Research Article

Antibiotic Resistance Pattern and Evaluation of Metallo-Beta Lactamase Genes Including \textit{bla}_{\text{IMP}} and \textit{bla}_{\text{VIM}} Types in \textit{Pseudomonas aeruginosa} Isolated from Patients in Tehran Hospitals

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1. Introduction

\textit{Pseudomonas aeruginosa} is one of the commonest causes of infection in burn patients and an important agent for hospital acquired infections and death in immunocompromised such as cystic fibrosis and cancer patients [1]. This bacterium is often resistant to many antimicrobial agents. The cause of resistance can be efflux pumps, decreased outer membrane permeability, and secretion of beta-lactamase enzymes [2]. Several kinds of beta-lactamase enzymes have been recognized. These enzymes were initially seen in Gram-negative bacteria which were detected in periplasmic space [3]. Metallo-beta lactamases are classified in group B of Ambler classification. This group is divided into three subclasses: BI, BII, and BIII. The BI subclass is divided into four categories according to their molecular structures: the IMP, VIM, GIM, and SPM types [4]. The first MBLs enzymes were IMP-1 which was initially found in \textit{S. marcescens} in Japan (1991), VIM-1 originally detected in Italy (1997), SPM-1 first detected in Brazil (1997), and finally GIM detected in Germany (2002) [5, 6]. Carbapenems are effective antibiotics against \textit{Pseudomonas aeruginosa} infections. But because the genes for MBLs are often carried on plasmids and class I integron, they can rapidly spread among different species of this bacterium and
other bacteria [7, 8]. MBLs can potently hydrolyze all beta-lactam antibiotics except aztreonam. These enzymes require zinc ion as cofactor [9]. Hence, their activity is inhibited by chelators like ethylenediaminetetraacetic acid (EDTA), sodium mercaptoacetic acid (SMA), 2-mercaptopropionic acid (MPA), and dipicolinic acid (DPA). Sulbactam, tazobactam, and clavulanic acid which are often used to inhibit beta-lactamase enzymes are not effective against MBLs [2, 10]. Several phenotypic methods are available for detection of MBLs producing bacteria. All these methods are based on the ability of metal chelators such as EDTA to inhibit the activity of MBLs. The double disk synergy test method was employed in this investigation [9]. The goal of this study was to determine the antibiotic resistance pattern in P. aeruginosa species isolated from nine hospitals in Tehran, Iran, and evaluate the prevalence of MBLs genes, bla-IMP, and bla-VIM, in imipenem resistance strains.

2. Materials and Methods

2.1. Collection of Strains. A total of 212 strains of P. aeruginosa were collected during six-month period from October 2011 to March 2012 from Motahari, Shariati, Hashemi Nejad, Kasra, Hazrat Rasoul, Milad, Mehr, Tebbi Kodakan, and Baghiatallah hospitals in Tehran, Iran. These strains were isolated from wound, blood, urine, trachea, sputum, pleural fluid, eye, catheter, and larynges samples. 148 isolates were obtained from male patients and 64 isolates from female patients. These isolates were subcultured on Brucella agar and their identification was performed by both biochemical methods such as oxidase test, catalase test, OF test, growth at 42°C, and PCR using specific primers for oprL gene (oprL is a constitutively produced peptidoglycan-associated lipoprotein which contains covalently bound fatty acyl chains) [11]. Bacterial strains were preserved in Trypticase soy broth.

2.2. Antibiotic Susceptibility Tests. Antimicrobial sensitivity tests were performed on Mueller-Hinton agar (Biolab-Hungary) by Kirby-Bauer disk diffusion method [12] and interpreted according to CLSI (Clinical and Laboratory Standards Institute) standard tables. Pseudomonas aeruginosa ATCC27853 was used as control for the susceptibility tests. The antibiotic disks used were Imipenem (10 μg), Ciprofloxacin (5 μg), Gentamicin (10 μg), Tetracyclin (30 μg), Ceftazidime (30 μg), Cefotaxime (30 μg), Azithromycin (15 μg), Tobramycin (10 μg), Ticarcillin (75 μg), and Piperacillin (100 μg) (Patdan Teb, Iran). At first the bacteria were cultured into TSB and incubated at 35°C for 24 hours. After 24 hours, microbial suspension was prepared equivalent to the turbidity of 0.5 McFarland standard. With sterile swabs they were plated on MH agar. The antibiotic disks were placed on the plate and incubated at 35°C for 24 hours. Following incubation, the diameters of the zone of inhibition were measured.

2.3. Minimum Inhibitory Concentration (MIC). Determination of MIC was performed for imipenem resistant strains by agar dilution method according to CLSI standards. Isolates with MIC value of ≥16 μg/mL were screened as MBLs producing strains. Pseudomonas aeruginosa ATCC27853 was used as a control strain for the susceptibility testing.

2.4. Detection of MBLs Producing Isolates by Double Disk Synergy Test (DDST) Method. Imipenem resistance isolates were investigated for MBLs producing strains by DDST method. The bacterial suspension with turbidity equivalent to 0.5 McFarland standard was prepared and cultured on Mueller-Hinton agar [13]. For preparation of IMP-EDTA disk, 750 μg of EDTA solution was added to 10 μg imipenem disk and dried in an incubator [13]. At first, the bacterial suspension with turbidity equivalent to 0.5 McFarland was prepared and cultured with sterile swab on MH agar. Then, two 10 μg imipenem and imipenem-EDTA disks were placed on the agar surface. After 18 hours of incubation at 35°C, the inhibition zone of imipenem disk and IMP-EDTA were measured. An increase of seven mm or more in the zone diameter for IMP-EDTA disk in comparison with imipenem disk alone was considered as a MBLs producing isolate [14].

2.5. DNA Extraction. DNA from P. aeruginosa isolates was extracted by boiling method. In this method, a number of bacterial colonies were inoculated in 10 mL of LB broth and incubation at 37°C for 16 hours. 1.5 mL of the LB broth culture was centrifuged at 13,000 x g at room temperature for 10 min. The bacterial pellet was suspended in 300 μL sterile water. The cells in the suspension were lysed by heating at 100°C for 10 min and the leftover cells were removed by centrifugation at 13,000 x g at room temperature for 10 min. The supernatant was transferred into new tubes and used as template DNA for PCR reactions. For purity assurance, the template DNA was electrophoresed on agarose gel [10].

2.6. PCR Reaction for Confirmation of Pseudomonas aeruginosa Strains (oprL Gene). PCR reaction for identification of P. aeruginosa strains (oprL gene) was performed in a final volume of 25 μL: PCR Buffer (10x) 2.5 μL, MgCl₂ (50 mM) 0.75 μL, dNTPs (10 mM) 1 μL, forward (5’-ATG-GAA-A-TG-CTG-AAA-TTC-GG-G-3’), and reverse (5’-CTT-CTT-CTG-GTC-GGA-GC-3’) primers [11] 500 bp (10 pmol/μL) 1 μL + 1 μL, Taq DNA polymerase (5 U/μL) 1 μL, distilled water 16.75 μL, and Template DNA 1 μL. The thermocycler program for oprL gene consisted of 5 min initial denaturation at 94°C, 35 cycles of denaturation at 94°C for 1 min, annealing at 60°C for 1 min, extension at 72°C for 1 min, and final extension at 72°C for 5 min.

2.7. PCR Assays for Detection of MBLs Genes

2.7.1. Primers. For design of primers, the nucleotide sequences of bla-VIM and bla-IMP genes in P. aeruginosa were obtained from Gene bank and aligned with Clastalw2 software (alignment program). After identification of commonality region, Gene Runner program was used for primer design. Finally for confirmation of primer specificity, they were subjected to BLAST program.
PCR reactions for \( \text{bla}^{\text{IMP}} \) and \( \text{bla}^{\text{VIM}} \) genes were performed in a final volume of 25\( \mu \text{L} \) containing the following.

\( \text{bla}^{\text{IMP}} \). PCR Buffer (10x) 2.5\( \mu \text{L} \), MgCl\(_2\) (50 mM) 1\( \mu \text{L} \), dNTPs (10 mM) 1\( \mu \text{L} \), forward (5'GTTTGAAGAAGTTAAGGG-GTGG3') and reverse (5'ATAATTGGCGGACTTTTGCC3') primers (designed) 459 bp (10 pmol/\(\mu \text{L}\)) 1 + 1\( \mu \text{L} \), Taq DNA polymerase (5 U/\(\mu \text{L}\)) 1\( \mu \text{L} \), template DNA 3\( \mu \text{L} \), and distilled water 14.5\( \mu \text{L} \). The thermocycler program for \( \text{bla}^{\text{IMP}} \) gene consisted of 4 min initial denaturation at 94°C, 35 cycles of denaturation at 94°C for 1 min, annealing at 61°C for 1 min, extension at 72°C for 1 min, and final extension at 72°C for 5 min.

\( \text{bla}^{\text{VIM}} \). PCR Buffer (10x) 2.5\( \mu \text{L} \), MgCl\(_2\) (50 mM) 1\( \mu \text{L} \), dNTPs (10 mM) 1\( \mu \text{L} \), forward (5'TGGTGTTTGGTGCGCA-TATCG3') and reverse (5'GAGCAAGTCTAGACGCC-GCG3') primers (designed) 595 bp (10 pmol/\(\mu \text{L}\)) 1 + 1\( \mu \text{L} \), Taq DNA polymerase (50 U/\(\mu \text{L}\)) 1\( \mu \text{L} \), template DNA 2\( \mu \text{L} \), and distilled water 15.5\( \mu \text{L} \). The thermocycler program for \( \text{bla}^{\text{VIM}} \) gene by thermocycler was 4 min initial denaturation at 94°C, 35 cycles of denaturation at 94°C for 1 min, annealing at 62°C for 1 min, extension at 72°C for 1 min, and final extension at 72°C for 10 min.

Water was used as negative control and \( P. \text{aeruginosa} \) strains producing MBLs genes (\( \text{bla}^{\text{VIM}} \) and \( \text{bla}^{\text{IMP}} \)) (provided from Pasteur Institute, Iran) were used as positive controls for MBL detection.

The PCR products were confirmed by gel electrophoresis in 1% (w/v) agarose gel (HT bioscience, UK) in TBE buffer and visualized with ethidium bromide staining and photographed with UV waves through Gel Documentation (Technogen, Iran) (Figures 2, 3, and 4).

3. Results

In total, 212 \( P. \text{aeruginosa} \) isolates were collected. After performing initial bacteriological tests, they were confirmed to be \( P. \text{aeruginosa} \) by PCR assay. They were obtained from clinical specimens such as wound (\( n = 78 \)), urine (\( n = 62 \)), blood (\( n = 39 \)), trachea (\( n = 16 \)), sputum (\( n = 7 \)), pleural fluid (\( n = 5 \)), eye (\( n = 2 \)), catheter (\( n = 2 \)), and larynges (\( n = 1 \)). The majority were from patients in burn unit (\( n = 58 \)) and the least were from patients in cardiac unit (\( n = 1 \)).

3.1. Antibiotic Susceptibility. Antibiotic susceptibility of the 212 isolates in the initial disk diffusion method against 10 antibiotics is presented in Figure 1. The isolates showed high resistance to tetracycline (86%) and the most effective antibiotic was ciprofloxacin (44%).

3.2. MIC. Determination of MIC for imipenem by agar dilution method indicated that 47.16% (\( n = 100 \)) of the strains were resistant to imipenem (MIC ≥ 16\( \mu \text{g/mL} \)).

3.3. Detection of MBLs Producing Isolates by Double Disk Method. In the double disk method performed on the 100 imipenem resistance isolates, 70 strains were shown to be positive by this phenotypic method.

3.4. Molecular Analysis. The PCR assays indicated that 20 (9%) of these strains contained the IMP gene, whereas 70 (33%) of them harbored the VIM gene.

4. Discussion

\( P. \text{aeruginosa} \) is an opportunistic human pathogen [15, 16]. Different antibiotics are commonly used for the treatment of \( P. \text{aeruginosa} \) infections, such as aminoglycosides, beta-lactamases, and quinolones [16–18]. Carbapenems are potent beta-lactam antibiotics against MBLs producing and multidrug resistance \( P. \text{aeruginosa} \) [19]. There have been many recent reports that clinical isolates of \( P. \text{aeruginosa} \) and Gram-negative bacilli are becoming resistant to carbapenems.
in many countries [19]. In recent years, MBLs have been identified from clinical isolates with increasing frequency across the world and strains that produce these enzymes have been responsible for prolonged treatment and acute infections [20]. A study from Japan showed that patients infected with MBLs producing P. aeruginosa needed to receive multiple antibiotics and infections leading to death due to IMP producing P. aeruginosa were more common than those with bla-IMP negative P. aeruginosa [21]. MBLs producing P. aeruginosa is a serious intimidation in hospital locations especially in burn units. These strains can create significant problem in treatment and spread of resistance among other bacteria [22]. Resistance to carbapenems via acquirement of MBLs genes among P. aeruginosa strains have increased rapidly in Asia, Europe, and South America. This has led to a drastic change in the pattern of antibiotics usage against multidrug resistant P. aeruginosa [23]. Detection of MBLs producing strains can be effective for optimal treatment of patients particularly in burned and hospitalized patients and control the spread of resistance [6]. Resistance to imipenem is increasing in Iran in recent years [9, 22, 24–27]. The differences in the reported values between the present study and those reported earlier may be due to the difference in geographical regions, difference in kind of infections, the enormous usage of antibiotics, or difference in antibiotic therapy regimens in the selective hospitals in this study than those in other studies. Among the 100 isolates which were resistant to imipenem, 70 (70%) were found to be MBLs producers. In the other strains which were resistant to imipenem but were MBLs negative, resistance to imipenem may be due to efflux systems, decreased outer membrane permeability, or production of Ampc enzymes. For confirmation of MBLs producing strains, PCR is an important and accurate method [10]. In this study all isolates were screened for VIM and IMP genes by PCR. 20 isolates had IMP gene and 70 isolates had VIM gene. Of the 11 isolates that were negative with phenotypic method, 4 harbored IMP gene and 7 isolates had VIM gene as detected by PCR. Also, 3 isolates that were positive with DDST method were negative with PCR. This shows that there may be genes other than VIM and IMP responsible for MBLs trait.

Yazdi et al. isolated 126 P. aeruginosa strains from nonburn patients in Iran in 2007. Production of MBL in these isolates was determined by E-test, followed by PCR to detect bla-IMP and bla-VIM. Among 70 imipenem resistant P. aeruginosa strains, 8 strains produced MBLs by E-test all of which carried bla-VIM. None of them were carriers of bla-IMP gene [24]. In another study in Iran during 2008, Shahcheraghi et al. collected 243 P. aeruginosa strains from nonburn patients. 22 strains were MBLs positive; 15 of them had bla-VIM and none was bla-IMP positive [25]. In a study carried out in India between 2005 and 2007, among 61 P. aeruginosa strains collected, 20 strains produced MBLs. Of the 20 MBLs confirmed strain by E-test, 17 strains were subjected to PCR testing. 15 of these strains were bla-VIM positive and two isolates were negative for both bla-VIM and bla-IMP and all were negative for bla-IMP [28]. In 2008, Khosravi and Mihani collected 100 P. aeruginosa in Iran. Production of MBLs was determined both by E-test and PCR method. Among 41 imipenem resistant P. aeruginosa, 8 strains were shown to be MBLs producer by E-test and all of these 8 strains carried bla-VIM and none of them had bla-IMP [22]. In another study in Turkey, 100 P. aeruginosa strains were collected from patients in a Turkish university hospital. One (1%) isolate was found to carry bla-VIM gene, whereas 9 (9%) carried bla-IMP gene. Among 9 isolates that carried bla-IMP gene, only 4 isolates were shown to be MBL producer by E-test [29]. In our study, the percent of strains that carried bla-VIM and bla-IMP genes was higher than those reported in previous studies. The reasons maybe an overall increase in the extent of acquirement of MBLs genes among P. aeruginosa. More MBLs genes are found to be located on the class I integron and can therefore easily transfer between
\textit{P. aeruginosa} strains [7]. In the majority of studies in Iran and other countries vim-type MBL was the most prevalent gene reported [30–32].

5. Conclusion

This study illustrated that the majority of \textit{P. aeruginosa} strains were resistant to various antibiotics. The high rate of antibiotic resistance among \textit{P. aeruginosa} strains is very alarming and can be responsible for serious infections. So identification of MBLs producing strains and taking efforts to reduce the rate of transfer between different strains are important goals for treatment of \textit{P. aeruginosa} infections.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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