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Imipenem-resistant *Pseudomonas Aeruginosa* Strains Carrying VIM-TYPE metallo-beta-lactamases Isolated from Intensive Care Unit, Shahid Beheshti Hospital, North of Iran

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Abstract

Background: Pseudomonas aeruginosa is the causing agent of many hospital infections and metallo-beta-lactamases (MBL) are being reported with increasing frequency. The aim of this study was to determine the frequency of metallo-β-lactamases (MBL) and VIM-1 gene in multidrug-resistant strains of *P. aeruginosa* isolates and to compare the methods of phenotypic and molecular detection.

Materials and Methods: In 2011- 2012, 50 samples of non – duplicate *P. aeruginosa* were isolated from intensive care units and tested for MBL production using phenotypic methods. Minimal Inhibitory concentrations (MICs) were determined by commercial micro dilution panels. The presence of metallo-β-lactamase (MBL) genes was established by polymerase chain reaction (PCR) with specific primers targeting the bla (VIM) genes.

Results: We used 50 clinical isolates amongst which 18 (%36) were found resistant to imipenem. Productions of MBL were detected in 15 (30%) isolates applying phenotypic method. PCR assay showed that 9 (18%) isolates carried aVIM-1 gene. MBL- producing strains were shown 100% resistant to cefepime, ceftazidime, ceftriaxone, cefotaxime and imipenem. Amikacin and ofloxacin appeared to be the most active antimicrobial agent.

Conclusion: These findings demonstrate the emergence of bla (VIM-1) producing *P. aeruginosa* in North of Iran. VIM metallo-beta-lactamases producing *P. aeruginosa* strains can cause serious infections that are difficult to treat, therefore, there is a need for rapid identification and the timely implementation of infection control measures in combination with systematic surveillance to monitor its potential clonal spread.

Keywords: *Pseudomonas aeruginosa*; metallo-beta-lactamases; Imipenem-resistant; bla (VIM-1) gene

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Introduction

Pseudomonas aeruginosa is the causing agent of many hospital infections, particularly in ICU wards. This organism is naturally resistant to many antimicrobial agents and it also can acquire resistance against available antibiotics through multiple mechanisms (1). Because of an increase in the antibiotic resistance in these bacteria, especially multi drugs ones; many problems have been created after the treatment of infections by the pathogens (2, 3). Standard therapy for *P. aeruginosa* infections includes broad specbeta-lactamases resistant penicillins, cephalosporins, carbapenems, and monobactams. Among beta-lactams, imipenem is the selective antibiotic against this bacterium, but *P. aeruginosa* can hydrolyze this antibiotic through metallo-beta-lactamases (MBL) (4, 5). Based on molecular structure, MBL are divided into the following types: DIM, KHM, GIM, SIM, SPM, AIM, VIM, and IMP, among which VIM and IMP are more distinct and pronounced in Enterobacteriaceae (6, 7) but the prevalence of MBL

genotypes varies in different parts of the world. VIM enzymes have been grouped into three main clusters designated VIM-1, VIM-2, and VIM-7 (8). During the past decade, the VIM type of MBL has become prevalent in *P. aeruginosa*, and this prevalence has been reported in numerous studies from different parts of Iran and other countries (9-12). Among MBLs, the first member of the VIM- type of enzymes, VIM-1, was identified in *P. aeruginosa* in Italy, but then it was emerged in Enterobacteriaceae and became a major problem in some settings. The phenotypical and genotypical characterization of MBL would be helpful in understanding the resistance mechanisms as well as its possible spread. Because MBL-producing *P. aeruginosa* is increasing globally, there is an urgent need to evaluate antibiotic regimens for the treatment of infections caused by these strains. Therefore, this research has been carried out with the purpose of identifying MBL-producing *P. aeruginosa* and to detect MBL-encoding VIM-1 genes among imipenem-non susceptible isolates.

Materials and Methods

Bacterial Isolates

The research followed the tenets of the Declaration of Helsinki; informed consent was obtained, and the research was approved by ethics committee of Babol University of Medical Sciences.This cross-sectional study was conducted during 2011 to 2012 in Shahid Beheshti Hospital in Babol. Isolates were obtained from clinical and surveillance specimens of patients in intensive care units. Samples were cultured in EMB and blood agar media (Merck. Germany) and incubated at 37 ºC for 24 hours. The gram negative bacilli were analyzed using differential tests in order to identify the *P. aeruginosa* strains.

The tests included: mobility, pigment production, growth at 42 ºC, oxidase, and catalase tests. Then, the purred colonies from each sample were identified as *P. aeruginosa* with differential tests, collected in BHI broth (Heart Infusion Broth, Difco) and two 1.5 ml micro tubes in which one of them contained 1 mL distilled water and the other one 1 mL physiological serum. These samples were kept in -20 ºC for performing sensitivity test and DNA extraction.

Antimicrobial Susceptibility Testing

Minimum Inhibitory Concentrations (MICs) were determined by micro broth dilution method according to the recommendations of the standard protocol of CLSI 2012 (13). The antibiotics were purchased from Sigma Chemical Company including cefepime, ceftriaxone, cefazolin, ceftazidime, ceftizoxime, imipenem, ticarcillin, cefotaxime, amikacin, ofloxacin, ciprofloxacin, and gentamicin.

Phenotypic Detection of MBL Activity

All strains that showed reduced susceptibility to imipenem ≥ 8 g/ml for imipenem and ≥ 32g/ml for ceftazidime were screened for MBL production. Also, all isolates with reduced susceptibility to imipenem and resistance to ceftazidime were subjected to a phenotypic analysis by EDTA combination disk test. Briefly, an overnight culture of clinical isolate was diluted with peptone water (Oxoid, USA) to 10^5 CFU/mL and spread on Mueller-Hinton (MH) agar (Difco) plate using cotton swab. Two IPM disks were placed on the surface of the agar at distances of 4-5 cm away from each other. Then, 5µL of 750 µg/mL EDTA solution was added to one of the IPM disks. The inhibition zones displayed around the IPM and the IPM-EDTA disks were compared after 14 to 16hrs incubation at 37ºC. The difference of ≥ 7mm between the inhibition zone diameter of the IPM-EDTA disk and that of the IPM alone disk was considered to be a positive test for the presence of MBLs (14). The procedure was repeated twice to ensure the reproducibility of results.

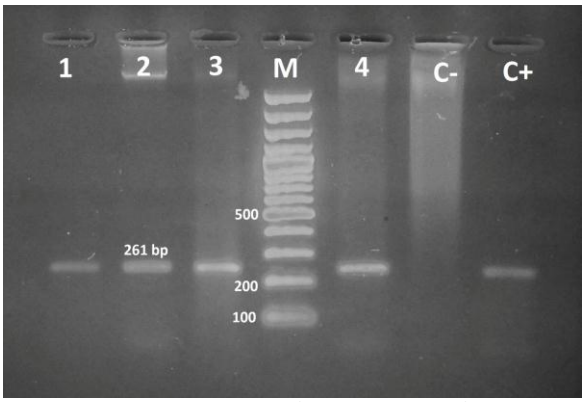

Figure 1. Gel-Electrophoresis of PCR products of VIM-TYPE MBLs of *P. aeruginosa* isolates: Lane 1 -3, 4: positive isolate, LaneM: DNA size marker, Lane C^+: positive control, Lane C^-: negative control.

PCR assay

DNA template from imipenem resistant isolates (MIC ≥8 µg/ml) were extracted by a Kit (High Pure PCR Template Preparation Kit, Roche Germany), and used as template in PCR assay to amplify blaVIM-1 by using thermocycler (Eppendorf, Hamburg, Germany). The primer sequences were as follows: forward 5'-AGTGGTGAGTATCCGACAG-3' and reverse 5'-ATGAAAGTGCGTGGAGAC-3' for amplification of blaVIM-1 gene, which could produce 261 bases per nucleotide length segment (15). The PCR reaction was performed in 50 µL volumes which contained 10 µL extractions of DNA (equal to 1 µg), 5 pmol/L from each primer, 1.5 mmol/L MgCl2, 0.2 mmol/L dNTPs and 1.5 unit of Taq DNA polymerase enzyme.

Table 1. Performance of phenotypic methods for detecting VIM enzymes and antimicrobial susceptibility testing results of *P. aeruginosa*

Phenotypic Method	**MBLenzyme**	**MIC(µg/ml)**												
		Cefotaxim	**Amikacin**	**Gentamicin**	**Cefepim**	**Cephazolin**	**Ofloxacin**	**Ceftizoxim**	**Ciprofloxacin**	**Ticarcillin**	**Ceftriaxo**	**Imipene**	**ceftazidim**	**ceftriaxon**
MBL- producing														
T3	VIM- I	12.5	400	100	400	100	25	12.5	25	≥400	250	25	50	100
T11	VIM- I	12.5	≥400	200	400	200	1.6	12.5	0.5	≥400	1.6	6.25	400	200
W1	VIM- I	200	100	≥200	100	≥200	0.5	200	50	100	0.5	25	50	200
W18	VIM- I	200	≥400	≥200	400	≥200	3.12	100	12.5	≥400	3.12	200	100	50
G18	VIM- I	1600	50	100	400	400	12.5	1600	50	200	800	100	1600	800
C11		200	100	200	400	400	25	200	25	400	100	100	50	100
E18		800	12.5	100	800	800	25	800	12.5	200	800	200	400	800
D18		1600	100	200	400	800	25	1600	100	200	800	100	200	800
H18		800	50	100	1600	1600	25	800	50	3.12	800	200	400	800
F13		100	100	100	400	100	3.12	100	100	200	200	25	400	200
J21		1600	25	50	1600	50	25	1600	6.25	100	1600	12.5	100	1600
I20		1600	100	200	1600	1600	50	1600	25	100	1600	12.5	1600	1600
D20		1600	50	200	1600	1600	12.5	≥1600	12.5	100	1600	50	1600	1600
J20		1600	25	100	1600	≥1600	50	≥1600	12.5	100	≥1600	25	1600	1600
S14		400	100	25	100	25	<0.25	400	25	100	0.25	100	200	200
MBL- Negative														
S16	VIM- I	200	12.5	12.5	12.5	12.5	0.5	200	1	12.5	0.5	0.5	50	6.25
U2	VIM- I	100	≥400	≥200	400	≥200	6.25	100	0.5	≥400	6.25	100	100	400
W6	VIM- I	200	200	200	200	200	0.5	200	<0.25	200	0.5	25	200	100
U6	VIM- I	100	12.5	100	12.5	100	6.25	100	0.25	12.5	6.25	0.5	200	6.25

After performing PCR reaction, electrophoresis of PCR products was conducted in 1.5% agarose gel for 60 minutes. Then, the results were evaluated under UV light on the UV gel document. After PCR, the specific band with associate weight was considered as a fragment of int l gene.

Statistical Analysis

Data was analyzed using SPSS statistical software version 18.

Results

In this study 50 clinical isolates were used study of which 18 (%36) were found resistant to imipenem, 7(14 %) isolates had an intermediate pattern, and 25 (50%) were susceptible to imipenem. Phenotypic method showed productions of MBL in 15(30%) isolates. PCR assay showed that 9 (18%) isolates carried aVIM-1 gene (Figure1). Of the 35 isolates that were negative in the phenotypic screening test, forth were positive for type VIM-1. The imipenem MICs for strains ranged from 0.5 to 200 μg/ml; 23 isolates were resistant to both imipenem and ceftazidim, and 27 were susceptible to either imipenem or ceftazidim. MBL- producing strains were shown to be 100% resistant to cefepime, ceftazidime, ceftriaxone, cefotaxime and imipenem. Amikacin and ofloxacin appeared to be the most active antimicrobial agent, with MIC 0.5 and 0.25. Antimicrobial susceptibilities of the MBL-positive and negative isolates are shown in Table 1.

Discussion

Imipenem-resistant Pseudomonas aeruginosa (IRPA) is a current and significant concern, especially because of the limited therapeutic options for this pathogen. Epidemiological studies carried out throughout the world has proven that the prevalence of MBLs has been increasing significantly. In the present study, we from 50 isolates of *P. aeruginosa*, 18 (36%) and 7 (14%) showed resistant and intermediate patterns to imipenem, respectively. The prevalence of IRPA among other Iranian studies has ranged from 23%- 56% in different geographic regions (16-18). In several studies, decreased susceptibility to imipenem has been accepted as the sole criterion for further phenotypic or molecular investigations in order to detect MBLs (19).

MBLs now account for up to 40% of worldwide IRPA cases; as well as**,** enzyme types may change by geographical area (20). The most significant ways to become resistant against imipenem is MBL production, of which the vim and ipm MBL families are very common between Gram negative rods (21). In our study, screening for MBLs by phenotypic methods showed that 15 (30%) isolates were MBL positive, but PCR results confirmed presence of MBL genes only in 9 (18%) isolates. Variations between phenotypic and molecular methods for detection of MBLs have also been reported in previous investigations. Previous studies in Iran showed that in Urmia 17.3% of *P. aeruginosa* isolates and Tabriz and 19.51% from Ahwaz were VIM-TYPE positive (9). These data are in accordance with the MBL-producing isolate proportions recently reported. For example, Pena *et al*. in a study from Portugal, found that 29.85% (40.134) of *P. aeruginosa* isolates were MBL positive using imipenem-EDTA combined disk method, but PCR confirmed MBL genes (VIM type) in 26 (19.40%) isolates (22).

The reason for such inconsistency is not well known, but it has been suggested that interference of other resistance mechanisms with MBL detection by phenotypic methods or presence of other unrecognized MBL resistance genes might be involved (23, 24). Chu *et al* also reported that methods using EDTA are highly sensitive but not specific, as was observed in our study (25). These findings suggest that caution must be taken in using only EDTA as the inhibitor agent when analyzing MBL production, as this method may lead to false positive results. In contrast, the EDTA disk test was able to discriminate between all MBL-positive and MBL-negative isolates by using a breakpoint of ≥14 mm. Of note, the same test interpreted with a breakpoint of ≥7 mm as suggested by Pitout *et al* falsely identified all MBL-negative isolates as MBL positive (26). We observed 35 isolates that were negative in the phenotypic screening test, forth were positive for type VIM-1. All *P. aeruginosa* isolates with reduced susceptibility to imipenem and resistance to ceftazidime were analyzed for genomic relatedness by PCR. These data therefore suggest that the optimal breakpoint may depend on the strain collection studied. An initial screening by combined susceptibility testing of imipenem and ceftazidime, followed by a confirmatory EDTA combination disk test, thus represents a valid and less expensive alternative to the molecular investigation of MBL genes.

VIM metallo-b-lactamase producing Pseudomonas strains can cause serious infections that are difficult to treat, therefore, MBL-producing *P. aeruginosa* requires rapid identification and the timely implementation of infection control measures in combination with systematic surveillance to monitor its potential clonal spread.

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Conflict of Interest

The authors declare that they have no conflict of interest in this work.

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