Prevalence of metallo-beta-lactamase genes blaVIM-1 and blaSPM-1 in Pseudomonas aeruginosa Clinical Isolates in Isfahan, Iran

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ABSTRACT

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Background and objectives: Class B beta-lactamases, termed metallo-beta-lactamases (MBLs) such as VIM and SPM are an increasingly serious clinical problems. They have a very broad substrate profile that includes penicillins, expanded spectrum cephalosporins, and carbapenems, except for monobactams such as aztreonam. MBLs producing Pseudomonas aeruginosa isolates have been responsible for resistant to carbapenems and several nosocomial outbreaks. The aim of this study was to determine the possibility of existence of MBLs, blaVIM-1 and blaSPM-1 metallo-beta-lactamase genes among clinical isolates of P. aeruginosa collected from Tehran hospitals.

Material and Methods: In this study, 252 clinical isolates were collected from patient at hospitals of Isfahan. The isolates were identified as P. aeruginosa by using biochemical tests. The resistance to different antibiotics was evaluated by disk diffusion method according to CLSI (Clinical and Laboratory Standards Institute) and MICs of imipenem were determined using E-test method. All of imipenem-resistant isolates screened for the presence of MBLs by using the Combined disk (IMP+EDTA). PCR assay was performed for detection of blaVIM-1 and blaSPM-1 beta-lactamase genes.

Results: From 106 P. aeruginosa isolates that collected from Isfahan hospitals in 2012 and 2013, 62 (58.5%) isolates were imipenem-resistant (minimum inhibitory concentration (MIC) ≥32 μg/mL). Among these imipenem-resistant isolates, 26 (42%) MBL-producing P. aeruginosa isolates were screened using IMP-EDTA. A specific PCR test showed that none of MBL positive isolates has blaVIM-1 or blaSPM-1 genes.

Conclusion: Considering the high prevalence and clinical importance of BL-producing isolates, rapid identification of them and use of the appropriate infection control measures are necessary to prevent further spread of infections by these organisms. We found that none of MBL-producing isolates in this study carries blaVIM or blaSPM-1 genes; therefore, other genes in MBLs family should be investigated.

Introduction

Pseudomonas aeruginosa is an opportunistic Gram-negative pathogenic bacterium associated with a range of nosocomial infections (e.g. septicemia and pneumonnia, bacterimia, meningitis, urinary tract and wound infections). Despite the use of potent antibiotics, invasive P. aeruginosa infection is associated with high mortality. In the past decade, acquired multidrug resistance, relating to selective antibiotic pressure, has emerged in several countries; and in some cases, infections caused by multidrug resistant P. aeruginosa have been untreatable (1, 2, 14). First report of the presence of P. aeruginosa in hospital
was in 1972 in Pennsylvania (13). Carbapenems, including meropenem and imipenem, are the most effective antibiotic against this organism isolated from patients. However, resistance to carbapenems has emerged by different mechanisms such as impermeability to drug due to loss of OprD porin, the up-regulation of an active efflux pump system present in the cytoplasmic membrane of these organisms or production of metallo-beta-lactamases (MBLs) that hydrolyze all carbapenems (3-4, 5). Based on the molecular studies in 1980, the β-lactamases were classified into 4 distinct classes by Ambler: A, C, D included serine enzymes possessing a serine moiety at their active site, and class B that is wholly compromised of metallo-enzymes and requires zinc ions for its enzymatic activity. From 1995 to 1997, Bush attempted to expand and modify MBLs classification (group 3) by studying the rate and criteria of imipenem and other β-lactams hydrolyzing. Therefore, Bush categorized these enzymes into three subgroups including 3a enzymes distinguished by their broad-spectrum activity, 3b that shows a special avidity to carbapenems, and 3c with a poor hydrolyzing activity (6, 7, 8). Chelators as EDTA in vitro but other chelators such as sulbactam, tazobactam, inhibit hydrolyzing activity of MBLs and clavulanic acid are not able to inhibit MBLs (11). During current years, the latter MBLs have been frequently studied showing a rapid spread across the world (3). Metallo-β-lactamases-producing *P. aeruginosa* isolates were first reported in Japan in 1991, and since then have been detected in various countries (7). VIM was first detected in Verona, Italy; SPM in Sao Paolo, Brazil (8). Since then, other kinds of MBLs including VIM, SIM, SPM, GIM, AIM were detected around the world (8, 10). The production of MBLs is important because several outbreaks caused by MBL-producing strains have been seen (11). Metallo-β-lactam genes are usually part of an integron structure and are carried on transferable plasmids but can also be part of the chromosome. Because of the integrin associated gene cassettes, MBLs-producing *P. aeruginosa* isolates are often resistant to different groups of antimicrobial agents, which can be transferred to various types of bacteria (12). Currently, by growing rate of resistance against traditional antibiotics, treatment of patients with infections caused by MBLs-producing *P. aeruginosa* seems critical. Therefore, current study is perform to examine the prevalence MBL-producing *P. aeruginosa* and to detect MBL genes *bla*VIM-1 and *bla*SPM-1 among imipenem-resistant isolates obtained from various hospitals in Isfahan.

**Materials and Methods**

**Bacterial Strains**

In this cross sectional descriptive study 252 isolated samples were collected from 9 hospitals in Isfahan (Al-Zahra, Imam Musa Kazim, Seyyedolshohada, Amin, Isa benne Maeyam, Feyz, Ayatollah Kashani and Khanevade clinic) during August 2012-March 2013. The specimens included urine (n=86, 34.12%), blood (n= 43, 17.06%), wound (n=37, 14.68%), respiratory tract (n=27, 10.71%), Catheter (n=18, 7.14%), ear (n=12, 4.76%), eye (n=11, 4.36%), stool (n=11, 4.36%) and other sites (n=7, 2.77%). These isolates were collected from different wards including ICU, urology, respiratory and surgery wards. Replicated isolates from the same patients were excluded from the study. All isolates were identified by conventional. Identification of *P. aeruginosa* isolates was as below. Gram staining, colony morphologies, McConkeys agar, TSI test, oxidase reaction, and growth at 42 °C are the phenotypic and bacteriological test, which used in this study.

**Antibiotic Susceptibility Testing**

Antibiotic susceptibility testing performed as recommended by the Clinical and Laboratory Standards Institute using disks containing ceftazidime (CAZ: 30 μg), ceftriaxone (CRO: 30 μg), cefotaxime (CTX: 30 μg), ceftizoxime (ZOX: 30 μg), piperacillin (PIP: 100 μg), piperacillin/tazobactam (PT: 110 μg), gentamicin (GM: 10 μg), amikacin (AN: 30 μg), imipenem (IMP:10 μg), ciprofloxacin (CIP:5 μg) collistin (COL:10 μg), polimixin B (PB:300 unit), meropenem (MEM:10 μg) and aztreonam (ATM: 30 μg) (MAST, Merseyside, U.K). *P. aeruginosa* ATCC 27853 used as controls for antibiotic resistance (5).

**Minimum Inhibitory Concentration (MIC):**

MIC test performed by E-test method for imipenem resistant isolates on Mueller-Hinton agar medium. Then, Etest-imipenem strips applied, and the plates incubated at 35°C in air for 16 to 20 h (15). Nonsusceptible *P. aeruginosa* strains with MIC≥32 μg/ml referred to as Imipenem-resistant (16).

**Phenotypic Detection of MBL-Producing Isolates:**

Phenotypic detection of MBL production (EDTA-IMP) was carried out for imipenem-resistant strains by dissolving 186.1 gr of disodium EDTA. 2H2O in 1000 ml of distilled water, 0.5 M EDTA solution was provided and adjusted to pH 8 by adding NaOH. Then, 750 μg of prepared solution was added to imipenem disk, and it was dried in an incubator. EDTA-imipenem disk plus imipenem disk were placed in a plate contained Muller-Hinton agar and cultured *P. aeruginosa*. After 18-24 h of
incubation at 35°C an organism was considered MBL positive if the inhibition zone diameter increased by 7 mm or more towards the IMP plus EDTA in comparison to IMP disk alone (17).

**Molecular Analysis:**

For molecular diagnosis, the Total DNA from MBL positive *P. aeruginosa* isolates was extracted by boiling method; this causes cell wall to lysed (Franco et al, 2010). PCR assay was performed to detect *blaVIM-1* and *blaSPM-1* genes, 2 strain of *P. aeruginosa* that contained these 2 genes separately were used as an positive control (Table 1) (11).

**PCR tests were done under the following program:**

The cycling parameters for the *blaSPM-1* gene were: 95°C for 5 min, followed by 30 cycles of denaturation at 95°C for 1 min, annealing at 40°C for 1 min, extension at 68°C for 1 min and a final extension at 68°C for 5 min. The parameters used for the *blaVIM-1* gene were: 94°C for 3 min, followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min, extension at 72°C for 2 min and a final extension at 72°C for 7 min. The PCR products were loaded into a 2.0% agarose gel, stained with 1% ethidium bromide, electrophoresed and visualized under UV light. Positive controls were the MBL-producing *P. aeruginosa* clinical strains (containing VIM-1 and SPM-1) (18).

**Results:**

Among 106 strains collected, the majority were isolated from urine (36%) and minority of them were related to ear (1%) (Figure 1). Disk diffusion method showed that 62 isolates (58.5%) were resistant to imipenem and meropenem. Other antibiotic resistances show 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 phenotypic method, twenty-six (42%) of imipenem-resistant isolates produced MBL enzyme (increase of ≥7 mm in zone diameter of EDTA-IMP disk compared to imipenem disk) (figure 4). Molecular assay (PCR method) not detected *blaVIM-1* and *blaSPM-1* genes in MBL positive isolates in this study (Figure 5 and 6).

![Figure 1. The frequency of isolation of P. aeruginosa isolated from clinical specimen](image-url)
Figure 2. Resistance pattern of *P. aeruginosa* isolates to different antimicrobial agents

Figure 3. Measuring the MIC of imipenem-resistant isolates by Etest Method
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**Figure 4.** Phenotypic Detection of MBLs by Combined disk (EDTA+IMP) among *P. aeruginosa* Isolates

**Figure 5.** PCR assay for the Detection of *blaVIM-1* gene (product size: 830 bp) (Lanes 1-5: amplified products, Lane 6, 7: Positive control, Lane 8, 9: DNA ladder 50 bp, Lane 10, 11: Negative control)
The rapid spread of MBLs among major gram-negative pathogens, particularly *P. aeruginosa*, is an emerging threat and a matter of concern worldwide (19, 20). There is more concern about *P. aeruginosa* that is one of opportunistic pathogen inpatients with immune deficiency (20, 21). Imipenem-resistant *P. aeruginosa* 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 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 worldwide IRPA cases; furthermore, enzyme types may vary by regional areas (31). In this study, 108 *P. aeruginosa* strains obtained from different hospitalized patients in different Hospitals of Isfahan. 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-lactamases that need 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 (23). *blaVIM-1* gene was first reported in Italy (31). As far as recent studies have shown this enzyme has been spread significantly. Furthermore there have been known 20 various different *blaVIM* alleles all over the world (32). This gene has been reported in different areas in Iran, but our study showed that none of IRPA strains carry *blaVIM-1* in hospitals of Isfahan. In Italy, 3 studies have been done. According to Francesco luzzaro et al. (2004), the *blaVIM-1* gene detect in 1 isolate only from 506 isolates of IRPA (23). Cristiano Lagatolla was conducted 2 studies. In first study on 89 IRPA isolates, 54(84%) isolates had *blaVIM-1* gene (24). second study reported that 86 strains between 174 IRPA had *blaVIM-1* gene (25) . In other study In brazil by Fernanda at el. (2009), 31 *P. aeruginosa* isolates investigate but no exist *blaVIM-1* gene in these isolates (33). Also, Franco et al (2010), Show that no exist this gene in 238 isolates of *P. aeruginosa* in his study (30). Two studies were conducted in Spain.According to study by carvalho et al.(2005), 27 isolates of *P. aeruginosa* investigated that *blaVIM-1* gene not identified in these strains (26). Also, in another study on 236 *P. aeruginosa* isolates by Gutierrez et al. (2007), not detect *blaVIM-1* gene (27). In 2008, Khosravi and colleagues collected 100 *P. aeruginosa* isolates. Disk diffusion showed 41 of isolates were resistant to imipenem, that 8(19.51%) appeared to produce MBL, and 8 imipenem-resistant strains were *blaVIM-1* positive (28). 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 *blaVIM-1* gene were determined. 65 out of 69 imipenem-resistant *P. aeruginosa* showed to have MBL activity while only 13 of them had MBL gene. *blaVIM-1* not found finally (29). In 2012, ForozeshFard and colleagues collected 11 *P. aeruginosa* isolates. All isolates were susceptible to imipenem, and all of them were *blaVIM-1* negative (34). Compared to our study, *P. aeruginosa* strains in Isfahan hospitals showed higher resistance to imipenem (58.5%), and MBLs existed in high level, but *blaVIM-1* gene has a low incidence or even maybe not exist, which this Show that another MBLs gene including IMP, SPM, GIM, SIM and AIM probably involved in strains that were MBL positive by phenotypic test (Combined disk).
Nevertheless, several studies have been done about blaSPM-1 gene. In a study conducted by Magalas and partners in Brazil (2005), 48 *P. aeruginosa* samples were collected that among these isolates 24 strains were resistant to imipenem. Among resistant strains, 15 isolates were MBL-producing and all isolates were blaSPM-1 carriers (35). In a study by Franco and colleagues (2010) conducted on the blood isolates of *P. aeruginosa*, prevalence of imipenem resistant strains were reported 34%. Seventy-seven percent of strains were MBL-producing; among these isolates, 81% were positive blaSPM-1 (36). Sadeghi and colleague (2012), conducted a study on 108 isolates of *P. aeruginosa* in central province that blaSPM-1 gene did not show in these strains (36). In a study by Youssefi et al. (2010) in the northwest of the Iran country from 104 isolates of *Pseudomonas aeruginosa*, 39 isolates were MBL-producing by phenotypic test, but none of them had blaSPM-1 gene by PCR test (37). In a study by Shahcheraghi and colleagues (2009) at Imam Khomeini Hospital, Tehran that conducted on 243 samples of *P. aerogenesa*, 28 samples were resistant to imipenem and 22 samples were MBL enzyme production, but none of these strains shows blaSPM-1 by PCR tests (38). However, in this study among 26 strains those producing MBLs, none of them has blaSPM-1 gene. Thus, while resistant to imipenem in Iran country is in high level and positive MBL strain increased, strains by blaSPM-1 gene has not yet appeared in this country. These results indicate that other genes of MBL family present in this resistant strains. Combination therapy can be useful to prevent resistance during therapy. Regarding to horizontal transmission of integron-associated MBL genes, detecting MBL positive strains is essential. Moreover, invention of new methods for identifying MBL positive bacteria, and screening involving people must be done in hospitals regularly.

References:


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