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Full Length Research Paper

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

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Pseudomonas aeruginosa **is an opportunistic human pathogen which causes serious problems especially in people who have immunodeficiency. Recently, β-lactamase resistance in this bacterium has led some difficulties in treating bacterial infections. One of resistance mechanisms is production of metallo-β-lactamase (MBL). The aim of this study was to determine prevalence of imipenem-resistant *P. aeruginosa* carrying MBL genes.108 *P. aeruginosa* strains were obtained. Antibiotic susceptibility test was done by agar disk diffusion method. Minimum inhibitory concentration (MIC) was done by broth microdilution for imipenem-resistant strains. Then, EDTA-IMP test was carried out for screening MBL production in imipenem-resistant strains. Finally, polymerase chain reaction (PCR) was performed by Duplex-PCR to detect MBL genes including bla_{VIM-1}, bla_{VIM-2}, bla_{IMP-1}, bla_{IMP-2} and bla_{SPM-1} in imipenem-resistant strains. Among 108 *P. aeruginosa* strains, 40 (37%) strains were showed to be resistant to imipenem, MIC results determined 30 out of 40 (75%) were resistant to imipenem (MIC ≥ 16 µg/ml). EDTA-IMP test showed 20 out of 40 (50%) imipenem-resistant strains were MBL positive. Furthermore, by molecular studies, 50% of imipenem-resistant strains were detected to have bla_{VIM-1}, 56.6% bla_{VIM-2}, 6.6% bla_{IMP-1}. In addition, two other genes (bla_{IMP-2} and bla_{SPM-1}) were not detected in imipenem-resistant *P. aeruginosa* strains. This study showed resistance to imipenem like other countries is increasing, and there are *P. aeruginosa* strains carrying MBL genes in Markazi province.**

Key words: β-lactamase, bla_{IMP}, bla_{SPM}, bla_{VIM}, imipenem resistance, metallo-β-lactamase genes, *Pseudomonas aeruginosa*.

INTRODUCTION

Pseudomonas aeruginosa is one of the important pathogens most frequently responsible for nosocomial infections and it is an opportunistic human pathogen (Patzer and Dzierzanowska, 2007). This pathogen is the major cause of morbidity and mortality in immunocompromised patients such as cystic fibrosis, burn issues, cancer, and patients in intensive care units (Patzer and Dzierzanowska, 2007; Huang et al., 2007; Crespo et al., 2004; Castanheira et al., 2004; Farra et al., 2008.

First report of the presence of *P. aeruginosa* in hospital was in 1972 in Pennsylvania. (Kominos et al., 1972). Therefore timely and appropriate treatment of infections caused by this pathogen is very important. Carbapenems are effective antimicrobial agents against *P. aeruginosa* infections which belong to β-lactam family. The introduction of carbapenems was a significant development into medical science because these anti- bacterial agents

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have a wide range of activity and stability against majority of β-lactamase enzymes. Consequently, carbapenems are used due to treatment of serious bacterial infections caused by β-lactam-resistant bacteria (Lee et al., 2003). The most well-known antibiotics in this group are imipenem and meropenem; these two drugs have a high efficiency against *P. aeruginosa* (Castanheira et al., 2004; Farra et al., 2008; Franco et al., 2010; Wang and Mi, 2006) while resistance to imipenem is increasing worldwide (Bahar et al., 2010). After that, nosocomial outbreaks of carbapenem-resistant *P. aeruginosa* have been seen therefore, finding carbapenem-resistant *P. aeruginosa* has begun (Huang et al., 2007; Cornaglia et al., 2009).

There are some mechanisms that can cause carbapenem-resistance in *P. aeruginosa* which include high production of efflux pumps, loss of production of OprD _it is demonstrated as an imipenem specific porin_ production of β-lactamase. The last one is the major mechanism of resistance to β-lactam antibiotics (Falagas and Karageorgopoulos, 2009). β-lactamase are classified into four classes including class A, C, D which have serine in their active site, and class B or metallo-β-lactamase (MBL) which need one or two zinc in their active site (Bebrone et al., 2007). Hydrolyzing activity of MBLs is inhibited by chelators like EDTA *in vitro* but other chelators such as sulbactam, tazobactam and clavulanic acid are not able to inhibit MBLs (Pitout et al., 2005; Helfand and Bonomo, 2005; Arakawa et al., 2000).

The first case of transferable MBL was reported in *P. aeruginosa* GN17203 in Japan in 1988 (Watanabe, 1991). The production of MBLs is important because several outbreaks caused by MBL-producing strains have been seen (Crespo et al., 2004). MBLs are classified in three groups including B1, B2 and B3 (Gupta, 2008), in addition the first class includes B1 and acquired B1. Acquired B1 enzymes which include VIM, IMP, SPM, GIM and SIM, are broad spectrum MBLs (Bebrone et al., 2007; Watanabe, 1991). MBL genes are located in cassettes in integrons, integron 1 has specific recombination site. Gene cassettes cause integrons to be able to move to other integrons or some sites in the bacterial genome, these sites are placed in plasmids and transposons (Watanabe, 1991). For instance, bla_{IMP} and bla_{VIM} are plasmid born and they are able to be transferred from one organism to another (Dugal and Fernandes, 2011). There are some reports of ability of MBLs in hydrolyzing carbapenems and, *P. aeruginosa* strains carried MBL genes are clinical-threatening factors (Senda et al., 1996). Presence of these enzymes in bacteria leads resistance to all β-lactams while any inhibitor has not been discovered for these enzymes until now (Cavallo et al., 2000; Park et al., 2003). Therefore the importance of studying of MBLs, their outbreak and fast detection due to control and prevention from more outbreaks is undeniable. In this study frequency of MBL genes including bla_{VIM-1}, bla_{VIM-2}, bla_{IMP-1}, bla_{IMP-2} and bla_{SPM-1} in imipenem-resistant *P. aeruginosa* isolates was determined by Duplex-PCR.

MATERIALS AND METHODS

Bacterial strains

108 *P. aeruginosa* isolates were collected from hospitalized patients in Markazi Province of Iran between July 2010 and June 2011; their strains were separated from blood, wound, sputum, urine, etc. Only one isolate per patient was included in the study. These isolates were identified as a *P. aeruginosa* based on colonial morphology on blood agar plates, Gram stain characteristics, oxidase test and Oxidative-fermentative test. Strains were preserved in Trypticase soy broth media (TSB) (Merck-German) containing 20 %(v/v) glycerol.

Antibiotic susceptibility tests

Antibiotic susceptibility test was done by disk diffusion method (Kirby-Bauer) on Muller-Hinton agar plates (Merck-German) according to NCCLS (national committee for clinical laboratory standards) (Staneck, 1986). The antimicrobial disks used were amikacin (10 µg), ciprofloxacin (10 µg), ceftazidime (10 µg), gentamicin (10 µg), imipenem (10 µg), meropenem (10 µg) (provided by Himedia-India), *P. aeruginosa* ATCC 27853 were used as control for susceptibility testing. Bacteria were inoculated into TSB, and incubated at 35℃ for 2 to 4 h until it re ached the turbidity of a 0.5 McFarland standard. Then using a sterile swab were culture on Meller-Hinton agar plate, streaking in 3 directions over the entire agar surface. Finally Antimicrobial disks were placed on the plate and incubated at 35℃ for 18 to 24 h. Aft er incubation, measure the diameters of the zone of complete inhibition.

Minimum inhibitory concentration (MIC)

MIC was performed by microdilution broth method for imipenem-resistant strains with four antibiotics including imipenem, ceftazidime, cefipime and ciprofloxacin. In this method, 96-well plates were used, certain amount of Muller-Hinton broth (2x) and antibiotic were added to each well. Finally, bacterial concentration was adjusted to 5×10^5 CfU/ml, and was added to each well (Bailey et al., 2000). *P. aeruginosa* strains with MIC ≥ 4 µg/ml to ciprofloxacin, MIC ≥ 32 µg/ml to ceftazidime, MIC ≥ 4 µg/ml to cefipime and MIC ≥ 16 µg/ml to imipenem are referred to as resistant (Miyajima et al., 2008).

EDTA-IMP

Phenotypic detection of MBL production (EDTA-IMP) was carried out for imipenem-resistant strains by dissolving 186.1 gr of disodium EDTA. $2H_2O$ in 1000 ml of distilled water, 0.5 *M* EDTA solution was provided and adjusted to *pH* 8 by adding NaOH. Then, 750 µg of prepared solution was added to imipenem disk, and it was dried in an incubator. EDTA-imipenem disk plus imipenem disk were placed in a plate contained Muller-Hinton agar and cultured *P. aeruginosa*. After 18 h of incubation at 35℃ an organism was considered MBL positive if the inhibition zone diameter increased by 7 mm or more towards the IMP plus EDTA in comparison to IMP disk alone (Hemalatha et al., 2005; Yong et al., 2002).

Molecular analysis

Total DNA from *P. aeruginosa* isolates was extracted by boiling

method; this causes cell wall to lyses (Franco et al, 2010). Duplex-PCR was performed to detect bla_{VIM-1}, bla_{VIM-2}, bla_{IMP-1}, bla_{IMP-2} and bla_{SPM-1} gene, 16srRNA gene was used as an internal control (Table 1).

PCR tests were done under the following program:

3 min initial denaturation at 94℃, 30 cycles of den aturation at 94℃ for 1 min , annealing at (55 to 60)℃ for 30 s, extens ion at 72℃ for 1 min and final extension at 72℃ for 7 min. Amplifi cation reactions were performed in a final volume of 25 µL containing 200 µM concentrations of dNTPs (Fermentas-Canada) , 10 pM of each primer(eurofins-MWG/Operon, Ebersberg, Germany), 0.8 mM $MgCl_2$ (Fermentas-Canada), 0.5 U Taq polymerase (Fermentas-Canada) and 50 ng DNA templates. *P. aeruginosa* strains producing bla_{IMP}, bla_{SPM-1}, bla_{VIM-1} and bla_{VIM-2} genes (Kindly provided by Dr. Shahcheraghi from Pasteur Institute Iran), were used as positive control for MBL detection. The polymerase chain reaction (PCR) products were confirmed by gel electrophoresis in 2% agarose gels (Merck-Germany) and visualized with ethidium bromide staining and photographed with a UV transillumination camera.

RESULTS

Bacterial strains

Among 108 strains, 40 (37%) of them were isolated from blood, 27 (25%) from urine, 19 (17.6%) from sputum, 19 (17.6%) burn wound and 3 (2.8%) from abdominal fluids.

Antibiotic susceptibility

Disk diffusion agar showed 12 (11.1%) strains that were resistant to amikacin, 19 (17.6%) to ciprofloxacin, 32 (29.6%) to ceftazidime, 17 (15.7%) to gentamicin, 40 (37%) to imipenem, and 41 (38%) to meropenem.

MIC

MICs illustrated 30 (75%) strains were resistant to imipenem (MIC ≥ 16 µg/ml), 33 (82.5%) to ceftazidime (MIC ≥ 32 µg/ml), 37 (92.5%) to ciprofloxacin (MIC ≥ 4 µg/ml) and 40 (37%) to cefepime (MIC ≥ 4 µg/ml).

EDTA-IMP

20 out of 40 (50%) imipenem-resistant strains were shown to be MBL positive by EDTA-IMP test.

Molecular analysis

Molecular studies detected 50% bla_{VIM-1}, 56.6% bla_{VIM-2}, and 6.6% bla_{IMP-1} while none of the strains had bla_{IMP-2} and bla_{SPM-1} genes (Figure 1).

DISCUSSION

In this study, 108 *P. aeruginosa* strains were obtained from different hospitalized patients in Markazi province. Antibiotic susceptibility pattern was determined in these strains and imipenem-resistant strains were subjected to be tested for MIC, EDTA-IMP, and molecular analysis. Then, frequencies of MBL genes including bla_{VIM-1}, bla_{VIM-2}, bla_{IMP-1}, bla_{IMP-2} and bla_{SPM-1} were detected. 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 (Bebrone, 2007). In the recent years, nosocomial outbreaks of MBL-producing bacteria have been reported worldwide.

In 2005, Hemalatha and colleagues collected 50 *P. aeruginosa* isolates. Disk diffusion showed 8 (16%) of isolates that were resistant to imipenem, and 7 out of 8 imipenem-resistant strains were MBL positive. In addition all imipenem-resistant isolates showed high level of MICs (8 to128 µg/ml) (Miyajima et al., 2008). Compared to our study, imipenem-resistant strains in Markazi province showed higher resistance to imipenem (37%), while only 16% of strains isolated from Indian patients were showed to be resistant to imipenem by disk diffusion method. By contrast, 7 out of 8 strains were MBL positive in India but in our study just half of imipenem-resistant strains were MBL positive. In 2010, Saderi and colleagues obtained 100 *P. aeruginosa* from 100 burn patients in tehran. In that study the prevalence of MBL-producing *P. aeruginosa* and detection of four MBL genes (IMP-1, IMP-2, VIM-1, VIM-2) were determined. 65 out of 69 imipenem-resistant *P. aeruginosa* showed to have MBL activity while only 13 of them had MBL gene. Only VIM-2 was detected and other genes were not (Saderi et al., 2010). There are a lot of differences between this study and ours, one of the reasons may be difference in kind of collected samples because we obtained 108 samples from patients in various units but in this study all patients were from burn units.

In 1997, bla_{SPM-1} was discovered in Brazil (Gupta, 2008). Then, in 2006, Maria Renata Gomes Franco et al. did a research about MBL production in *P. aeruginosa* in Brazil (Franco et al., 2010). Phenotypic production of MBL was assigned in 69 imipenem-resistant *P. aeruginosa* strains isolated from blood, MBL production varied from 28 to 77%. PCR analysis detected MBL genes in 34% of imipenem-resistant strains (81 and 19%, respectively bla_{SPM-1} and bla_{VIM-2}). Primer used for bla_{SPM-1} in our study was the same as what was used in 2006 in Brazil, but amazingly bla_{SPM-1} was not detected in Markazi province. Despite of several Iranian studies, this gene has not been reported in Iran yet (Shahcheraghi et al., 2010).

The other MBL gene studied was bla_{VIM-1}. This gene was first reported in *P. aeruginosa* in Italy (Gupta, 2008). As far as recent studies have shown this enzyme has been spread significantly. Furthermore there have been known 20 various different bla_{VIM} alleles all over the world

Table 1. Nucleotide sequences of primers used for detection of genes.

Primer name and detected gene	Sequence	Size (bp)	References
bla_{VIM-1}	5 -AGTGGTGAGTATCCGACAG-3 5-ATGAAAGTGCGTGGAGAC-3	261	(Shibata et al., 2003)
bla_{VIM-2}	5-ACTCACCCCCATGGAGTTTT-3 5-ACGACTGAGCGATTTGTGTG-3	815	(Shibata et al., 2003)
bla_{IMP-1}	5-TGAGCAAGTTATCTGTATTC-3 5-TTAGTTGCTTGGTTTTGATG-3	740	(Franco et al., 2010)
bla_{IMP-2}	5-GTTTTATGTGTATGCTTCC-3 5-AGCCTCTTCCCATGTAC-3	678	(Franco et al., 2010)
bla_{SPM-1}	5-CCTACAATCTAACGGCGACC-3 5-TCGCCGTGTCCAGGTATAAC-3	674	(Franco et al., 2010)
16sr RNA	5-AGGAGGTGATCCAACCGCA-3 5-ACCTGGAGGAAGGTGGGGAT-3	367	-

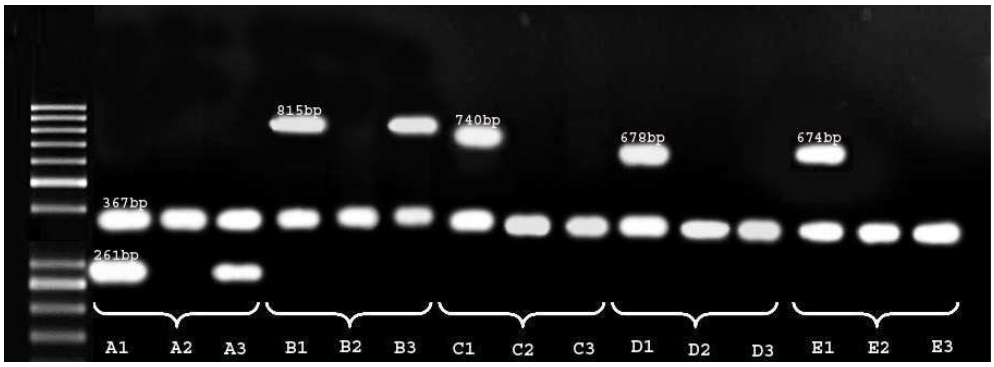

Figure 1. Electrophoresis of PCR products: Each three lanes belong to one gene. First lane of each series is positive control, the second is negative control which contains 16srRNA (367 bp) and the third is *P. aeruginosa* sample. A series stand for bla_{VIM-1} (261 bp), B series for bla_{VIM-2} (815 bp), C series for bla_{IMP-1} (740 bp), D series for bla_{IMP-2} (678 bp) and E series for bla_{SPM-1}(674 bp).

which include: Singapore, Saudi Arabia, Taiwan, Greece, Portugal (Gupta, 2008; Cardoso et al., 2008). Moreover, this gene has been reported in different areas in Iran, and our study showed that 50% of imipenem-resistant *P. aeruginosa* strains harbored bla_{VIM-1} and 56.6% of imipenem-resistant strains contained bla_{VIM-2} in Markazi province either.

In 2008, Khosravi and Mihani experimented activity of six carbapenem antibiotics against 100 *P. aeruginosa* in Iran. Then, production of MBL was determined by Etest. In addition, PCR was performed to recognize bla_{IMP} and bla_{VIM}. Among 41 imipenem-resistant *P. aeruginosa*, 8 strains were able to produce MBL by Etest, and in all of these 8 strains, 8 carried bla_{VIM}. By contrast none of them were not shown to have bla_{IMP} (Khosravi and Mihani, 2008). In another case in Iran in 2009, Bahar and colleagues assigned antibiotic susceptibility pattern of *P. aeruginosa* isolated from patients with burn issues. 23 Imipenem-resistant strains were MBL positive and all had bla_{VIM}, but none of them had bla_{IMP} (Bahar et al., 2010). In 2011, Peymani et al. could detect bla_{IMP} in *Acintobacter baumanii* for the first time in Iran. It might happen that bla_{IMP} was transmitted from *A. baumanii* to *P. aeruginosa*. It seems this study is the first report of occurrence of bla_{IMP-1} in *P. aeruginosa* in Iran. Additionally, with no heed to hygiene especially in hospitals in Iran, transmission of bla_{SPM-1} from *A. baumanii* to *P. aeruginosa* would occur. One of the problems in treating *P. aeruginosa* infections is that this pathogen has ability to become resistant during treatment. Combination therapy can be useful to

prevent resistance during therapy. Regarding to horizontal transmission of integron-associated MBL genes, detecting MBL positive strains is essential. Moreover, invention of new methods for identifying MBL positive bacteria, and screening involving people must be done in hospitals regularly.

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