Iranian Journal of Microbiology
Volume 7 Number 1 (February 2015) 2-6

ORIGINAL ARTICLE

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.

Received: May 2014, **Accepted:** November 2014

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-ß-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 phenotipically MBL producers. Additionally, the results of PCR method showed that 2 strains (1.6%) and 67strains (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 Ahavaz. 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). Resistantce to carbapenems is often associated with production of metallo-ß-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 ß- lactam antibiotics in which imipenem as a carbapenem is considered as the most appropriate antibiotic to be used against the mentioned organisms. Carbapenem

[*]Corresponding Author: Mohammad Rahimzadeh, PhD
Address: Department of Microbiology, School of Medicine, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran.
Tel: (0098- 611)3330074. 09149442060
Fax: +98- 611- 3332036.
Email: mohammadrahimzadeh62@yahoo.com

Table 1. Nucleotide sequences of primers used for detection of metalo-beta lactamase genes.

Primer name	Sequence	PCR Condition				
		Denaturing	Anneal	Extension	Cycles	Size(bp)
bla_{IMP-1}	5′ TGAGCAAGTTATCTGTATTC 3′ 5′ TTAGTTGCTTGGTTTTGATG 3	94°C, 60 s	57°C, 60 s	72°C,2 min	35	740
bla_{SPM-1}	5′CCTACAATCTAACGGCGACC 3′ 5′ TCGCCGTGTCCAGGTATAAC 3′	94°C, 60 s	40°C, 60 s	68°C, 60 s	30	674
bla_{VIM-2}	5′ AAAGTTATGCCGCACTCACC 3′ 5′ TGCAACTTCATGTTATGCCG 3′	94°C, 60 s	52.9°C, 60 s	72°C, 2 min	35	815

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 *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 *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 bla_{IMP}, bla_{VIM} and bla_{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 bla_{IMP-1}, bla_{VIM-2}, and bla_{SPM-1} metallo-beta-lactamases between imipenem-resistant *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 *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 *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), tobramicin (10µg), amikacin (30µg), ticarcillin(75µg), gentamicin (10µg), cefotaxime (30µg), and ceftizoxime (30µg) (MAST Co. UK). *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 *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 *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).

Table 2. Resistance pattern of *P. aeruginosa* isolations

Antibiotics	R	I	S	Percentage of resistance	Percentage of sensitivity
Imipenem (10µg)	122	7	107	51.4	45.3
Meropenem (10µg)	127	4	105	54	44.4
Ceftazidime (30µg)	151	0	85	63.9	36.1
Carbenicillin (100µg)	160	6	70	67.7	29.8
Tobramicin (10µg)	154	3	79	65.2	33.5
Amikacin (30µg)	118	12	106	50	45
Ticarcillin (75µg)	160	0	76	67.7	32.3
Gentamicin (10µg)	149	6	81	63.2	34.3
Cefotaxime (30µg)	161	58	17	68.2	7.3
Ceftizoxime (30µg)	187	40	9	79.2	3.9

R: Resistance I: Intermediate S: Sensitive

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_{IMP}, bla_{VIM} and bla_{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_{IMP-1} from *Seratia marcesens* (sequenced by Bioneer company), and bal_{VIM-2} 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 $bla_{\text{IMP-1.}}$ 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 $bla_{\text{VIM-2}}$ that cultured from urinary tract infection(n=1) and blood culture(n=1), whereas none of them were positive for $bla_{\text{SPM-1}}$ 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 trageting bla_{IMP}, bla_{VIM} and bla_{SPM}.

MBLs are a group of β-lactamase enzymes which need one or two zinc in their active site to cleave the amide bond of the β- lactam ring to inactive β-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_{IMP} and bla_{VIM} (15). Forty eight out of 83 (57.9%) imipenem-resistant *P. aeruginosa* showed MBL activity while 12% of them had only bla_{VIM} 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_{SPM-1} 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_{VIM-2} 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_{VIM} 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_{VIM-2} gene.

According to the report represented by Khosravi and Mihani (2008) from Ahvaz Jundishapour University of Medical Scince, of 41 imipenem resistant isolates, 8 carried bla_{VIM} but none of them had bla_{IMP} (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_{VIM} ,but none of them had bla_{IMP} (2). Peymani *et al.* (2011) detected bla_{IMP} gene in *Acintobacter baumanii* in Iran. Some investigators hypothesized that it might be possible that bla_{IMP} or bla_{SPM-1} may transmit from *Acintobacter 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.

ACKNOWLEDGEMENTS

This work was supported by Ahvaz Jundishapur University of Medical Sciences (Project B9102), and Infectious & Tropical Disease Research Center.

REFERENCES

1. Franco M, Caiaffa-Filho H, Burattini M, Rossi F. Metallo-beta-lactamases among imipenem-resistant *Pseudomonas aeruginosa* in a Brazilian university hospital. Clinics (Sao Paulo) 2010;65:825-829.
2. Bahar MA, jamali S, Samadi A. Imipenem resistant *Pseudomonas aeruginosa* strains carry metallo-beta-lactamase gene bla_{VIM} in a level I Iranian burn hospital. *Burn* 2010; 3636: 826-830.
3. Sepehriseresht S, Boroumand MA, Pourgholi L, Sotoudeh Anvari M, Habibi E, Sattarzadeh Tabrizi M. Detection of vim- and ipm-type metallo-beta- lactamases in *Pseudomonas aeruginosa* clinical isolates. *Arch Iran Med* 2012; 15:670 – 673.
4. Polotto M, Casella T, Oliveira MG, Rubio FG, Nogueira ML, Almeida MTG, *et al*. Detection of *Pseudomonas aeruginosa* harboring $bla_{CTX-M-2}$, bla_{GES-1} and bla_{GES-5}, bla_{IMP-1} and bla_{SPM-1} causing infections in Brazilian tertiary-care hospital. *BMC Infect Dis* 2012; 12:176.
5. Sadeghi A, Rahimi B, Shojapour M. Molecular detection of metallo-β-lactamase genes bla_{VIM-1}, bla_{VIM-2}, bla_{IMP-1}, bla_{IMP-2} and bla_{SPM-1} in *Pseudomonas aeruginosa* isolated from hospitalized patients in Markazi province by Duplex-PCR. *Afr J Microbiol Res* 2012; 6: 2965-2969.
6. Liakopoulos A, Mavroidi A, Katsifas E, Theodosiou A, Karagouni AD, Miriagou V, et al. Carbapenemase-producing *Pseudomonas aeruginosa* from central Greece: molecular epidemiology and genetic analysis of class I integrons. *BMC Infect Dis* 2013;13(505): 1-7.
7. Rizek C, Fu L, Dos Santos LC, Leite G, Ramos J,

Rossi F, Guimaraes T, Levin AS, Figueiredo Costa S. Characterization of carbapenem-resistant *Pseudomonas aeruginosa* clinical isolates, carrying multiple genes coding for this antibiotic resistance. *Ann Clin Microbiol* 2014; 13:1-5.

8. Nikokar I, Tishayar A, Flakiyan Z, Alijani K, Rehana-banisaeed S, Hossinpour M, et al. Antibiotic resistance and frequency of class 1 integrons among *Pseudomonas aeruginosa*, isolated from burn patients in Guilan, Iran. *Iran J Microbiol* 2013;5: 36-41.
9. Forbes BA, Sahm DF, Weissfeld AS.Bailey& Scott's Diagnostic Microbiology. 12th ed. London. Mosby Inc; 2007; pp: 340-350.
10. Clinical and Laboratory Standarad Institute. Performance standards for antimicrobial susceptibility testing. Twenty-second informational supplement M100-S22, Wayne, PA: CLSI; 2012, 188 pp.
11. Shahcheraghi F, Nikbin VS, Feizabadi M. Identification and genetic characterization of metallo-beta-lactamase producing strains of *Pseudomonas aeruginosa*in Tehran, Iran. *New Mirobiologica* 2010; 33: 243-248.
12. Silva FM, Carmo MS, Silbert S, Gales AC. SPM-1 Producing *Pseudomonas aeruginosa*: Analysis of the ancestor relationship using multilocus sequence typing, pulsed-field gel electrophoresis and automated ribotyping. *Microbial Drug Resistance 2011*; 17 (2): 215-220.
13. Sarhangi M, Motamedifar M, Sarvari J. Dissemination of *Pseudomonas aeruginosa* Producing bla_{IMP1}, bla_{VIM2}, bla_{SIM1}, bla_{SPM1} in Shiraz, Iran. *Jundishapur J Microbiol* 2013;6:e6920.
14. Bebrone C, Bogaerts P, Delbrück H, Bennink S, Kupper MB, De Castro R, et al. GES-18, a new carbapenem-hydrolyzing GES-type beta-lactamase from *Pseudomonas aeruginosa* that contains Ile80 and ser170 residues. *Antimicrob Agents Chemother* 2013; 57:396- 401.
15. Fallah F, Shams Borhan R, Hashemi A. Detection of bla(IMP) and bla(VIM) metallo-β- lactamases genes among *Pseudomonas aeruginosa* strains. *Int J Burn Trauma* 2013; 3:122-124.
16. Cardoso O, Alves AF, Leitao R. Metallo-beta-lactamase VIM-2 in *Pseudomonas aeruginosa* isolates from a cystic fibrosis patient. *Int J AntimicrobAgents* 2008; 31: 375- 379.
17. Khosravi AD, Mihani F. Detection of metallo-beta-lactamase producing *Pseudomonas aeruginosa* strains isolated from patients suffering from burns in Ahvaz, Iran. *Diagn Microbiol Infect Dis* 2008; 60: 125-128.
18. Peymani A, Nahaei MR, Farajnia S, Hasani A, Mirsalehian A, Sohrabi N. High prevalence of metallo-beta-lactamase producing *Acinetobacter baumannii* in a teaching hospital in Tabriz, Iran. *Jpn J Infect* 2011; 64: 69-71.