Molecular detection of metallo-β-lactamase genes, \( \text{bla}_{\text{IMP-1}} \), \( \text{bla}_{\text{VIM-2}} \) and \( \text{bla}_{\text{SPM-1}} \) in imipenem resistant \textit{Pseudomonas aeruginosa} isolated from clinical specimens in teaching hospitals of Ahvaz, Iran

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Received: May 2014, Accepted: November 2014

ABSTRACT

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

Material and Methods: 236 \textit{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, \( \text{bla}_{\text{IMP-1}} \), \( \text{bla}_{\text{VIM-2}} \) and \( \text{bla}_{\text{SPM-1}} \) 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 \textit{Pseudomonas aeruginosa} isolates contained \( \text{bla}_{\text{VIM-2}} \) and \( \text{bla}_{\text{IMP-1}} \) genes respectively. No \( \text{SPA-1} \) gene was found in the examined samples.

Conclusion: Resistance of \textit{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: \textit{Pseudomonas aeruginosa}, carbapenem resistant, \( \text{bla}_{\text{IMP-1}} \), \( \text{bla}_{\text{VIM-2}} \), \( \text{bla}_{\text{SPM-1}} \).
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 blaIMP, blaVIM and blaSPM 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 blaIMP-1, blaVIM-2 and blaSPM-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), ticarcilnin(75µg), gentamicin (10 µg), cefotaxime (30µg), and cefazoxime (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 1. Nucleotide sequences of primers used for detection of metalo-beta lactamase genes.

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Sequence</th>
<th>PCR Condition</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>Denaturing</td>
</tr>
<tr>
<td>blaIMP-1</td>
<td>5’ TGAGCAAGTTATCTGTATTC 3’</td>
<td>94°C, 60 s</td>
</tr>
<tr>
<td></td>
<td>5’ TTAGTTGCCTGGTTTATG 3’</td>
<td></td>
</tr>
<tr>
<td>blaVIM-2</td>
<td>5’ CCTACAATCTAAGCGGACCC 3’</td>
<td>94°C, 60 s</td>
</tr>
<tr>
<td></td>
<td>5’ TCCGGTAGTCGGATATAAC 3’</td>
<td></td>
</tr>
<tr>
<td>blaSPM-1</td>
<td>5’ AAAGTTATGCAGCACCTACCC 3’</td>
<td>94°C, 60 s</td>
</tr>
<tr>
<td></td>
<td>5’ TGCAACTTCAATGTTATGC 3’</td>
<td></td>
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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 mM Tris, 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*<sub>IMP</sub>, *bla*<sub>VIM</sub> and *bla*<sub>SPM</sub> 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*<sub>IMP</sub>, from *Seratia marcesens* (sequenced by Bioneer company), and *bal*<sub>VIM</sub> 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*<sub>IMP</sub>. 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*<sub>VIM</sub> that cultured from urinary tract infection(n=1) and blood culture(n=1), whereas none of them were positive for *bla*<sub>SPM</sub> 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 β-lactamase enzymes which need one or two zine 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 Tehran to detect *bla*<sub>VIM</sub> and *bla*<sub>VAP</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>VIM</sub> but none of them had *bla*<sub>IMP</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-1</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.

**ACKNOWLEDGEMENTS**

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

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