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OXA-10 and OXA-2 ESBLs among multidrug-resistant *Pseudomonas aeruginosa* isolates from North West of Iran

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ABSTRACT

Production of Extended spectrum β-lactamases (ESBLs) is a common mechanism of resistance in multidrug-*Pseudomonas aeruginosa*, but the frequency of different ESBLs may vary significantly in different parts of the world. The aim of this study was to investigate the prevalence of OXA-2/OXA-10 type ESBLs and class 1 integron among clinical isolates of *P. aeruginosa* in Tabriz, North West of Iran. A total of 110 *P. aeruginosa* isolates was entered in the study. Antibiotic susceptibility was determined by disk diffusion method. Production of ESBL was confirmed by combined disc method, and polymerase chain reaction was used for detection of OXA-2/OXA-10 beta-lactamases and class 1 integrons. Antibiotic susceptibility tests revealed that the highest resistance rate was against aztreonam (82%) and cefepime (77.3%), whereas the highest susceptibility was to imipenem (71%), meropenem (66.4%) and piperacillin/tazobactam (37.3%). In combined disc test, 68 isolates (61.8%) were ESBL producers. PCR analysis showed that 47 (42.7%) isolates carried class 1 integron, among them 22 (32.3%) contained blaOXA-10 and 7 (10.3%) contained blaOXA-2 genes. In conclusion, high prevalence of OXA-10 and OXA-2 type ESBLs were detected in the study region and that imipenem and meropenem were the most active agents against *P. aeruginosa* isolates.

Keywords: Antibiotics; Antimicrobials; Molecular epidemiology; *Pseudomonas*; Resistant isolates

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Introduction

Pseudomonas aeruginosa is a major cause of nosocomial infections including pneumonia, urinary tract infections, and bacteremia. The infections can be particularly severe in patients with impaired immune system, such as neutropenic or cancer patients (1, 2). Emergence of antibiotic resistance is a major problem in treatment of *P. aeruginosa* infections worldwide (3). There are four main groups of beta-lactam antibiotics including penicillins, cephalosporins, monobactams and carbapenems, all having a beta-lactam ring which can be hydrolysed by various beta-lactamases. These enzymes cleave the amide bond in the beta-lactam ring and inactivate the beta-lactam antibiotics (4-5). Extended-spectrum beta-lactamases (ESBLs) play an important role in the development of resistance to beta-lactam antibiotics in Gram-negative bacteria (6-7), leading to increased mortality and morbidity rate in intensive care units (ICUs) (4, 8-9).

Most ESBLs have evolved from TEM and SHV beta-lactamases, but in recent years, new families of ESBLs including OXA, CTX-M and PER enzymes as well as novel beta-lactamases have been emerged (3-4, 9). According to the scheme of Ambler et al. (1991) these enzymes are classified in four molecular classes from A to D (10-11). The OXA-type (oxacillin-hydrolysing) beta-lactamases is a growing family of beta-lactamases, which belongs to the Ambler class D (functional group 2d), possessing a serine residue at the active site, similarly to classes A and C beta-lactamases. Class D ESBLs have been found mainly in clinical isolates of *P. aeruginosa* (12-13). These enzymes are characterized by high hydrolytic activity against oxacillin, cloxacillin and methicillin. Furthermore, they are poorly inhibited by clavulanic acid (12-14). Most of OXA type beta-lactamase genes are plasmid-transposon or integron-located genes. Integrons are natural gene cloning and expression systems that incorporate open reading frames and convert them into functional genes. Class 1 integrons are the most prevalent gene cassettes among clinical isolates (15). Like β-lactamases, integron-related gene cassettes have been found mainly in *P. aeruginosa*, *Acinetobacter baumannii* and among species of Enterobacteriaceae family (16) with variable prevalence worldwide (17). Antibiotic resistance mediated by ESBLs has remarkably evolved during the last 20 years (9, 15) and organisms producing these enzymes become a major problem in most of healthcare settings. There are few reports about the prevalence of OXA-2, OXA-10 and class 1 integron in nosocomial strains of *P. aeruginosa* from Iran (18). The aim of the present study was to determine the antibiotic susceptibility patterns of *P. aeruginosa* strains isolated from nosocomial infections and to identify the prevalence of OXA-2/OXA-10 type ESBLs and class I integron among these isolates in Tabriz, northwest of Iran.

Materials and methods

Bacterial isolates

A total of 110 isolates of *P. aeruginosa* were collected from Emam Reza educational hospital of Tabriz during 2016-2018. This research was approved by the Regional Ethics Committee, Tabriz University of Medical Sciences, Iran. Different clinical specimens including urine, blood, bronchial secretion, sputum and wound were used for isolation of *P. aeruginosa* strains. The isolates were identified on the basis of standard bacteriologic tests. The samples were selected on the basis of growth of oxidase positive, lactose non-fermenting pale colonies on MacConkey medium which mostly were pigmented on nutrient agar. After obtaining the pure strains, the strains were subjected to biochemical identification tests to identify *Pseudomonas* spp. For this purpose, samples were inoculated in Triple Sugar Iron media (TSI), Citrate media, Peptone water, Urease media and kept in an incubator for 18 hrs at 37°C. Next day the results were noted on TSI, Citrate media and Urease media. Part of growth on Peptone water was subjected to Indole test with Kovac's Reagent and part for motility test by 'Hanging drop' method. A strain of *Pseudomonas* in the TSI medium showed alkaline slant, no reaction in butt. It showed negative reaction in indole and urease tests and positive in citrate test. Glucose is utilized oxidatively, forming acid only.

Disc diffusion method

Antibiotic sensitivity of the isolates was determined according to the Kirby-Bauer method on Mueller-Hinton (MH) agar (Merck, UK). The antibiotic discs (MAST Company) used in this study were ceftazidime (30 μg), cefepime (30 μg), aztreonam (30 μg), gentamicin (30 μg), ciprofloxacin (30 μg), amikacin (30 μg), piperacillin/tazobactam (100/10 μg), meropenem (10 μg) and imipenem (10 μg). The results were interpreted according to the CLSI standards (19).

Detection of ESBL positive isolates

Combined disc method was carried out using discs of ceftazidime (CAZ), ceftazidime clavulanic acid (CAZ/CLO), cefotaxime (CTX), cefotaxime/clavulanic acid (CTX/CLO), cefepim (CPM) and cefepim/ clavulanic acid (CPM/CLO). The test was performed by placing discs on MH plates at a distance of 20 mm (center to center) from each other. ESBL production was inferred in cases that the zones of clavulanate containing discs were ≥5 mm larger than those without clavulanate (20-23).

DNA extraction and PCR amplification of β-lactamase genes and class 1 integron

All isolates were subjected to polymerase chain reaction (PCR) assays to detect blaOXA2/10 genes and class 1 integron. To extract DNA, one to three fresh bacterial colonies was suspended in 100 µl of TE Buffer (Tris 10 mM, EDTA 1 mM) and boiled at 100°C for 10 min. After centrifugation for 10 min at 10,000 rpm, the supernatant was collected for PCR. Primers were designed for OXA-2, OXA-10 and class 1 integron genes as shown in Table 1. Each PCR reaction contained 200 ng DNA, 10 pmol of each forward and reverse primers (10 pmol of each), 1.5 mM $MgCl_2$, 200 µM dNTPs, 1x PCR buffer, 2 units of Taq DNA polymerase (Fermentas) and up to 25 µL dH_2O. PCR amplification was carried out in 94°C for 4 min, followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 60°C for 1 min and extension at 72°C for 50 sec with a final extension cycle at 72°C for 5 min. PCR products were electrophoresed on 1% agarose gel and stained by ethidium bromide. The DNA bands were visualized under an ultraviolet light (UV transilluminator) and were documented.

Statistical Methods

Descriptive statistical approach was used for analysis of the data using the software of SPSS version 16.

Table 1. Primers used in PCR amplifications.

Primer pairs	Sequence (5´–3´)	Product size (bp)
OXA-2-F	5'- AAGAAACGCTACTCGCCTGC -3'	485
OXA-2-R	5'- CCACTCAACCCATCCTACCC -3'	
OXA-10-F	5'-TATCGCGTGTCTTTCGAGTA-3'	774
OXA-10-R	5'-TTAGCCACCAATGATGCC-3'	
Int1-F	5'-GGTGTGGCGGGCTTCGTG-3'	459
Int1-R	5'-GCATCCTCGGTTTTCTGG-3'	459

Results

Bacterial isolates and patients

Among 110 *P. aeruginosa* isolates collected from Emam Reza Educational Hospital of Tabriz, 71 (64.5%) cases were from male and 39 (35.5%) cases were from female patients. The age range was 12 to 88 years old with the mean age of 47 years old. Of 110 isolates, 44.5% were from urine, 29.1% from wound, 10.9% from sputum, 10% from bronchial secretion and 5.5% from blood. The clinical specimens were collected from different hospital wards including: ICU (32.7%), Internal (29%), Urology (15.5%), Surgery (6.4%) and Trauma, Neurology and Emergency (5.5%).

Antimicrobial susceptibility tests

The results of antimicrobial susceptibility test showed that the highest resistance rates were against aztreonam (81.8%), cefepime (77.3%), gentamicin (74.5%), ciprofloxacin (72.7%), ceftazidime (72.7%) and amikacin (70%), whereas the lowest resistance rated were found to imipenem (22.7%), meropenem (29%) and piperacillin/tazobactam (37.3%) (Table 2).

Table 2. Antimicrobial susceptibility of 110 clinical isolates of *Pseudomonas aeruginosa*.

Antibiotic	Resistance No. (%)	Intermediate No. (%)	Sensitive No. (%)
Aztreonam (10µg)	90 (81.8)	13 (11.8)	7 (6.4)
Cefepime (30µg)	85 (77.3)	7 (6.4)	18 (16.3)
Gentamicin (10µg)	82 (74.5)	23 (21)	5 (4.5)
Ceftazidime (30µg)	80 (72.7)	2 (1.8)	28 (25.5)
Ciprofloxacin (30 µg)	80 (72.7)	10 (9.1)	20 (18.2)
Amikacin (30µg)	77 (70)	3 (2.7)	30 (27.3)
Piperacillin/tazobactam (100µg)	45 (40.8)	24 (21.9)	41 (37.3)

ESBL production

Combined disc test was used by placing the discs of CAZ-CAZ/CLO, CTX-CTX/CLO and CPM-CPM/ CLO on the MH plates. Results of the test showed that among 110 *P. aeruginosa* isolates, 68 (61.8%) isolates were positive for ESBL. There were 17 isolates resistant to all tested antibiotics; among them 15 isolates were ESBL producers whereas only 2 of them were ESBL negative. The resistance rate of ESBL producing isolates to different antibiotics were as follows: aztreonam (59%), cefepime (55.3%), ceftazidime (54.6%), gentamicin (54.5%), ciprofloxacin (53.5%) and amikacin (51.81%), whereas 23% of ESBL non-producers isolates showed resistance to aztreonam followed by cefepime (22%), gentamicin and ciprofloxacin (20.5%), ceftazidime (18.4%) and amikacin (18.2%).

Screening for class 1 integrons by PCR method

Screening for class 1 integron by PCR revealed that 47 (42.7%) *P. aeruginosa* isolates carried INT-1 gene (Fig. 1). Frequency of class 1 integrons were 18 (23.1) and 29 (90.6) in ESBL positive and ESBL negative isolates, respectively (P<0.01). Other factors associated with imipenem susceptibility among *P. aeruginosa* isolates were presented in Table 2.

Detection of blaOXA- 10/OXA2 resistance genes by PCR method

The results of PCR showed that 22 (20%) samples were positive with primers specific for blaOXA-10 (Fig. 2), whereas 7 (6.4%) isolates were positive with blaOXA-2 primers (Fig. 3). In addition, four (3.6%) isolates carried both blaOXA-2 and blaOXA-10 β-lactamases.

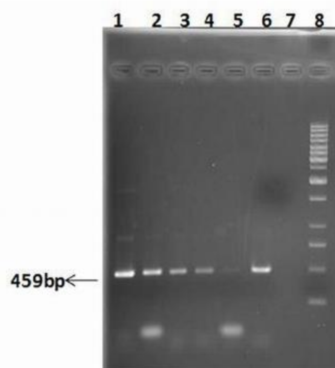

Figure 1. PCR amplification of class 1 integron gene (459 bp) in clinical isolates of *P. aeruginosa*. Lanes 1-5: class 1 integron positive isolates, Lane 6: positive control, Lane 7: negative control, and lane 8: 1 kbp DNA size marker.

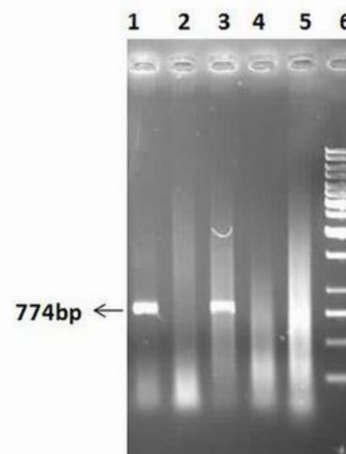

Figure 2. PCR detection of OXA-10 gene (774 bp) in *P. aeruginosa* clinical isolates. Lanes 1 and 3: OXA-10 positive isolates, lane 2 and4: OXA-10 negative isolates, lane 5: negative control and lane 6: 1 kbp DNA size marker.

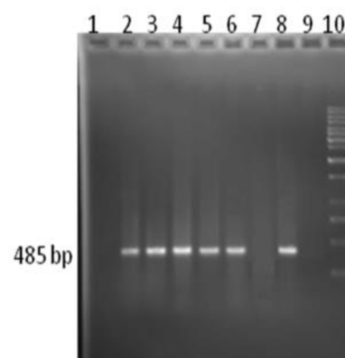

Figure 3. PCR for detection of OXA-2 gene (485 bp) in *P. aeruginosa* clinical isolates. Lanes 1 and 7: OXA-2 negative isolates, lanes 2- 6: OXA-2 positive isolates, Lane 8: positive control, lane 9: negative control and lane10: 1 Kbp DNA size marker.

Discussion

Pseudomonas aeruginosa is one of the most important nosocomial pathogens that causes various types of clinical infections in the world (2, 23). In recent years, emergence of MDR strains restricted the therapeutic options for treatment of infections due to *P. aeruginosa*. The results from this study showed a high-level resistance of *P. aeruginosa* isolates to most of tested antimicrobials. It was also demonstrated that most of *P. aeruginosa* isolates (88.7%) were multi-drug resistant strains, showing resistance to ≥3 different antibiotic classes. Our findings also revealed that prevalence of antibiotic resistance in *P. aeruginosa* isolates is very high in comparison to other studies (12, 15).

Pseudomonas aeruginosa has an inducible, naturally occurring cephalosporinase that confers low-level resistance to aminopenicillins and narrow-spectrum cephalosporins. Resistance to extended-spectrum cephalosporins is associated with the overexpression of this cephalosporinase. Alternatively, resistance to the broad spectrum cephalosporins may result from production of extended-spectrum ß-lactamases (ESBLs) (23-24). It has been shown that unlike other Gram-negative bacteria, production of TEM and HSV ESBLs are uncommon in *P. aeruginosa* (25) and other extended spectrum enzymes, such as PER-1 (26) OXA-2 and OXA-10 ESBLs (27-29) are involved in resistance to extended-spectrum cephalosporins in this bacterium. The results of this study revealed that among 68 (61%) ESBL positive isolates, 22 (32.3%) contained blaOXA-10 and 7 isolates (10.3%) contained blaOXA-2 genes. These findings are similar to results reported from Kerman province of Iran, where 38.33% of *P. aeruginosa* isolates were positive for ESBLs, of which 63% were positive for OXA-10 and 2.1% for OXA-2 ESBLs (28). Alipour et al. (2010) showed that among ESBL positive *P. aeruginosa* isolates in Ilam hospitals, 87.61% were positive for OXA-10 while only 4.76% of isolates were positive for OXA-2 ESBLs (30). A study conducted by Lee et al., in Korea (2005) demonstrated that among 252 *P. aeruginosa* isolates, 13.1% were positive for OXA-10 whereas OXA-2 found in 2.3% of isolates (1). Jiang et al., also found OXA-10 ESBL in 17.3% of 75 *P. aeruginosa* strains (20). These results show a notable variation in prevalence of ESBL genes in different countries indicating heterogeneous distribution of this class of resistance genes in various geographic regions.

Spread of ESBL-producing bacteria is a great problem in healthcare centers in Iran. However, it should be considered that the prevalence of ESBL genes varies in different geographical areas (31). It is clear that ESBL-producing organisms are widely distributed globally, but this rate is lower in several parts of the world than Iran. Therefore, the presence of ESBLs genes is a risk factor for the future use of antimicrobial treatment in Iran. ESBLs distribution and the facilitation of their spread in different regions may be caused by factors such as "mobility" of ESBL genes, strong selective pressure of antibiotic use, purchase antibiotics without prescriptions, lack of observing hand hygiene, use of antibiotics in animals, travel, and different weather conditions.

In conclusion, the results of this study demonstrated high prevalence of ESBL positive, MDR strains of *P. aeruginosa* in our hospital settings, where OXA-10 and OXA-2 ESBLs had a noteworthy prevalence. These findings emphasize on necessity of preventive measures to avoid further spread of such resistant strains.

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Editorial Note

Volume 7, issue 2 of Progress in Biological Sciences was initialy scheduled to be published in December 31, 2017. However, some administrative changes leaded to a major delay in processing of the manuscripts. This issue is actually published in May 1, 2020. Editor-in-chief apologizes deeply for any inconvenience caused especially to the authors.