

Occurrence of Ambler Class B Metallo- β -Lactamase Gene in Imipenem-Resistant *Pseudomonas Aeruginosa* Strains Isolated from Clinical Samples

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Article information	Abstract
<p>Article history: Received: 7 Dec 2012 Accepted: 5 Feb 2013 Available online: 5 May 2013 ZJRMS 2014; 16(2): 6-9</p> <p>Keywords: Beta-lactamase <i>Pseudomonas aeruginosa</i> Antibiotic resistance PCR</p> <p>*Corresponding author at: Department of Microbiology, Young Researchers and elite Club, Islamic Azad University, Falavarjan Branch, Isfahan, Iran E-mail: zey_golshani@yahoo.com</p>	<p>Background: Metallo-β-lactamase (MBLs) can hydrolyze a broad spectrum of beta-lactams, including penicillins, cephalosporins, and carbapenems. Genes encoding these enzymes are located on the plasmid that can easily be transferred to other bacteria. The aim of this study was to isolate and identify the <i>Pseudomonas aeruginosa</i> strains encoding VIM1 gene, in clinical samples, using the PCR technique.</p> <p>Materials and Methods: During a 4 month period, 100 strains of <i>Pseudomonas aeruginosa</i> from clinical specimens were collected. Standard tests were performed to identify strains of <i>Pseudomonas aeruginosa</i>. Resistance to antibiotics was examined and then the PCR was used to detect VIM1 gene.</p> <p>Results: In this study, the highest rates of resistance to antibiotics, amikacin and cefotaxime was observed (65% and 62%), the lowest resistance to antibiotics piperacillin (48%) and imipenem and cefepime with 55% resistance was reported. DDST method was performed for 37 strains for the MBL detection. Among the 37 isolate, 30 strains were MBL-producing with imipenem-EDTA method. Twelve strains (18%) were carriers of VIM1 gene using the PCR method.</p> <p>Conclusion: In the present study, the prevalence of strains producing MBL genes in strains of hospitals is a growing trend; correct prescription of medications can prevent the spread of resistant pathogens. It is suggested that molecular methods for rapid detection of resistance genes can be used to prevent the spread of this genes.</p> <p>Copyright © 2014 Zahedan University of Medical Sciences. All rights reserved.</p>

Introduction

Pseudomonas aeruginosa is a gram-negative, obligate aerobic rod-shaped and highly prevalent opportunistic pathogen. One of the most worrisome characteristics of *P. aeruginosa* is its low antibiotic susceptibility, which is attributable to a concerted action of multidrug efflux pumps with chromosomally encoded antibiotic resistance genes. Extended-spectrum cephalosporins (ESCs) resistance in *P. aeruginosa* is often associated with the overproduction of a naturally produced cephalosporinase [1, 2]. Carbapenems (imipenem and meropenem), the most effective antibiotics used in multi-resistant strains of pseudomonas that often, they are inhibited by metallo- β -lactamase (MBLs). High resistance of bacteria producing ambler class B enzymes, lead to, lack of efficacy of antibiotics and can cause severe infections such as septicemia and pneumonia [3]. MBLs based on molecular structure can be divided into six types, including AIM, SPM, SIM, GIM, IMP and VIM. MBL enzymes that first identified in *Serratia marcescens* was IMP-1, in 1991. MBL producing *P. aeruginosa* was first identified in Japan. VIM-type MBLs in *P. aeruginosa* are more common than others. The gene coding for this enzyme in most cases, is found on a cassette of genes and mobile elements. Generally, these enzymes are not able to hydrolyze aztreonam [4-6].

Cations such as zinc (Zn) are essential for the activity of MBL, but chemicals such as EDTA and DPA are able to inhibit these enzymes. In addition to *P. aeruginosa*, this genes identified in bacteria such as *E. coli*, *Klebsiella pneumoniae* and *Acinetobacter baumannii* [7-10]. MBL may also cause resistance to carbapenem and aminoglycosides. Genes encoding these enzymes are located on plasmids that can transfer easily to the enterobacteriaceae family and between species [8-11]. The aim of this study was isolation of *Pseudomonas aeruginosa*, and identification of strains carrying the gene encoding MBL, in clinical samples, in the Isfahan city, by the PCR techniques.

Materials and Methods

In this study, 100 clinical isolates were collected from different hospitals (Al-Zahra, Shariati, Imam Mosa Kazem). Strains from different clinical samples were collected, such as urine, Bransh, blood, wound infection, nasal swabs and ICU. The samples were transported to the laboratory and examined. Standardized tests including oxidase, production of colorless colonies on macConkey agar, blue- green pigment on Mueller Hinton agar, and growth at 42°C. Then, for further tests were kept in BHI

broth containing 18% glycerol [12]. Antibiotic susceptibility was confirmed by Kirby-Bauer disk diffusion method on Muller-Hinton medium, according to the Clinical Laboratory Standard Institute (CLSI) guidelines. After preparing bacterial suspension equivalent to 0.5 McFarland turbidity, this suspension were inoculated on Mueller Hinton agar medium using a sterile swab. The antibiotic discs with respect to distance from each other were placed on the medium and the plates were incubated at 37°C for 18-24 hours. Diameter of inhibition zone was measured for each strain and compared to that of standard strain and the results were interpreted as sensitive, intermediate resistant, or resistant, based on CLSI guidelines. Quality control was performed utilizing strains from the Iranian type culture collection *P. aeruginosa*. (PTCC: 1074). Discs which are used, included imipenem, cefepime, cefotaxime, piperacillin, ceftriaxone, ceftazidime, tobramycin, amikacin, gentamicin, and ciprofloxacin (Hi-media Laboratories, Mumbai) [11, 12].

Screening of strains for MBL production: In order to isolate strains producing MBLs, first, 0.5 M EDTA solution, was prepared, by dissolving 186.1 g EDTA powder in 1000 ml distilled water and PH was adjusted by NaOH on 8. Then autoclaved, volume of the solution containing the 750 µg EDTA, added to imipenem discs and after drying, were kept at 4 degrees. Then imipenem resistant strains were cultured on Mueller Hinton agar. In the next step, two discs, one containing imipenem alone and the other containing imipenem and 0.5 M EDTA, was placed on Mueller Hinton agar plate. The plates were incubated at 37 °C for 24 hours. Diameter of inhibition zone was measured. If the diameter of the inhibition zone around the imipenem-EDTA disk was equal or greater than 7 mm, the strain is reported as an MBL [9-11].

DNA extraction from strains: Phenol-chloroform extraction of DNA was used [12, 13]. The primers were designed to blaVIM1 gene. Primer was ordered to Cinaclon companies for synthesis. Primers set are as follows:

P.F: 5-CGTGATGGTGTGATGAGTTGC-3

P.R: 5-GCACAACCACCGTATAGCAC-3

PCR assay: PCR reaction was performed to detect the target gene in a final volume 25 µl, as follows, Buffer PCR 2.5µl, MgCl₂ 0.75 µl, dNTPs 0.5 µl, 1 µl of each primer, 1 µl target DNA and 1U Taq DNA polymerase. The reaction conditions were as follows: pre-denaturation at 94°C for 4 minutes, followed by 25 amplification cycles of 94°C for 1 minute, 55°C for 1 minute and 72°C for 1.5 minutes, with a final extension step of 72°C for 10 minutes. PCR products by agarose gel 1.5% containing ethidium bromide were electrophoresed at constant voltage 80, And the products were analyzed by UV transilluminator. Distilled water was used as negative control. One strain of *P. aeruginosa* producing VIM1 gene was used as positive controls [12-14].

Results

One hundred strains of *Pseudomonas aeruginosa* were collected from clinical samples of patients. Most samples were obtained from the urine and Bransh. Table 1 summarizes the susceptibility testing results obtained with *P. aeruginosa*. The most active compound against this was piperacillin (48%). Rate of resistance to ciprofloxacin was 56%, gentamicin 59%, tobramycin 61%, amikacin 65%, imipenem 55%, cefepime 55%, ceftazidime 57%, ceftriaxone 60%, cefotaxime 62%, and piperacillin was 48%. 55 strains were resistant to imipenem by disk diffusion method, that among them, 37 strains showed MIC₂ ≥ 4 µg/ml, to this antibiotic. All of 37 strains, In order to determine the production of MBL, were investigated by DDST method. Of these 37 strains, 30 strains were producing of MBLs with imipenem-EDTA method (Fig. 1).

In this study, all of resistant strains to multiple antibiotics (imipenem, ceftazidime, cefotaxime), were test to determine VIM1 gene by PCR method. Of 63 bacteria resistant to multiple antibiotics, that PCR testing was performed on all of them, 12 strains (18%) were carriers of the VIM1 gene (Fig. 2).

Table1. Antibiotic resistance pattern of pseudomonas aeruginosa isolated from clinical specimens

Antibiotic	Sensitivity(%)	Resistant(%)
Ciprofloxacin	44	56
Gentamicin	41	59
Tobramycin	39	61
Amikacin	35	65
Imipenem	45	55
Ceftazidime	43	57
Cefepime	45	55
Ceftriaxone	40	60
Cefotaxime	38	62
Piperacillin	52	48

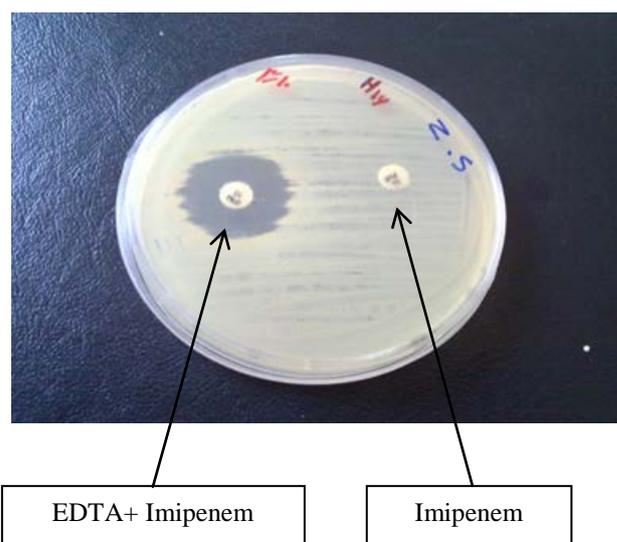


Figure 1. Phenotypic tests for detection of MBL-producing strains, Increasing the diameter of the inhibition zone around the disc combination imipenem- EDTA, equal or greater than 7 mm, the strain is reported as an MBL producer

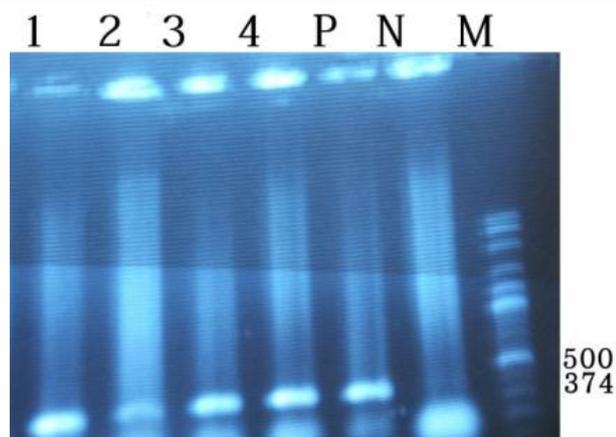


Figure 2. Electrophoresis of PCR product on agarose gel, P=positive control, N=negative control, M=DNA marker (100bp) Strains of 1, 3, 4 was producing of VIM1 gene (374 bp)

Discussion

Patterns of antibiotic resistance in *P. aeruginosa* strains in different countries and different regions of a country are different. Therefore, identification of resistant strains to multiple antibiotics to prevent the spread of resistance genes appears to be essential in hospitals. Today, with the prevalence of resistant bacteria to multiple antibiotics, control of nosocomial infections often lead to failure, leading to death of the patients. This could be due to lack of sufficient information about the dominant flora and antibiotic resistance in patients may remain unknown [13-15]. Determination of antibiotic resistance of these strains showed that resistance to carbapenems is increasing. The survey on 100 clinical isolates, showed that resistance to ceftazidime, ciprofloxacin and imipenem was 57%, 56% and 55%, respectively.

Jamali et al. studied on 186 patients (72% male and 28% female). The lowest resistance to imipenem was observed 61.8%. The highest resistance to cefotaxime and ceftazoxime was observed (100%), also resistance rate to ceftazidime was 91%, amikacin 73%, piperacillin 75% and ciprofloxacin 68% reported [14]. The results showed high prevalence of antibiotic resistance in burn patients compared with other patients in hospitals. VIM1 gene among the resistant strains to imipenem was observed in 20% and 12% of total strains, this showed that prevalence of VIM1 genes in Isfahan (18%) is higher than in Tehran, Probably due to the higher consumption of antibiotics in this region.

Furthermore, in the present study, minimum resistance to piperacillin (48%) have been reported. This difference reflects the higher sensitivity of the bacteria to imipenem in burn patients compared to other bacteria. In the present study, among 37 strains, 30 strains were producer of MBL enzymes, while in the Shahcheraghi study, among 28 strains, 22 strains, with Imipenem-EDTA method. Fazeli and colleagues, studied on 111 burn patients in the Imam Musa Kazem hospital, and reported that, all strains to ceftazidime and ticarcillin, and more than 94% of strains

to imipenem, ciprofloxacin, and piperacillin were resistant, that 55% of them were MBL producers, and all of these strains were resistant to imipenem [15]. In 2008, Khosravi et al., by E-Test method, reported that 19% of strains and in Shahcheragh et al. 72% were carrier [16]. Mirsalehian and colleagues studied on 170 clinical isolates of *P. aeruginosa*, that among them, 52% of strains were resistance to imipenem, 81% to amikacin and 84% to ticarcillin. Of the 90 isolates resistant to imipenem, 10 isolates (11%) were MBL producers by E-test method.

All strains carrying this gene by PCR method [2]. As can be seen, the prevalence of MBL in the present study (18%) was greater than the Mirsalehian study (11%). In France also, Walsh et al. reported that, 46% of the strains and in Korea 31% of strains were MBL producers [17]. MBLs Phenotypic detection by the imipenem-EDTA combined disc, usually the results are not so reliable, due to various reasons. As in various studies, the results of the phenotypic and genotypic detection are different from each other [18]. Therefore, to avoid the false positive results, PCR technique seems be necessary for the detection of MBL. In the present study, using PCR, 12 strains (18%) were carriers VIM1 gene, that similar to the results of Khosravi et al. study, the prevalence of this gene was 19.5%, respectively.

This study showed that the rate of antibiotic resistance in strains of *P. aeruginosa* isolated from clinical specimens in hospitals was increased, but compared with burn patients, the prevalence of resistance is less. By comparing the results, we can say, the prevalence of antibiotic resistance in hospitals of Tehran was greater than Isfahan, that it may be due to greater use of antibiotics in this city and, consequently, lead to increase resistance genes. For this reason, the identification of organisms with multiple resistance, for control and prevent of infection is an imperative. It is recommended, correct prescription of medications can prevent the spread of resistant pathogens.

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Authors' Contributions

All authors had equal role in design, work, statistical analysis and manuscript writing.

Conflict of Interest

The authors declare no conflict of interest.

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