

Full Length Research Paper

Outbreak of ambler class A and D b-lactamase in multidrug-resistant *Pseudomonas aeruginosa* strains isolated from non burn patients

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Pseudomonas aeruginosa is a leading cause of infections including pneumonia, urinary tract infections and bacteremia, especially in patients with weakened immune systems. This pathogen is intrinsically resistant to many antibacterial agents, such as most beta lactams. The aim of this study was to survey antibiotic resistance pattern and frequency of bla VEB 1 and bla OXA10 genes in multidrug-resistance *P. aeruginosa* isolated from non burn patients in Isfahan hospitals. Total of 100 clinical isolates of *P. aeruginosa* from different samples were collected. Antibiotic susceptibility for antimicrobial agents was carried out according to the Clinical and Laboratory Standards Institute (CLSI) guidelines and the frequency of these genes was studied by polymerase chain reaction (PCR). Among the isolated strains, resistant rate to the antibiotic ciprofloxacin was 56%, gentamicin 59%, tobramycin 61%, amikacin 65%, imipenem 55%, cefepime 55%, ceftazidime 57%, ceftriaxone 60%, cefotaxime 62% and piperacillin was 48%, respectively. *P. aeruginosa* demonstrated the highest resistance rate to amikacin (65 %), 55% of isolates were resistance to imipenem and cefepime whereas 63% were MDR (resistant to three or more classes of antibiotics). A multidrug-resistant (MDR) phenotype occurred frequently in *P. aeruginosa*. PCR was performed for all the resistant strains, prevalence of structural genes for VEB-1 and OXA-10 in isolates were 38 (60%) and 40 (64%), respectively. Proper infection control practices are essential to prevent spreading and outbreaks of ESBL-producing *P. aeruginosa* in hospitals.

Key words: *Pseudomonas aeruginosa*, ambler class A and D b-lactamase, antibiotic resistance.

INTRODUCTION

The blaVEB-1 and blaOXA10 extended spectrum β-lactamases (ESBLs)-genes belong to ambler class A and D and associated with multiple antibiotic resistances, in the form of a gene cassette. blaVEB was identified first in an *Escherichia coli* isolated from a child in France (Poirel et al., 1999). The blaVEB-1 gene is considered to be emerging, since its presence has been reported in many

Gram-negative organisms from different parts of the world during the last decade. It has been identified in *Enterobacteriaceae* and *Pseudomonas aeruginosa* in many countries, such as France, Spain, Algeria, Turkey, Canada, Korea and Thailand (Naas et al., 2008). The OXA-type enzymes are frequently observed in *P. aeruginosa* and possess high-level hydrolytic activity

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Table 1. Primer designed sets.

| Primer | Sequence | Product size (bp) |
|------------|----------------------------|-------------------|
| blaVEB1-F | 5'-GTGGAGTCCGATTAAGAGG-3' | 449 |
| blaVEB1-R | 5'-CATCATTAGTGGCTGCTGC -3' | |
| blaOXA10-F | 5-ATTATCGGCCTAGAACTGG-3 | 170 |
| blaOXA10-R | 5-CTTACTTCGCCAACTTCTCTG-3 | |

against oxacillin and methicillin and their activities are poorly or not inhibited by clavulanic acid. These enzymes confer resistance to amoxicillin and cephalothin (Bush et al., 1995). Class D OXA-type β -lactamases, have extreme sequence variation, with the identities varying from 16 to 99% between individual enzymes. ESBLs are commonly encoded on plasmids that confer resistance to most cephalosporins, such as cefotaxime, ceftriaxone and ceftazidime (Nordmann and Guibert, 1998). Other ESBLs such as TEM and SHV types have been identified in the past (Bradford, 2001). TEM ESBLs are also extremely reported. These enzymes are constantly found in *E. coli* and *Klebsiella pneumoniae* and recently reported in *P. aeruginosa* at low frequency. These enzymes belong to the family of class A (TEM, SHV, CTX-M) β -lactamase. Today, there are more than 157 TEM, 101 SHV, 65 CTX-M and 5 VEB variants (Poole, 2004). Antibiotic resistance in bacteria carrying these enzymes occurs in several ways, including efflux pumps, production of β -lactamases and outer membrane modifications (Zavascki et al., 2010). Many *P. aeruginosa* strains can produce several types of ESBLs that enable them to be resistant to many strong antibiotics (Jones, 2001). ESBLs are inactivated by the β -lactamases inhibitor (Drawz and Bonomo, 2010) for example clavulanic acid (Yu et al., 2006). So far, prevalence of blaVEB1 genes, in few studies have been shown. This study was performed to investigate the prevalence of Ambler class A and D β -lactamases in non burn isolates of *P. aeruginosa*.

MATERIALS AND METHODS

Bacterial sources

100 strains of *P. aeruginosa* were isolated from different infected patients in care unit of the hospitals (SHariati, Al Zahra, Imam Musa), in Isfahan within six months period (February to July 2012). Identification of the isolates was done according to standard microbiology tests including colonial morphology, oxidase positivity, the presence of characteristic pigments and growth at 42°C (Tam et al., 2010).

Antibiotic susceptibility tests

Antimicrobial susceptibility test was performed for all isolates. Antibiotic disks included ciprofloxacin, gentamicin, tobramycin, amikacin, imipenem, cefepime, ceftazidime, ceftriaxone, piperacillin

and cefotaxim. A sterile loop was used to pick colonies of *P. aeruginosa*, then emulsified in 5 ml of sterile Mueller Hinton broth to match with 0.5 McFarland (1.5×10^8 cfu/ml). A sterile swab using, the surface of Mueller Hinton Agar (Oxoid, Basingstoke, UK) in a Petri dish was evenly inoculated with the suspension and then the disks were put. The plate was then incubated at 37°C for 18 h. The diameters of the zones of inhibition were measured in millimetres. Disks (Himedia, Mumbai) were used. *P. aeruginosa* ATCC 1310 was used as quality control strain in susceptibility determination. Multidrug-resistant (MDR) isolates were defined as those showing resistance to three or more classes of antipseudomonal agents (carbapenems, fluoroquinolones, penicillins/cephalosporins and aminoglycosides) (Jarlier et al., 1998).

DNA extraction

DNA extracted with phenol/chloroform briefly, 1 ml of 24 h grown *P. aeruginosa* in Brain-Heart Infusion Broth (BHI) (Scharlau, Spain) were transferred into 1.5 ml sterile microfuge tubes and centrifuged at 13,000 g for 10 min. The pellets were dissolved in 500 μ l of lysis buffer (NaCl 1M, Tris-HCl 100 mM, EDTA 0.5 M), 20 μ l SDS (10%), 3 μ l of proteinase -K (20 mg/ml) and incubated at 55°C for 2 h. Then, phenol/chloroform/isoamylalcohol (25:24:1 volume/volume) were added, vortexed, and centrifuged at 12,000 g for 10 min. The supernatants were transferred to sterile tubes. 1 ml of 95% cold ethanol was added and stood for 1 h in refrigeration condition (4°C). DNA was then precipitated in each tube by centrifugation at 12,000 g for 10 min. The precipitated DNA was dissolved in 50 μ l of 10 mM Tris EDTA - buffer (TE) (Shacheraghi et al., 2010).

PCR reaction

PCR was used to detect blaVEB1 and OXA10 genes, in the multidrug resistant bacterial strains using the following primer designed sets (Table 1).

Total volume of reaction was 25 μ l. PCR Master consisted of 1X-PCR reaction buffer (Sinaclon), MgCl₂ 0/7 μ l, dNTP (0/5 μ l), 1 μ l of each primer, 10 mM Tris-HCl, Taq DNA polymerase (0/2 μ l) and 1 μ l of DNA template. Amplification was carried out in a thermocycler (Eppendorf Mastercycler®, USA). Agarose gel electrophoresis (1%) of PCR products was carried out in horizontal bed apparatus using 1 mM Tris-Borate- EDTA (TBE) buffer (pH-7) at 85 V for 1 h and the bands were then stained with 0.5 μ g/ml ethidium bromide for 10 min. The reaction conditions were as follows: pre-denaturation at 94°C for 4 min, followed by 25 amplification cycles of 94°C for 1 min, 55°C for 1 min and 72°C for 1.5 min, with a final extension step of 72°C for 10 min. The gels were washed and observed under U.V. gel documentation (UV DOC, England) at 280 nm. 100 bp DNA ladder was used to confirm the size of specific bla gene. Simultaneously, a positive control was run for bla genes (*P. aeruginosa* strains containing blaVEB-1 and bla OXA-10 were prepared from stock collection of Institute pasture of Iran). *P. aeruginosa* PTCC 1074 was used as a sensitive strain (negative control) (Shacheraghi et al., 2010).

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TTCGCGCGCCCGGAACCCGATGGGACGGTATAGCCCCAGTGCCATGAGCAATGGGTAAGTGACTTGACCTTCAG
TGGGGCAATACAAGTTTCAGCTGTTCCCGTATTTCAACAAATCGCCAGTGAAGTTGGCCGAAGTAAGTTATAACACC
TTCTTTTTCCCTTCTTTCTTTCTCTCCCTTCTCCTGACTTCTCCCTTCCATCCCCTCTCTCCTCCCTGGTTTCCC
CTTCTTCCTTTACCTCCTTCTCTCCGCCCCCCCCAGCCACCTCCCCTCTGCTACCTCGCCTTCCTTCCGTCTCC
CTTTCTCCACTTGCGCCCTCCTGCTTCTCCCCCCCCCTCCTCCCTTCTCTTCCCCTTCCCCCTTATCCTTTCTCCCC
GCTTCTGTCCTTACCCCTGCTCCTTCTTCTGCTGTCCCCGCTCCGCCCCCCCTTCTGCTCTTCTCCCTCCTC
CCCTTTTCGCGCTCCTCTCCTTCTCCTCTATCTCCCGTTGCTCTCCGTCGTGTTCTGTTCTCCCTCTTCTTCTCCTG
CTTCCGTCTCCCTTTTCTGTTCCGTTCTTCTCTCTTCTCTTCTTCTGTTTGGCACTTCATGCCTCCTATCACCC
TTGTTTTCCCCTCTTCTCTCCTTCTCTCCTCCCCCTTCTCCCTCTTTTTTTCTTGC

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Figure 1. Sequences of *bla_{oxa}-10* of *P. aeruginosa* strains isolated from patients in Isfahan hospital. The nucleotide sequences was analyzed with CROMASPRO-2 and MEGA 4 software and confirmed by blast system in the internet.

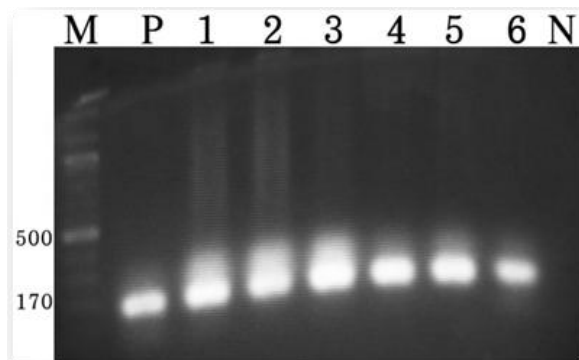


Figure 2. Agarose gel electrophoresis of PCR product of class D betalactamase *bla_{OXA}-10* detected from *P. aeruginosa* isolates. M = Molecular weight marker (100-bp), P = positive control, 1, 3 to 6 = strains producing OXA10 genes (170 bp), N = negative control.

Sequencing of the PCR products

DNA sequencing was subjected to direct sequencing of both the strands performed by the Macrogen Company (Seoul, Korea) (Al Naiemi et al., 2006). The nucleotide sequences were analyzed with CROMASPRO-2 and MEGA-4 softwares (Figure 1).

Statistical analysis

SPSS version 11.5 was used to analyze the data, and a Fisher exact test was used for the categorical data. P-value of ≤ 0.05 was considered significant.

RESULTS AND DISCUSSION

The prevalence of multidrug-resistant *P. aeruginosa* producing ESBLs is increasing worldwide (Bonomo and Szabo, 2006). Antibiotic resistance that can be related to plasmid or chromosome occur in *P. aeruginosa* isolated from patients in different hospitals in Iran (Shakibaie, 2002). Our results reported the highest resistance rate to amikacin and cefotaxime, with 65, 62% and lowest resistance rate to piperacillin, imipenem and cefepime

with 48 and 55%, respectively. In our study, amikacin resistance value was higher than values reported in study conducted by Amutha et al. (2009). In the study of Prashant et al. (2011), highest resistance rate to ceftazidime (53%) and lowest resistance rate to imipenem (12%) was reported. In the present study, resistance to gentamicin: 59%, tobramycin: 61%, ceftazidime: 57%, ceftriaxone: 60% and cefotaxime: 62%, was observed, also PCR was performed for all the resistant strains, the frequency of *bla_{VEB1}* gene was 38 (60%) and *bla_{OXA10}* gene was 40 (64%) among 63 strain with multidrug resistance (Figure 2), that have significant increase as compared to the previous studies.

In Saudi Arabia, Tawfik et al. (2012) reported that resistance rate to ceftazidime piperacillin, piperacillin/tazobactam, cefepime, aztreonam, imipenem, amikacin, gentamicin and ciprofloxacin were 22.43, 100, 71.14, 88.57, 48.57, 70.0, 82.5, 87.5 and 90.0%, respectively. The prevalence of ESBL and MBL in *P. aeruginosa* was 69.44 and 42.85%, respectively. Also, prevalence of structural genes for VEB-1, OXA-10 and GES ESBLs in isolates was 68, 56 and 20%, respectively. VIM gene was detected in 15 (100%) of

MBL-producing isolates. As can be seen, the frequency of this genes in Iran and Saudi Arabia, are almost identical to each other. While, in the case of antibiotic resistance, there is a significant difference. Mirsalehian et al. (2010) in Tehran (Iran) showed that, aztreonam, Imipenem and meropenem were the most effective anti-pseudomonal agents. Also, they reported that 148 (87.05%) of the isolates were MDR and 67 (39.41%) of the isolates were ESBL producer. Fifty (74.62%), 33 (49.25%) and 21 (31.34%) strains among 67 ESBL-producing strains amplified blaOXA-10, blaPER-1 and blaVEB-1, respectively. There was significant differences between the results of the present study and that of Mirsalehian et al. (2010), which may be due to differences in the specimens type (burn and non burn). Class D OXA β -lactamases were more frequently detected than class A in *P. aeruginosa* from Korea (21.0 versus 6.3%), which contrasts with data from Europe (31.3 versus 64.9%) and our study (60 versus 64%). In the study of Lee et al. (2005) in Korea, among the 252 isolates, 53 (21.0%) isolates harboured OXA-type enzyme. Among them, blaOXA-10, like our study, was most prevalent, and OXA-4, OXA-2, OXA-30 and OXA-17 also had a high frequency. 6 isolates (2.4%) harboured two different β -lactamases and one isolates harboured three enzymes. PER-1, VEB-1, TEM, SHV, CTX-M and GES-1 enzymes were not detected. Bert et al. (2002) reported that four isolates of *P. aeruginosa* produced ESBLs according to the double disc synergy (DDST) (phenotypic) method. PCR showed that 68 (26.3%) isolates was carrier of OXA-10 group including: 31 carried bla_{OXA-10}, one carried bla_{OXA-14} and 36 carried a new variant between bla_{OXA-13} and bla_{OXA-19}. The bla_{OXA-2} gene was identified in 13 (5%) isolates. According to the results, we can say that the prevalence of these genes was much lower in 2002. The most prevalent beta-lactamase genes were blaVIM-2 and blaOXA-10 in the study of Suk et al. (2007). Lee et al. (2005) found that in 64 (25.4%) isolates, there was prevalence of OXA-10 (13.1%), OXA-4 (4.3%), OXA-30 (2.0%), OXA-2 (2.3%) and OXA-17 (0.4%); their distribution varied between provinces. The cross-class resistance rates to other antibiotics was significantly higher in class A and D β -lactamase producers than in non-producers. Suk et al. (2007) reported that 2.9% of isolates were multidrug-resistant (MDR), PCR amplification, and sequencing of the PCR products showed that the blaOXA-10, blaVIM-2, blaOXA-2, blaOXA-17, blaPER-1, blaSHV-12, and blaIMP-1 genes were carried by 34.3, 26.9, 3.0, 3.0, 1.5, 1.5 and 1.5% of 67 MDR *P. aeruginosa* isolates, respectively. As compared to our study, the frequency is less. Prevalence of blaOXA10 and VEB1 in the study of Shacheraghi et al. (2010) in Tehran was 92%, in burn patients. Finally, the prevalence of MDR *P. aeruginosa* encoding blaOxa10 In Iran (64 to 92%) was higher, when compared with that from Korea (13%), France (26%) and India. Although, the prevalence of this genes in Tehran

(92%), is higher than that in our study, probably it is due to the high prevalence of these resistant genes in burn patients.

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