

Association Between Metallo- β -lactamases and Integrons with Multi-Drug Resistance in *Pseudomonas aeruginosa* Isolates

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Pseudomonas aeruginosa is among the most important pathogens in the nosocomial infections. A genetic mobile element, the integron, is one of the major agents involved in dissemination of multi-drug resistance among gram negative bacteria. During a descriptive study from October 2009 to August 2010, some 130 *P. aeruginosa* clinical isolates were collected from different wards of three hospitals in Tehran. The Minimal inhibitory concentration (MIC) of 4 antibiotics conventionally used in clinical settings against the isolates was determined by E-test method. Also, the existence of integron classes and metallo- β -lactamases (*bla*_{VIM-1}, *bla*_{IMP-1}, and *bla*_{VIM-2}) were investigated by PCR assay. Out of 130 isolates, 74 (56.9%) carried class 1 integron. None of the isolates harbored integrons class 2 and 3. Also, the *bla*_{VIM-1} gene was detected in 10 (13.3%) high level ceftazidime and imipenem-resistant isolates that carried class 1 integrons. The *bla*_{IMP-1} and *bla*_{VIM-2} genes were not detected in any isolates. In the present study, the antibiotic resistance rates in class 1 integron-positive isolates of *P. aeruginosa* were significantly higher than those lacking this integron, e.g., 82.6% resistance versus 17.3% sensitivity to ceftazidime. Also, 13.3% of ceftazidime and imipenem resistant isolates was metallo- β -lactamase producer. This indicates that all metallo- β -lactamase genes are correlated with class 1 integrons. These results imply that the *bla*_{VIM-1} gene has been presumably dispersed into *P. aeruginosa* isolates with the help of class 1 integron element.

Key words: β -lactam, Integron, Antibiotic resistance, Metallo- β -lactamase, *Pseudomonas aeruginosa*

INTRODUCTION

Antimicrobial chemotherapy of *Pseudomonas aeruginosa* infections is a major problem, especially in hospitals. In humans, *P. aeruginosa* causes a broad spectrum of infections particularly among immunocompromised patients and persons with severe burns, diabetes mellitus or cystic fibrosis [1]. Some strains of this pathogen have been found resistant to all previously known antibiotics [2]. *P. aeruginosa* resistance is acquired either through mutations or via horizontal transfer of mobile DNA elements. The intensive use of antimicrobial agents has facilitated the rapid emergence of increased resistance in this species. Recently, integrons, a newly discovered class of mobile DNA elements, was found to be responsible for multi-drug resistance among gram negative bacteria [3]. These elements are distinct from all other genetic elements, i.e., they are able to utilize site-specific recombination to

acquire and integrate circular gene cassettes [4]. The gene cassettes in the variable segment of integrons, contain an antibiotic resistance gene and a recombination site (59-bp, also called *attC*), which is recognized by an integrase [5]. To date, ten different classes of integrons have been described of which five carry antibiotic resistance genes [6-8]. Class 1 integrons are the most prevalent among clinical isolates [9]. On the other side, gram-negative bacteria produce class B metallo- β -lactamases (MBLs), which hydrolyze a wide range of broad-spectrum β -lactams and are not inhibited by clinical β -lactam inhibitors [10, 11].

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IMP (active on Imipenem), VIM (Verona Integron-encoded Metallo- β -lactamase) and SPM (Sao Paulo Metallo- β -lactamase) metallo- β -lactamases types have been developed worldwide and their genetic determinants are often associated with class 1 integrons [12, 29]. The aim of this study was to evaluate the resistance profiles of *P. aeruginosa* to 4 antibiotics as well as detecting the presence of resistance determinants including class 1, 2, 3 integrons and *bla*_{IMP-1}, *bla*_{VIM-1,2} genes.

MATERIALS AND METHODS

Clinical isolates. Some 130 clinical isolates of *P. aeruginosa* were collected from different wards of three hospitals in Tehran during years 2003-2004. The isolates were stored as suspension in a 10% sterilized skim milk solution containing 15% glycerol at -70°C.

Antibiotic Susceptibility Testing. Antibiotic susceptibility testing was performed according to the Clinical and Laboratory Standards Institute (CLSI) recommendations [13], using E-test method on Mueller-Hinton agar (Merck, Germany). The E-test strips used were as followed: ceftazidime (256 μ g/ml), gentamicin (256 μ g/ml), ciprofloxacin (32 μ g/ml), imipenem (32 μ g/ml), (ABBioMerieux, Solna, Sweden). *E. coli* (ATCC 25922) and *P. aeruginosa* (ATCC 27853) were used as reference strains for antibiotic resistance.

DNA Extraction. DNA extraction from single colonies of *P. aeruginosa* was obtained by boiling method as described by others [14]. To do this, single colonies from overnight cultures were suspended in 100 μ l sterile double distilled water and incubated in a hot block at 95°C for 10 min. Cellular debris was removed by centrifugation at $\times 11000$ g for 2 min, and the supernatant was transferred to 20°C until used.

Screening of MBL_S Producers. Identification of *Pseudomonas* MBL_S with β -lactams and carbapenems hydrolyzing activity were performed as 57.7% and 57% of the strains were resistant to ceftazidime and imipenem with MICs ≥ 128 μ g/ml

and ≥ 32 μ g/ml, respectively. The screening for MBL_S detection was evaluated by using disk synergy method, with one Kerby-Bauer disk containing 30 μ g of CAZ or 10 μ g IPM and a blank disk containing 930 μ g of EDTA (0.5 M) as MBL inhibitor. Two disks were placed on inoculated agar plates within a distance of 1.0 to 2.5 cm. Growth-inhibitory zones between two disks were appeared after incubation for 16-20 h at 35°C. An increase of ≥ 7 mm in zone diameter was considered as metallo-beta lactamase producing isolates [15, 16].

PCR detection of MBL_S and integrases genes. Specific PCRs for IMP-1 and VIM-1, 2 metallo- β -lactamases were performed on all isolates, which had positive results for screening test using EDTA disks. PCR detection of *bla*_{IMP-1}, *bla*_{VIM-1}, and *bla*_{VIM-2} genes was performed as described by others [17, 18]. *P. aeruginosa* ATCC 27853 was used as a negative control for MBL_S detection. PCR amplification for the detection of integrase genes was carried out under following condition: initial denaturation at 94°C for 5 min, followed with a cycle program (94°C for 30 sec, 55°C for 45 sec, 72°C for 2 min) for 30 cycle and final extension at 72°C for 10 min. For amplification of the *intI3* gene, the annealing temperature was increased to 57°C. Reaction mixture with final volume of 25 μ l contained 20 pmol of each primer, 200 μ M of dNTPs, 3.5 μ l of 10x reaction buffer (containing 1.5 mM MgCl₂), 0.5 U of *Taq* DNA polymerase, and 2 μ l of template DNA. The six primer sets used in this study are shown in Table 1. PCR products were separated on 1% agarose gel and visualized by ethidium bromide fluorescence.

RESULTS

Antibiotic susceptibility testing. Determination of MIC on 130 isolates revealed that 70 (53.8%) strains carried multi-drug resistant phenotype with MICs ≥ 16 μ g/ml for gentamicin and imipenem, ≥ 4 μ g/ml for ciprofloxacin, and ≥ 32 μ g/ml for ceftazidime. Interpretive criteria for antimicrobial susceptibility testing were adopted from manufacturers. Table 2 summarizes the resistant pattern of the isolates.

Table 1. Primers used for PCR amplification of integrase and MBL genes in this study

Gene	Sequence	The expected size of amplicon (bp)	Reference
<i>intI1</i> -F	5' -GCA TCC TCG GTT TTC TGG-3'	457	[20]
<i>intI1</i> -R	5' -GGT GTG GCG GGC TTC GTG-3'		
<i>intI2</i> -F	5' -CAC GGA TAT GCG ACA AAA AGG T-3'	789	[20]
<i>intI2</i> -R	5' -GTA GCA AAC GAG TGA CGA AAT G-3'		
<i>intI3</i> -F	5' -ATC TGC CAA ACC TGA CTG-3'	922	[20]
<i>intI3</i> -R	5' -CGA ATG CCC CAA CAA CTC-3'		
<i>bla_{IMP-1}</i>	F (5' -ACC GCA GCA GAG TCT TTG CC-3') R (5' -ACA ACC AGT TTT GCC TTA CC-3')	587	[20]
<i>bla_{VIM-1}</i>	F (5' -ATG TTA AAA GTT ATT AGT AGT-3') R (5' -CTA CTC GGC GAC TGA GCG AT-3')	801	[18]
<i>bla_{VIM-2}</i>	F (5' -ATG TTC AAA CTT TTG AGT AAG-3') R (5' -CTA CTC AAC GAC TGA GCG AT-3')	801	[20]

Table 2. Association between the existence of integron and antibiotic resistance among *P. aeruginosa* clinical isolates

Antibiotic (Abbreviations: CAZ, Ceftazidime; GEN, Gentamicin; CIP, Ciprofloxacin; IPM, Imipenem)	Integron-positive isolates (n= 56)	Integron-negative isolates (n= 74)	<i>p</i> -Value	% resistance of total (n=130)
	%R	%R		
CAZ	82.6	17.3	<0.05	57.7
GEN	72.9	20.7	<0.05	63
CIP	81	18.9	<0.01	59.2
IPM	76.6	23.3	<0.01	57

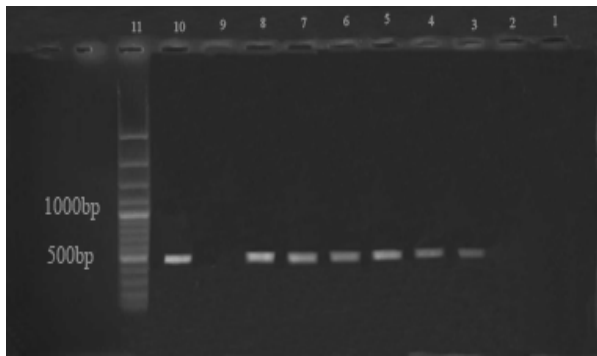


Fig. 1. Occurrence of *intI1* gene in clinical isolates of *P. aeruginosa*; lanes 1, negative control; lanes 2 and 9, negative strains; lanes 3, 4, 5, 6, 7, 8 and 10 positive strains; lane 11, 100 bp Plus DNA ladder.

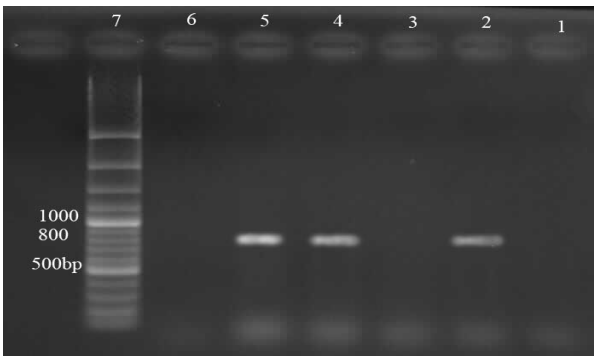


Fig. 2. PCR amplification with specific primers for *bla_{VIM-1,2}* and *bla_{IMP-1}* genes; lanes 1 and 3 negative strains; lanes 2 and 4 positive strains; lane 5, positive control; lane 6, negative control; lane 7, 100 bp Plus DNA ladder.

Detection of integrons. A total of 74 (56.9%) strains carried class 1 integrons. None of the isolates

carried class 2 and/or 3 integrons. Our results also indicated an association between antibiotic resistance to ceftazidime, gentamicin, ciprofloxacin,

imipenem with class 1 integron. The numbers of resistant isolates in the integron-positive and integron-negative groups were calculated using χ^2 test in SPSS software. Integron-positive strains with $P < 0.05$ were considered statistically to be resistant to the tested antibiotics (Table 2).

MBL detection. PCR revealed the presence of *bla*_{VIM-1} genes in 10 (13.3%) high-level ceftazidime (MIC ≥ 128) and imipenem -resistant (MIC ≥ 32) isolates. No *bla*_{IMP-1} and *bla*_{VIM-2} genes were detected in the present study

DISCUSSION

Emerging antimicrobial resistance to most classes of antibiotics including carbapenems is evident. The main concern, now, is that the increasing use of carbapenems as the last option may result to pandrug-resistant species in the future [19]. MBL-producing, gram negative bacteria are among the most important nosocomial pathogens, and further proliferation of these strains in clinical settings poses a serious global problem in the future [20]. The mobility of β -lactamase genes is associated with the class 1 integrons, and their dissemination throughout bacterial populations is a great concern. The present study on the existence of integrons and metallo- β -lactamase revealed that 56.9% of the isolates contained class 1 integron. These results are in agreement with the other published reports showing that the clinical isolates of *P. aeruginosa* harbors high prevalence of the integrons class 1 [21, 22]. Also, 13.3% of ceftazidime and imipenem resistant isolates showed to be MBL producer; this indicates that all MBL genes are correlated with class 1 integrons. Another study from Ahvaz, Iran, reported 41 imipenem-resistant *P. aeruginosa* isolates detected by PCR method; 8 isolates (19.51%) appeared to produce MBL and were positive for *bla*_{VIM} genes [23, 27]. Resistance of *P. aeruginosa* isolates to the broad-spectrum cephalosporins may be mediated by the extended-spectrum beta-lactamases (ESBLs). The high prevalence of multidrug-resistant *P. aeruginosa* isolates reported from burn patients in Tehran, Iran,

which were positive for *bla*_{PER-1}, *bla*_{VEB-1} (Ambler class A serine beta lactamases), and *bla*_{OXA-10} (Ambler class D) genes emphasize the importance of drug resistant strains in burn hospitals [24]. The ESBL genes detected among Gram-negative bacteria in the ICU setting of Sanandaj hospitals, Iran, were SHV, TEM, CTX-M (class A), OXA-1 and OXA-2 [25]. There is a need to institute a strict hospital infection control policy and regular surveillance of bacterial resistance to antimicrobial agents. In agreement with our findings, previous studies in Iran only detected *bla*_{VIM} gene among isolates and no MBL-producing isolates were positive for *bla*_{IMP} gene by PCR [26, 27]. A report on patients with level I burn from Iranian burn hospitals showed a mortality rate of 82.6% caused by infection with VIM-producing *P. aeruginosa*. MBL was produced by 23 imipenem-resistant isolates and *bla*_{VIM} gene was detected in all of these isolates. None of the isolates carried *bla*_{IMP} gene [28]. The important role of integrons in epidemiology of MBLs has been demonstrated in Japan, Greece, and Taiwan [20, 29, 30]. Since genes for *bla*_{VIM} have also been dispersing into various non-glucose fermentative bacteria such as *P. aeruginosa* and *Acinetobacter baumannii*; hence special precautions against the further global dissemination of integron-associated MBL genes among the nosocomial gram negative species should be brought into consideration. In view of the emerging threat of integron-positive and VIM-producing *Pseudomonas* strains in Iranian hospitals, a strategy should be designed to restrict the inappropriate prescription of the antibiotics.

ACKNOWLEDGMENTS

We thank Vice Chancellor of Alzahra University (Ms. Mobasheri) for the financial support and Dr. Sara Gharavi for revising of article.

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