 39% in all patients and 48% in intensive care unit patients (13).

Thus, drugs effective against *P. aeruginosa* infections are limited to aminoglycosides (e.g., gentamicin, amikacin), fluoroquinolones (ciprofloxacin remains the most active), selected β-lactams (e.g., ceftazidime, carbapenems), and one β-lactam / β-lactamase inhibitor combination (piperacillin / tazobactam) (8, 14, 15).

*P. aeruginosa*, like other Gram-negative pathogens, is known to acquire resistance by producing various β-lactamases (such as VEB, OXA, PER, BEL, KPC, VIM and IMP), which are mostly plasmid-mediated β-lactamases. Among plasmid-mediated β-lactamases, extended-spectrum β-lactamases (ESBL) are generally known to hydrolyze cephemycins and/or carbapenems which cannot be easily hydrolyzed by traditional β-lactamases (8, 16). VEB-type ESBLs were the predominant ESBL reported in *P. aeruginosa* (8). The production of AmpC variants with improved activity against oxyiminocephalosporins (e.g., ceftazidime),
cefepime, and carbapenems (including imipenem), first described in the Enterobacteriaceae and referred to as extended-spectrum AmpC (17), have been reported in clinical isolates of *P. aeruginosa* (18, 19).

OXA enzymes (molecular class D β-lactamases) (20) are mostly narrow-spectrum β-lactamases that confer resistance to aminopenicillins and carboxypenicillins and narrow-spectrum cephalosporins (21). Although several OXA-type enzymes are ESBLs (22).

PER-1-type β-lactamase is an ESBL that hydrolyzes various β-lactams except for carbapenems, oxacillin and cephemycins (23, 24). BEL-1 (25) is not frequently observed in *P. aeruginosa*, but it was reported in ESBL positive *P. aeruginosa* in a Belgium study (26, 27).

KPC is a class A β-lactamase with activity against carbapenems. Only KPC-2 and KPC-5 have been reported in *P. aeruginosa*. Interestingly, KPC-2 is more active against carbapenems than is KPC-5 while the latter shows better activity against ceftazidime (8, 28). Class B MBLs are by far the major determinants of β-lactamase mediated resistance to carbapenems and the major cause of high level resistance to these agents, VIM and IMP β-lactamases (molecular class B) are carbapenemases (35). The predominance of VIM vs. IMP in *P. aeruginosa* appears to be geographical, with IMP-type MBLs predominating in Asia where it was first discovered and VIM-type enzymes predominating in Europe though both enzymes are now disseminated globally, with VIM-2 in particular well established on five continents (30, 31).

OXA group-1 β-lactamases especially OXA-10 has the most frequency among all OXA-type β-lactamases in Iran (32, 33, 34).

In Iran, the prevalence of VIM β-lactamases and IMP β-lactamases were 11-23% and 0-5% respectively (35-38).

Prevalence of PER-1 type extended-spectrum β-Lactamase in clinical strains of *Pseudomonas aeruginosa* isolated from Iran is 17 to 27.5% (39, 40).

According to many surveys in Iran, the most prevalent ESBL genes were *bla*<sub>oxa</sub>-10 and *bla*<sub>veb</sub> genes. The prevalence of *veb* gene is about 31% (32, 33, 40).

BEL β-Lactamases have never been studied in Iran. This study for the first time surveys these genes.

Integrons and transposons have been repeatedly detected in multi-drug resistant *P. aeruginosa* isolates (41, 42). Integrons are genetic elements encoding the components of a site specific recombination system that recognizes and captures mobile gene cassettes, mostly resistance determinants (43). Such elements may be located within transposons, which in turn actively contribute to the dissemination of resistance determinants for aminoglycosides and β-lactams among Gram-negative species (44). Moreover, it has been demonstrated that integrons and transposons are associated with the spread of resistance to third-generation cephalosporins when they encode extended-spectrum β-lactamases (ESBLs) such as VEB-1, GES-1 or TEM-21 (42).

The present study reviews the prevalence of different groups of β-lactamase genes (*bla*<sub>bel</sub>, *bla*<sub>per</sub>, *bla*<sub>kpc</sub>, *bla*<sub>vim</sub>, *bla*<sub>imp</sub> and *bla*<sub>oxa</sub> group-1) and the existence of class-1 integron in clinical isolates of *P. aeruginosa* as a principle pathogen in nosocomial infections at Children's Medical Center Hospital in Tehran, Iran.
Materials and Methods

Sample collection and clinical data

During a 3 month period, from November 2011 to January 2012, 72 clinical isolate (P. aeruginosa) collected from pediatric patients younger than 18 years old who had one positive sample at Children’s Medical Center Hospital (the major pediatric hospital in Tehran, Iran).

These samples included thirteen blood specimen, thirty five urine specimens, five wound specimens, twelve tracheal specimens, two throat specimens, three CSF specimen and other biological fluids (two specimens). Over the study period, all the isolates were identified by using microscopic observation and biochemical tests, and then strains were confirmed by PCR for oprL gene, which is specific for P. aeruginosa. Isolates were analyzed for susceptibility to anti-Pseudomonas antibiotics.

To avoid duplicates only one isolate was selected from each patient, unless isolates showed different resistance profiles.

Antimicrobial Susceptibility tests and detection of ESBLs and MBLs by phenotypic methods

Antibiotic susceptibility of P. aeruginosa isolates was determined by the disc diffusion method on Mueller-Hinton agar (Oxoid, Basingstoke, UK). Susceptibility was defined according to the Clinical and Laboratory Standards Institute (CLSI) guidelines (45). The following β-lactam antibiotics were used: piperacillin 100 g, piperacillin / Tazobactam 100/10 g, meropenem 10 g, imipenem 10 g, ceftazidime 30 g, cefepime 30 g, ceftazidime/clavulanic acid 30/10 g, amikacin 30 g, ciprofloxacin 5 g and aztreonam 30 g discs (MAST, Bootle, Merseyside, UK). P. aeruginosa ATCC PAO1 was used as reference strains in susceptibility test.

To detect possible ESBL production, double disc synergy test (DDST) was performed with discs containing ceftazidime (30 mg) alone and in the presence of clavulanate (10 mg). Ceftazidime resistant isolates were suspected to be ESBL producers when a positive double disc synergy test was observed (increase in ceftazidime inhibition zone of > 5mm in the presence of clavulanic acid as compared with when tested alone) (CLSI guidelines) (45).

To screen for Metallo-β-lactamase producing strains, initial screening tests and phenotypic confirmatory tests were performed. For this reason, determination of resistance to meropenem (45) and then phenotypic detection of MBL in all the isolates was performed by double-disc synergy test with discs containing imipenem and EDTA (46).

PCR amplification for detection of P. aeruginosa, β-lactamasegenes and class-1 integron

The presence of class 1 integron was tested by PCR using primers specific for the integron integrase genes (intI1) (51).

The total DNA from P. aeruginosa isolates was extracted by DNA extraction kit (DNeasy blood and tissue, QIAGEN). PCR was carried out in a 50 l volume by using 25 l Emerald Amp MAX HS PCR Master Mix (Takara Shuzo, Shiga, Japan), 50ng of genomic DNA of the test strain and 0.2 M of each primer.

PCR with the following cycling parameters was performed: initial denaturation at 94°C for 240 s; 30 cycles of denaturation at 94°C for 30 s, annealing for 30 s and extension at
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72°C for 60s; and a final extension at 72°C for 300s. Published primers (25, 47-51) or sets of primers which were designed in this study were used (Table 1).

Table 1. Oligonucleotides used as primers for PCR amplification of class-1 integron, \( \beta \)-lactamase and oprL genes

<table>
<thead>
<tr>
<th>Name</th>
<th>Target</th>
<th>Sequence (5’&gt;&gt;&gt;3’)</th>
<th>Annealing temperature (°C)</th>
<th>Amplicon Size (bp)</th>
<th>Reference</th>
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<td>blaOXA-group-1</td>
<td>TCAACAAATCGCCAGAGAAG</td>
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<td>276</td>
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<tr>
<td>PER-F</td>
<td>blaPER</td>
<td>CCTGACGATCTGGGAACCTT</td>
<td>56</td>
<td>641</td>
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<tr>
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<td>KPC-F</td>
<td>blaKPC</td>
<td>AGTTCTGCTGACTCTC</td>
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<td>BEL-F</td>
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<td>VIM-F</td>
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Results

Seventy-two patients (38 females and 34 males) were included in this study. Samples were comprised various specimens such as blood, urine, wound, tracheal, throat, CSF and other biological fluids. Of these, 60 (83.3%) isolates were resistant to ceftazidime (inhibition zone diameter of antibiotic disc ≤ 14 mm) and resistance to piperacillin (inhibition zone diameter of antibiotic disc ≤ 17 mm) and resistance to cefepime (inhibition zone diameter of antibiotic disc ≤ 14 mm). The ceftazidime resistant strains were subjects for double disc synergy tests (DDST).

DDST using ceftazidime alone and with clavulanic acid, allowed the detection of 60 potential ESBL producers among 72 isolates (observation of the positive synergy test).

The presence of genes for various ESBLs was investigated using PCR. All of the isolates were evaluated for the presence of ESBL-encoding genes (blaPER, blaBEL and blaKPC) by PCR. Also all the isolates were evaluated for blaOXA-group-1 and blaAmpC and examined for the presence of class-1 integron (intI-1). As a result, all of the isolates had ampC gene and none of them had blaPER, blaBEL and blaKPC, blaOXA-group-1 and class-1 integron.

Whole clinical isolates (100%) were susceptible to meropenem and imipenem, and phenotypic tests for MBLs were performed for all isolates. None of them were positive and they were not suspected for MBL. In order to confirm it, PCR for two MBL genes (blaVIM, blaIMP) was performed for all isolates. Results showed that there were not any MBL genes in these isolates. Furthermore all isolates were susceptible to ciprofloxacin, 60 (83.3%) isolates were susceptible to amikacin and 57 (79.2%) isolates were susceptible to aztreonam. We found that most of the P. aeruginosa strains isolated from Children's...
Children's Medical Center Hospital, had high level of resistance to ceftazidime (The inhibition zone diameter of ceftazidime disc for most of the isolates was zero) (Table 2). Of all isolates, 16.7% of clinical isolates were identified as multidrug resistant (MDR) which showed resistance to piperacillin, ceftazidime, cefepime, aztreonam and amikacin.

| Table 2. Antimicrobial susceptibility of 72 P. aeruginosa by disc diffusion method |
|---------------------------------|-----------------|-----------------|-----------------|
| Antibiotics                     | Con. (μg)       | Sensitive No. (%) | Resistance No. (%) |
| Pipecillin                      | 100             | 12 (16.7)        | 60 (83.3)        |
| Pipecillin/tazobactam           | 100/10          | 66 (91.6)        | 6 (8.4)          |
| Ceftazidime                     | 30              | 12 (16.7)        | 60 (83.3)        |
| Ceftazidime/clavulanate         | 30/10           | 72 (100)         | 0 (0)            |
| Cefepime                        | 30              | 12 (16.7)        | 60 (83.3)        |
| Imipenem                        | 10              | 72 (100)         | 0 (0)            |
| Meropenem                       | 10              | 72 (100)         | 0 (0)            |
| Aztreonam                       | 30              | 57 (79.2)        | 15 (20.8)        |
| Ciprofloxacin                   | 5               | 72 (100)         | 0 (0)            |
| Amikacin                        | 30              | 60 (83.3)        | 12 (16.7)        |

Discussion

This study is the first report on β-lactamase genes among P. aeruginosa clinical isolates in pediatric hospital in Iran and the ESBL screening results demonstrated a high frequency of ESBLs in P. aeruginosa isolates examined.

According to our data, 83.3% of P. aeruginosa isolates from major pediatric hospital in Tehran, Iran, were ceftazidime resistant. The results of investigation by Pourakbari et al at Children’s Medical Center Hospital, among the Gram-negative isolates, P. aeruginosa were the most frequently isolated species (52). Previous research at Children Hospital in Tabriz, Iran showed 50% ceftazidime resistant (CAZres) isolates (53). In another study in Tehran, Iran among burned patients with infection by P. aeruginosa, the rate of resistance to ceftazidime was 91.8% (54). Therefore, the rate of P. aeruginosa CAZres strains in Children Hospital was less than general hospital (usually admitted adults). But in our study, the prevalence of P. aeruginosa CAZres isolates (83.3%) obtained from children at Children's Medical Center Hospital was really high and it seems unusual findings.

The results of current study, confirmed the potent of ESBL in 83.3% of the isolates based on DDST.

In present study, all clinical isolates (100%) were susceptible to meropenem and imipenem. But in another study among burned patients in Iran, 32.1% were resistant to imipenem (54) and as discussed by Ranjbar et al, 97.5% of isolates found among burned patients in Tehran, Iran, were imipenem resistant (55). In our study 100% and 82.6% of isolates were susceptible to ciprofloxacin and amikacin, respectively. Similar to our results, in a survey at Children Hospital in Tabriz, Iran the rate of resistant isolates to ciprofloxacin was low (53). While in another study in Iran among burned patients with P. aeruginosa infection, 77.2% and 47.2% of isolates were resistant to ciprofloxacin and amikacin, respectively (54, 55).

Investigation for four types of β-lactamases (class D and class A ESBLs) including \( \text{bla}_{\text{OXA}} \), \( \text{bla}_{\text{PER}} \), \( \text{bla}_{\text{BEL}} \) and \( \text{bla}_{\text{KPC}} \) (car-
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bacapenemase) by PCR method, showed that none of them existed in our clinical isolates.

It is the first time that BEL and KPC is investigated in Iran and, fortunately, none of them detected. According to previous study in Iran, OXA-10 was more frequently detected than other classes of β-lactamase in P. aeruginosa, especially in adults (32, 33). Therefore, we searched for OXA group-1 including OXA-10, in P. aeruginosa isolates obtained from children, but there was not any β-lactamase gene from this group among the isolates.

PER type enzymes were reported in different studies in Iran. All of them were detected in P. aeruginosa isolates from adults, but among our samples from children, PER was not detected. Carbapenems (e.g., meropenem, imipenem) are an important class of anti-pseudomonas β-lactam owing to their stability to most β-lactamases and are of particular use in treating infections associated with ESBL- and AmpC-producers (8). In our study, the clinical isolates of P. aeruginosa in pediatric hospital, showed no resistance to imipenem and meropenem and MBL phenotypic tests were negative. It suggested that none of the isolates have MBL which was confirmed by PCR for blaVIM and blaIMP. But as discussed by Owlia, Ranjbar and Shahcheraghi, notable number of imipenem resistant isolates of P. aeruginosa (mostly obtained from adults) have been detected in burned patients in Iran (32, 38, 54).

According to our investigation on antibiotic susceptibility in E. coli and Klebsiella pneumonia isolates from children in a pediatric hospital (data not published yet), the susceptibility of these isolates was higher than the susceptibility of same isolates from adults in other hospitals (samples obtained from adults) (56).

Our findings demonstrated the growing problem of extended-spectrum β-lactams resistance such as CAZres in pediatric P. aeruginosa isolates. Also, multi-drug resistant (resistance to piperacillin, ceftazidime, cefepime, aztreonam and amikacin) P. aeruginosa isolates were detected at Children’s Medical Center Hospital. In contrast to wide spread of β-lactamase genes and other resistance mechanisms in P. aeruginosa strains isolated from adult patients, all of the studied isolates were susceptible to carbapenems (meropenem, imipenem) and ciprofloxacin. It seems that, the absence of class-1 integron (intl-1) prevents the isolates from acquiring β-lactamase genes that cause resistance to carbapenems. Also, the absence of class-1 integron, probably, helps to decrease horizontal gene transfer among bacteria that prevents creation of resistant strains.

Conclusion

In conclusion, our findings showed that absolute susceptibility to carbapenems and ciprofloxacin and high rate of aminoglycoside susceptible strains among P. aeruginosa clinical isolates in pediatric hospital have important implications for empirical antimicrobial therapy. Thus, it helps to decrease the mortality rate of nosocomial infections by ESBL producing P. aeruginosa within children.

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**Conflict of Interest**

None declared conflicts of interest.

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