Molecular detection of metallo-β-lactamase genes, $bla_{IMP-1}$, $bla_{VIM-2}$, and $bla_{SPM-1}$ in imipenem resistant *Pseudomonas aeruginosa* isolated from clinical specimens in teaching hospitals of Ahvaz, Iran

Mojtaba Moosavian$^{1,2}$, Mohammad Rahimzadeh$^2$

$^1$Health Research Institute, Infectious and Tropical Diseases Research Center, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran. $^2$Department of Microbiology, School of Medicine, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran.

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**ABSTRACT**

**Background and Objectives:** Carbapenem resistant *Pseudomonas aeruginosa* is a serious cause of nosocomial infections. The main purpose of the study is to determine the prevalence rate of imipenem resistant *Pseudomonas aeruginosa* carrying metallo-β-lactamase (MBL) genes.

**Material and Methods:** 236 *Pseudomonas aeruginosa* isolates were collected from teaching hospitals of Ahvaz University of Medical Sciences during a period of 9 months in 2012. These strains were identified using conventional microbiological tests. The susceptibility of isolates to antibiotics were assessed using disk diffusion test. The IMP-EDTA combination disk phenotypic test was performed for detection of MBL producing strains. Finally, polymerase chain reaction (PCR) was performed to detect MBL genes, $bla_{IMP-1}$, $bla_{VIM-2}$ and $bla_{SPM-1}$ in imipenem resistant strains.

**Results:** Out of 236 examined isolates, 122 isolates (51.4%) were resistant to imipenem. The IMP-EDTA combination test showed that among 122 imipenem resistant strains, 110 strains (90%) were phenotypically MBL producers. Additionally, the results of PCR method showed that 2 strains (1.6%) and 67 strains (55%) of imipenem resistant *Pseudomonas aeruginosa* isolates contained $bla_{VIM-2}$ and $bla_{IMP-1}$ genes respectively. No $SPM-1$ gene was found in the examined samples.

**Conclusion:** Resistance of *P. aeruginosa* isolates to imipenem due to MBL enzymes is increasing in Ahvaz. Because of clinical significance of this kind of resistance, rapid detection of MBL producing strains and followed by appropriate treatment is necessary to prevent the spreading of these organisms.

**Keywords:** *Pseudomonas aeruginosa*, carbapenem resistant, $bla_{IMP-1}$, $bla_{VIM-2}$, $bla_{SPM-1}$.

**INTRODUCTION**

*Pseudomonas aeruginosa* originally is an environmental bacterium that is considered as an opportunistic pathogen which infects hospitalized and immune-compromised patients. The carbapenem-resistant *P. aeruginosa* causes serious infections, such as nosocomial pneumonia which based on the reports is increasing in the hospitalized patients (1). Resistance to carbapenems is often associated with production of metallo-β-lactamases (1, 2). Nosocomial infections caused by *Pseudomonas aeruginosa* remains the major cause of mortality, particularly because of emergence of multidrug-resistant strains (2).

The most effective antibiotics that can be used against *Pseudomonas aeruginosa* are β-lactam antibiotics in which imipenem as a carbapenem is considered as the most appropriate antibiotic to be used against the mentioned organisms. Carbapenem-resistant *P. aeruginosa*
resistance occurs because of decrease in antibiotics absorption due to lack of an outer membrane porin, as oprD, exclusion from the cell by efflux pump, decrease in outer membrane permeability and production of MBL (2, 3). According to some recent reports, infection with metallo-beta-lactamase producing \emph{P. aeruginosa} strains has increased mortality. Nowadays the emergence of antibiotic resistance strains is one of the challenges in treating patients, such as MBLs producing \emph{Pseudomonas aeruginosa} (4, 5).

Carbapenemases can be classified into two main molecular families: those with serine at their active site, known as serine carbapenemases, and those with at least one zinc atom at their active site known as metallo-carbapenemases, which are considered as subgroup of metallo-beta-lactamases (MBLs). The VIM, IMP and SPM types are the most clinically significant carbapenemases which encoded by \textit{bla}_{\text{IMP}}, \textit{bla}_{\text{VIM}} and \textit{bla}_{\text{SPM}} genes (6). At least 14 different VIMs and 23 different IMP MBLs have been identified so far. MBLs also divided into several families as follows: IMP, VIM, SPM, GIM, SIM, DIM, AIM, KHM, NDM and KPC. Most of them, if not all, genes encoding IMP, VIM and SPM types as well as GIM are found as gene cassettes in class 1 integrons, although IMP MBL genes are also found on class 3 integrons (6 and 7).

The aim of this study was to evaluate the existence of encoding genes of \textit{bla}_{\text{IMP}}, \textit{bla}_{\text{VIM}} and \textit{bla}_{\text{SPM}} metallo-beta-lactamases between imipenem-resistant \emph{P. aeruginosa} strains which were isolated from clinical specimens in Golestan and Imam Khomeini hospitals in Ahvaz, Iran.

**MATERIALS AND METHODS**

**Bacterial strains and antibiotic susceptibility tests.** During a period of 9 months from October 2011 to June 2012, the bacterial colonies suspected to \emph{Pseudomonas} were collected from hospitalized patients in Golestan and Imam Khomeini, in Ahvaz, Iran. Theses bacteria had been isolated from different clinical specimens such as urine, wound, blood, trachea and other clinical specimens. After transporting the samples to the microbiology laboratory in Medical School, the colonies were again inoculated into MacConkey agar medium and pure colonies were identified as \emph{P. aeruginosa} based on Gram staining and biochemical tests such as oxidase, catalase, Oxidative-fermentative test, growth on media such as TSI, SIM, cetrimide agar and growth at 42°C (8). Isolates were preserved in Trypticase soy broth media (TSB) containing 20 % glycerol and stored at -70°C until used (8, 9).

**Susceptibility testing.** The susceptibility pattern of isolates to different antibiotics were examined using disk diffusion method (Kirby-Bauer) on Muller-Hinton agar plates according to guidelines of CLSI (10). The antimicrobial disks were included: imipenem (10μg), meropenem (10μg), ceftazidime(30μg), carbenicillin (100μg), tobramycin (10μg), amikacin (30μg), ticarcillin(75μg), gentamicin (10μg), cefotaxime (30μg), and ceftizoxime (30μg) ( MAST Co. UK). \emph{Pseudomonas aeruginosa} ATCC27853 were used as a control strain (11).

**MBL phenotypic test.** Combination disk diffusion test (CDDT) was used for phenotypic detection of MBLs producing \emph{P. aeruginosa} strains. In brief, 5μl of 0/5M EDTA (935μg) plus 10μg of imipenem were placed on the Muller Hinton agar plates which were inoculated with \emph{P. aeruginosa}. After 18-24h of incubation at 37°C, an organism was considered MBL positive, if growth inhibition zone was increased 7 mm or more in comparison with IMP disk alone (11).
Extraction of DNA. DNA was extracted from P. aeruginosa colonies using a simple boiling method. A few colonies from an overnight culture of P. aeruginosa isolates were suspended in 500 μl of TE buffer (10 m MTris, 0.5 mM EDTA) using vortex. The suspension was heated in a boiling bath at 95°C for 10 min. After centrifugation at 14000 × g for 4 min, the supernatant was used as a source of template for amplification (12).

Molecular analysis. Polymerase chain reaction (PCR) was carried out for detection of bla\textsubscript{IMP}, bla\textsubscript{VIM} and bla\textsubscript{SPM} genes on a thermal cycler (Eppendorf, Germany). The primer pair sequences used in this study and the PCR conditions are detailed in Table 1 (1). The PCR products depend on molecular size were separated on 1% and 1.5% agarose gel and then were stained with ethidium bromide. The separated bands visualized under UV light in a Gel documentation box (VilberLourmat, French). Positive controls used in this test were SPM-1 producing P. aeruginosa 16 strain (provided by Prof. Patrick Nordmann), bla\textsubscript{IMP}, from Seratia marcesens (sequenced by Bioneer company), and bal\textsubscript{VIM2} from Klebsiella pneumoniae (sequenced by Bioneer company). P. aeruginosa ATCC 27853 was used as a negative control (13).

RESULTS

Bacterial strains, antibiotic susceptibility and MBL phenotypic test. In this study a total of 236 clinical isolates of P. aeruginosa that were cultured from the clinical specimens were examined. Out of 236 clinical isolates, 99 (41.9%) were isolated from urine, 47 (19.9%) from trachea, 39 (16.6%) from lesion, 20 (8.5%) from blood, 17 (7.3%) from pus, 7 (2.9%) from eye and 7 (2.9%) from ear.

Based on the susceptibility test results, 122 isolates (51.4%) were resistant to imipenem. The rates of resistance to other antibiotics is shown in the Table 2. Of 122 imipenem resistant isolates, 110 (90%) were MBL producer as determined by CDDT. All MBL producing isolates were resistant to the examined antibiotics.

Genomic analysis. The results of amplified genes by PCR showed that 67 (55%) MBL-producing isolates contained bla\textsubscript{IMP}. These 67 isolates belonged to 2 general hospitals and were cultured from urinary tract infection (n=32), tracheal aspirates (n=15), pus (n=13), blood culture (n=5), ear infection (n=1) and eye infection (n=1). The results of PCR assay for 122 imipenem resistant isolates showed that only two isolates (1.6%) harbored bla\textsubscript{VIM2}, that cultured from urinary tract infection(n=1) and blood culture(n=1), whereas none of them were positive for bla\textsubscript{SPM1} gene.

DISCUSSION

Pseudomonas aeruginosa is an opportunistic pathogen causing serious diseases in immunocompromised patients. It has been recognized as most common
bacterium in different wards of hospitals throughout the world (1). In recent years, nosocomial infections with MBL producing strains of this organism have emerged.

In this study, susceptibility pattern of 236 clinical isolates of P. aeruginosa was determined and subsequently the imipenem resistant isolates were assessed for MBL using IMP-EDTA disks and molecular analysis targeting bla<sub>IMP</sub>, bla<sub>VIM</sub> and bla<sub>SPM</sub>. MBLs are a group of ß-lactamase enzymes which need one or two zinc in their active site to cleave the amide bond of the ß- lactam ring to inactive ß-lactam antibiotics (14). In 2012, Plotto et al. surveyed 56 P. aeruginosa isolates by disk diffusion method and showed that 54/56 (96.4%) of isolates were resistant to imipenem. They also showed that 17/56 (30.3%) of imipenem-resistant strains were positive for production of MBL (4) which is less than rate we found in the current study (37%), but higher than rate reported from Brazil (12.4%). By contrast, 76.8% of strains were MBL positive in Brazilian study (1) but in our study 90% of imipenem-resistant isolates were positive for production of MBL.

In 2012, Fallah et al. checked 100 P. aeruginosa isolates from Shahid Motahari hospital in Teheran to detect bla<sub>IMP</sub> and bla<sub>VIM</sub> (15). Forty eight out of 83 (57.9%) imipenem-resistant P. aeruginosa showed MBL activity while 12% of them had only bla<sub>VIM</sub> gene (15). The main reason for such difference between their results and ours can be attributed to the differences between the clinical specimens: we obtained 236 samples from patients of different wards but all isolates from Shahid Motahari belonged to burn units.

Franco et al. (2010) surveyed MBL production in P. aeruginosa isolates in Brazil (1). Although primer pair sequence of bla<sub>SPM-1</sub> used in our study was the same as what was used by Franco et al (2010) in Brazil, but this MBL gene was not detected in our study, confirming the results obtained in another study from Iran (14).

bla<sub>VIM-2</sub> was reported from Italy for the first time (6) but has been spread significantly in other countries including Iran. Furthermore, up to now 20 different bla<sub>VIM</sub> alleles have been identified in Singapore, Saudi Arabia, Taiwan, Greece, and Portugal (6,16). Our study showed that 1.6% of imipenem-resistant P. aeruginosa isolates contained bla<sub>VIM-2</sub> gene.

According to the report represented by Khosravi and Mihani (2008) from Ahvaz Jundishapur University of Medical Science, of 41 imipenem resistant isolates, 8 carried bla<sub>VIM</sub> but none of them had bla<sub>IMP</sub> (17).

In another report from Iran, Bahar et al. (2010) showed that all 23 imipenem-resistant strains were positive for MBL and were positive for bla<sub>IMP</sub> but none of them had bla<sub>VIM</sub> (2). Peymani et al. (2011) detected bla<sub>IMP</sub> gene in Acinetobacter baumanii in Iran. Some investigators hypothesized that it might be possible that bla<sub>IMP</sub> or bla<sub>SPM</sub> may transmit from Acinetobacter baumanii to P. aeruginosa (18).

In conclusion, regarding to horizontal transmission of integron-associated MBL genes, detecting MBL positive strains is necessary. Moreover, by using new methods for rapid identification of MBL positive bacteria in the patients, we could prevent spreading of metallo-beta lactamase strains to other patients.

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