Molecular detection of metallo-β-lactamase gene *blaVIM*-1 in imipenem-resistant *Pseudomonas aeruginosa* strains isolated from hospitalized patients in the hospitals of Isfahan

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Abstract

Background: Pseudomonas aeruginosa is an opportunistic human pathogen that causes serious problems, especially in people, who have immunodeficiency. In recent times, metallo-β-lactamase (MBLs) resistance in this bacterium has led to some difficulties in treating bacterial infections. The metallo-beta-lactamase family of genes, including *blaVIM*-1, is being reported with increasing frequency worldwide. The aim of this study is the detection of the metallo-β-lactamase gene *blaVIM*-1 in imipenem-resistant *P. aeruginosa* (IRPA) strains isolated from hospitalized patients.

Materials and Methods: In this study, 106 *P. aeruginosa* samples were isolated from various nosocomial infections. The isolates were identified, tested for susceptibility to various antimicrobial agents by the Kirby-Bauer disk diffusion method, and all the imipenem-resistant isolates were screened for the presence of MBLs by using the combined disk (IMP-EDTA). The minimal inhibitory concentration (MIC) of imipenem was determined by E-test on the Mueller-Hinton agar. To detect the *blaVIM*-1 gene, the isolates were subjected to a polymerase chain reaction (PCR).

Results: Of all the *P. aeruginosa* isolates, 62 (58.5%) were found to be imipenem-resistant *P. aeruginosa* (MIC ≥32 μg/ml). Twenty-six (42%) of the imipenem-resistant isolates were MBL positive. None of these isolates carried the *blaVIM*-1 gene using the PCR assay.

Conclusion: The results demonstrated the serious therapeutic threat of the MBL-producing *P. aeruginosa* populations. The rate of imipenem resistance due to MBL was increased dramatically. Early detection and infection-control practices are the best antimicrobial strategies for this organism. None of MBL-producing isolates in this study carry the *blaVIM*-1 gene; therefore, another gene in the MBL family should be investigated.

Key Words: *blaVIM*-1, imipenem, metallo-β-lactamase, Pseudomonas aeruginosa

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INTRODUCTION

Pseudomonas aeruginosa is one of the important pathogens most frequently responsible for nosocomial infections and it is an opportunistic human pathogen.[1] 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.[2-5] They exhibit intrinsic resistance to several antimicrobial agents, including most b-lactams, and they often carry acquired resistance determinants that further reduce their spectrum of susceptibility.[6-8] Antimicrobial resistance in this species is a problem of growing concern and limits our therapeutic alternatives. Carbapenems are commonly used as last-resort drugs for the treatment of infections caused by multidrug-resistant *P. aeruginosa* isolates. However, intensive use of carbapenems in the treatment of nosocomial *P. aeruginosa* infections has facilitated the emergence of mechanisms that confer resistance to carbapenems, such as, diminished permeability, overexpression of the intrinsic efflux systems, and production of carbapenemases.[9] Acquisition of class B metallo-b-lactamases (MBLs) constitute a growing family of carbapenem-hydrolyzing beta-lactamases, among *P. aeruginosa* strains.[10] These enzymes efficiently hydrolyze all beta lactam compounds, except aztreonam.[11] These enzymes belong to Ambler class B and Bush group 3 and require divalent cations, usually zinc (Zn), as a cofactor for enzyme activity. They are inhibited by metal chelators, such as, Ethylenediaminetetraacetic acid (EDTA), but are not affected by therapeutic beta-lactamase inhibitors like sulbactam, tazobactam or clavulanic acid.[12] Metallo-β-lactam genes are usually a part of an integron structure and are carried on transferable plasmids, but can also be part of the chromosome. On account of integron-associated gene cassettes, MBL-producing *P. aeruginosa* isolates are often resistant to different groups of antimicrobial agents, which can be transferred to various types of bacteria.[13] Therefore, MBL-producing strains are important from an infection-control perspective. The MBLs are divided into the following six groups based on molecular structure: IMP, VIM, SIM, SPM, GIM, and AIM.[14] The the three main clusters of the VIM MBL, among 11 variants of this type, have been identified so far, represented by *blaVIM*-1, VIM-2, and VIM-7.[15] Metallo-β-lactamases producing *P. aeruginosa* isolates were first reported in Japan in 1991, and since then have been detected in various countries.[16] The present study was completed to detect *blaVIM*-1 producing *P. aeruginosa*, isolated from patients admitted to the hospitals of Isfahan.

MATERIALS AND METHODS

One hundred and six isolates of *P. aeruginosa* were collected from the hospitalized patients in Isfahan city of Iran, between October 2012 and November 2013. These strains were separated from blood, wound, sputum, urine, eye infection, catheters, ear, peritoneum, and burning specimen Only one isolate per patient was included in the study. These isolates were identified

as *P. aeruginosa* based on the colonial morphology on Sytrymyd agar and Pseudomonas agar, Gram stain characteristics, Oxidative-fermentative test, oxidase test, growth in 42ºC, and pigment production tests. Strains were preserved in Brain–Heart infusion broth (Merck-German) containing 20% glycerol.[17]

Antibiotic susceptibility tests
The antibiotic susceptibility test was done by the disk diffusion method (Kirby-Bauer) on Muller-Hinton agar plates (Merck-German) according to the CLSI (Clinical and Laboratory Standards Institute) guidelines. The antimicrobial disks used were amikacin (10 µg), ciprofloxacin (10 µg), ceftazidime (10 µg), gentamicin (10 µg), imipenem (10 µg), meropenem (10 µg), cefotaxime (10 µg), cefepime (10 µg), and aztreonam (10 µg), which were obtained from MAST (Merseyside, UK). *P. aeruginosa* ATCC 27853 were used as control for, susceptibility testing. The bacteria were inoculated into BHI, and incubated at 35ºC for two-to-four hours until it reached the turbidity of a 0.5 McFarland standard. Then using a sterile swab they were cultured on Muller-Hinton agar plate. Then, antimicrobial disks were placed on the plate and incubated at 37ºC for 18 to 24 hours. After incubation, the diameters of the zone of complete inhibition were measured.[18,19]

Minimum inhibitory concentration
Minimum inhibitory concentration (MIC) test was performed by the E-test method for imipenem-resistant strains. Mueller-Hinton agar plates, the recommended medium, were streaked by using cotton swabs. The E-test strips were impregnated with imipenem and then applied on the plates, which were incubated at 35ºC in air for 16 to 20 hours.[20] *P. aeruginosa* strains with MIC $\geq$8 µg/ml to imipenem are referred to as resistant.[21]

Ethylenediaminetetraacetic-imipenem test
Phenotypic detection of MBL production (EDTA-IMP) was carried out for imipenem-resistant isolates (by dissolving 186.1 g of disodium EDTA in 2H2O in 1000 ml of distilled water, 0.5 M EDTA solution was provided and adjusted to pH 8 by adding NaOH. Next, 750 µg of prepared solution was added to the imipenem disk. The EDTA-imipenem disk plus the imipenem disk were placed in a plate containing Muller-Hinton agar and cultured *P. aeruginosa*. After 18–24 hours of incubation at 37ºC an organism was considered MBL positive if the inhibition zone diameter increased by 7 mm or more toward the IMP + EDTA, in comparison to the IMP disk alone.[22]

Molecular analysis
Total DNA from the *P. aeruginosa* strains was extracted by the boiling method; this caused the cell wall to

denaturate and lysis.[23] PCR assay was performed to detect the *blaVIM-1* gene. A strain of *P. aeruginosa* known to produce VIM-1 metallo-beta-lactamase was used as a positive control in all cases.[17]

Polymer chain reaction tests were done under the following program

Polymer Chain Reaction for detection of the *blaVIM-1* gene was performed on total DNA using VIM-1 primers (Forward: 5 × TTA TGG AGC AGC AAC CGA TGT 3 × and Reverse: 5 × CAA AAG TCC CGC TCC AAC GA 3×). The size of the amplification product was 830 bp.[24] The cycling parameters for the *blaVIM*-1 gene were: 94ºC for three minutes, followed by 35 cycles of denaturation at 94ºC for one minute, annealing at 55ºC for one minute, extension at 72ºC for two minutes, and a final extension at 72ºC for seven minutes. The PCR products were loaded into a 2.0% agarose gel, stained with 1% ethidium bromide, electrophoresed, and visualized under ultraviolet (UV) light.[24] The positive controls were the MBL-producing strains. Amplification 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 MgCl2 (Fermentas-Canada), 0.5 U Taq polymerase, and 50 ng DNA templates.[25]

RESULTS

Among 106 strains collected, the majority was isolated from urine (36%) and minority was related to the ear (1%) [Figure 1]. The disk diffusion method showed that 62 isolates (58.5%) were resistant to imipenem and meropenem. Other antibiotic resistances are shown in Figure 2. MIC test illustrated that MIC in all of imipenem-resistant *P. aeruginosa* (IRPA) strains were MIC ≥32 µg/ml [Figure 3]. In the phenotypic method, 26 (42%) imipenem-resistant isolates produced the MBL enzyme (increase of ≥7 mm in a zone diameter of EDTA-IMP disk compared to the imipenem disk) [Figure 4]. The molecular assay (PCR method) did not detect the *blaVIM-1* gene in this study [Figure 5].

DISCUSSION

The rapid spread of MBLs among major gram-negative pathogens, particularly *P. aeruginosa*, is an emerging threat and a matter of concern worldwide.[26,27] There is more concern about *P. aeruginosa,* which is one of the opportunistic pathogens inpatients with immune deficiency.[27,28] Imipenem-resistant *P. aeruginosa* (IRPA) is a current and significant concern, especially because of the limited therapeutic options for this pathogen. MBL enzymes may play a critical role in IRPA, given that there is a high
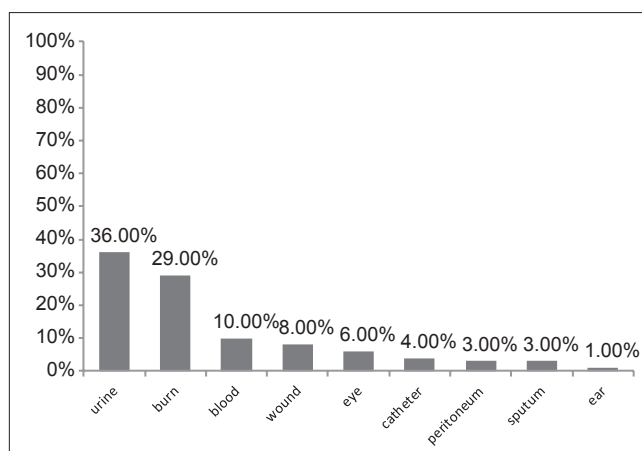

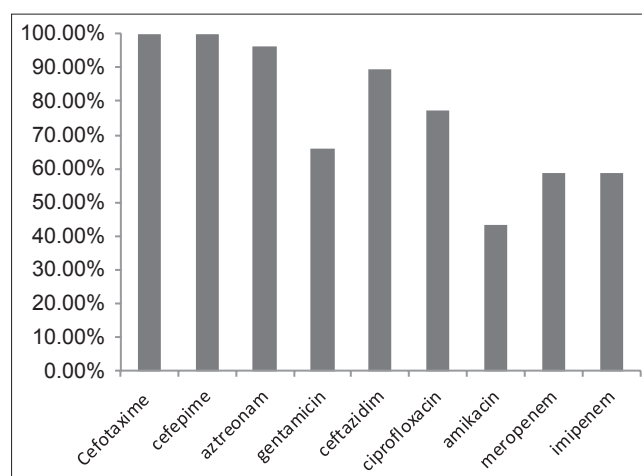**Figure 1:** The frequency of isolation of *P. aeruginosa* isolated from a clinical specimen

Figure 2: Resistance pattern of *P. aeruginosa* isolates to different antimicrobial agents
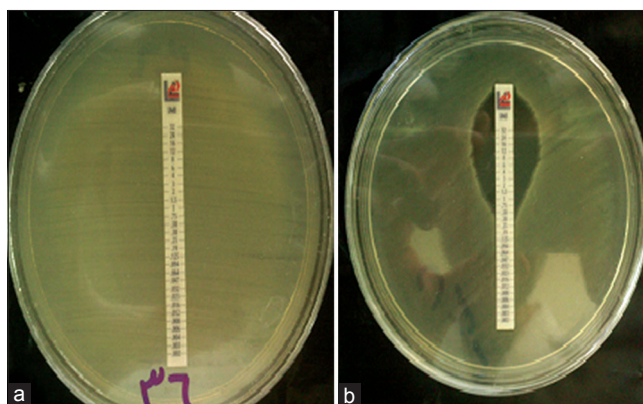

Figure 3: Measuring the MIC of imipenem-resistant *P. aeruginosa* isolates and (a) Compared with the control strain (b) by the E-test Method

possibility of these carbapenemases being spread among nosocomial isolates. The prevalence of MBLs has been increasing significantly. MBLs now account for up to 40% of the IRPA cases worldwide; furthermore, the enzyme types may vary by regional
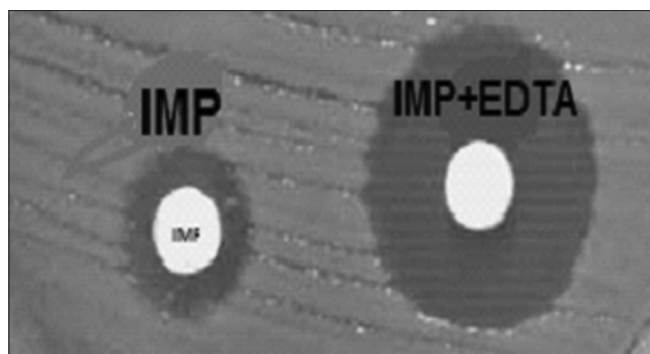
Figure 4: Phenotypic Detection of MBLs by Combined disk (EDTA+IMP) among the *P. aeruginosa* Isolates

areas.[24] In this study, 108 *P. aeruginosa* strains were obtained from different hospitalized patients from the different hospitals of Isfahan. The antibiotic susceptibility pattern was determined in these strains and imipenem-resistant strains were subjected to be tested for MIC, EDTA-IMP, and molecular analysis. Metallo-beta-lactamases are a group of β-lactamase enzymes that have one or two zinc (Zn) in their active site to cleave the amide bond of the β-lactam ring to inactive β-lactam antibiotics. In the recent years, nosocomial outbreaks of MBL-producing bacteria have been reported worldwide.[29] The *blaVIM-1* gene was first reported in *P. aeruginosa* in Italy.[25] As far as recent studies have shown, this enzyme has spread significantly. Furthermore, there are 20 various known varieties of *blaVIM* alleles all over the world.[19] This gene has been reported in different areas in Iran, but our study showed that none of IRPA strains carry *blaVIM*-1 in the hospitals of Isfahan.

In Italy, three studies have been done. According to Francesco luzzaro *et al.* (2004), the *blaVIM-1* gene was detected in only one isolate from the 506 isolates of IRPA.[30] Cristiana Lagatolla, conducted two studies. In the first study on 89 IRPA isolates, 54 (84%) isolates had the *blaVIM-1* gene.[31] The second study reported that 86 strains between 174 IRPA had the *blaVIM-1* gene.[32] In another study in Brazil, by Fernanda *et al.,* (2009), 31 *P.aeruginosa* isolates were investigated, but no *blaVIM-1* gene existed in these isolates.[17] Also, Franco *et al.* (2010), showed that this gene did not exist in 238 isolates of *P.aeruginosa* in his study.[24] Two studies were conducted in Spain. According to a study by Carvalho *et al.* (2005), 27 isolates of *P.aeruginosa* were investigated, where the *blaVIM-1* gene was not identified in these strains.[33] Also, in another study on 236 *P.aeruginosa* isolates by Gutierrez *et al.* (2007), the *blaVIM-1* gene was not detected.[34] In 2008, Khosravi and colleagues collected 100 *P.aeruginosa* isolates. Disk diffusion showed that 41 of the isolates were resistant to
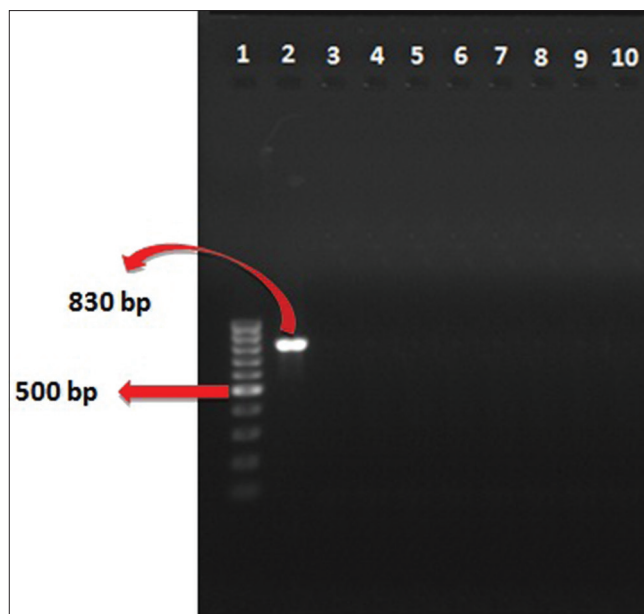

Figure 5: PCR assay for the Detection of the *blaVIM*-1 gene (product size: 830 bp) (Lane 1: DNA ladder 100 bp, Lane 2: Positive control, Lane 3: Negative control, Lanes 4-10: Amplified products)

imipenem, eight (19.51%) appeared to produce MBL, and eight imipenem-resistant strains were *blaVIM-1* positive.[35] 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 the *blaVIM-1* gene were determined. Sixty-five out of 69 imipenem-resistant *P. aeruginosa* showed MBL activity, while only 13 of them had the MBL gene. The *blaVIM-1* was not found finally.[35] In 2012, Forozesh Fard and colleagues collected 11 *P.aeruginosa* isolates. All the isolates were susceptible to imipenem, and all of them were *blaVIM*-1 negative.[18] Compared to our study, the imipenem-resistant strains in Isfahan Hospitals showed higher resistance to imipenem (37%), and MBLs existed in a high level, but the *blaVIM-1* gene had a low incidence or even maybe did not exist, which shows that another *MBL* genes were probably involved.

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

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