J Arch Mil Med. 2015 May; 3(2): e26977. DOI: 10.5812/jamm.3(2)2015.26977

Published online 2015 May 30. **Research Article**

Detection of *blaSPM-1* Metallo-β-Lactamase Gene in Imipenem-Resistant *Pseudomonas aeruginosa* Strains Isolated From Hospitalized Patients in Isfahan Hospitals

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Received: January 14, 2015; **Revised:** February 2, 2015; **Accepted:** February 16, 2015

Background: *Pseudomonas aeruginosa* is an opportunistic human pathogen, which causes serious problems especially in people who have immunodeficiency. Recently, metallo-β-lactamase (MBLs) resistance in this bacterium has led to some difficulties in treating bacterial infections. The *blaSPM-1* is one of the MBL gene families, which induces resistance to the carbapenem class antibiotics; this gene has not been previously assessed in Iran.

Objectives: Detection and quantification of *blaSPM-1*- metallo-β-lactamase gene among resistant *Pseudomonas aeruginosa* strains (imipenem), isolated from patients in Isfahan hospitals.

Patients and Methods: A total of 180 samples were isolated from various nosocomial infections. These isolates were identified as *Pseudomonas aeruginosa* by using biochemical tests. In order to determine their bacterial drug resistance-pattern the Kirby-Bauer disk diffusion method was utilized. Presence of MBLs in imipenem isolates was detected using the combine disk technique (IMP-EDTA). Similarly, an E-test on Mueller-Hinton agar was used to determine the minimal inhibitory concentration (MIC) of imipenem isolates. The imipenem isolates were then subjected to polymerase chain reaction (PCR) to detect the *blaSPM-1* gene. Data were analyzed using the SPSS software (version 16, SPSS Inc., Chicago, IL, USA).

Results: In total, 96 isolates of *Pseudomonas aeruginosa* were collected. Of all isolates, 34 (35.41%) were found to be imipenem-resistant *P. aeruginosa*. The MIC levels in all imipenem-resistant strains were MIC ≥ 32 μg/mL. Thirteen (38.23%) of the imipenem-resistant *P. aeruginosa* isolates were MBL positive. None of the isolates carried the *blaSPM-1* gene, as indicated by the PCR assay.

Conclusions: The rate of imipenem resistance due to MBL has increased dramatically. Early detection and infection-control practices are the best antimicrobial strategy for this organism.

Keywords: Pseudomonas aeruginosa; Imipenem; Nosocomial Infection; *blaSPM-1*; Metallo-β-Lactamase

1. Background

Pseudomonas aeruginosa is an opportunistic gram-negative pathogenic bacterium associated with a range of nosocomial infections (e.g. septicemia and pneumonia, bacteremia, meningitis, urinary tract and wound infections). *P. aeruginosa* infections are associated with high mortality, despite the use of potent antibiotics. Selective antibiotic pressure, due to acquired multi-drug resistance, has emerged in several countries. Moreover, there have been some untreatable cases arising due to infections caused by multi-drug resistant *P. aeruginosa* (1-3). *P. aeruginosa* was first reported in a Pennsylvanian hospital during 1972 (4). Carbapenems including meropenem and imipenem are the most effective antibiotics against *P. aeruginosa* isolated from patients. However, resistance to carbapenems has emerged by various mechanisms such as impermeability of the drug due to loss of OprD porin, the up-regulation of an active cytoplasmic efflux pump system or production of enzymes or compounds such as metallo-beta-lactamases (MBLs) that can hydrolyze all carbapenems (5-7). According to molecular studies conducted in the 1980s, β-lactamases were classified to four distinct classes namely, A, B, C and D. Classes A, C and D consist of serine enzymes at their active site and these possess a serine moiety. On the other hand enzyme activity of class B requires zinc ions and their active site consists of metallo-enzymes. During the years 1995 to

1997, attempts have been made to expand and modify classification of MBLs. The rate and criteria of imipenem and hydrolysis of other beta-lactams were analyzed for a better understanding. These enzymes were categorized to three subgroups including 3a, 3b and 3c enzymes. Enzymes of the 3a subgroup were distinguished from the other two by their broad-spectrum activity; 3b enzymes revealed special avidity to carbapenems; and 3c enzymes were associated with poor hydrolyzing activity (8-10). Chelators such as EDTA in vitro and other chelators (e.g. sulbactam and tazobactam) inhibit hydrolyzing activity of MBLs while clavulanic acid is not able to inhibit MBLs (11). Clavulanic acid MBLs are the frequent focus of studies worldwide (5). Metallo-β-lactamase-producing *P. aeruginosa* isolates were first reported in Japan in 1991, and since then have been detected in various countries (9). In addition to the detection of VIM in Verona, Italy and SPM MBL in Sao Paolo, Brazil, other MBLs have been identified (10, 11). The production of MBLs is important because several outbreaks caused by MBL-producing strains have been reported (12). Metallo-β-lactam genes are usually part of an integron structure and are carried on transferable plasmids while they can also be part of the chromosome. Furthermore, MBLs-producing *P. aeruginosa* isolates are often resistant to antimicrobial agents due to the presence of integrin associated gene cassettes. These cassettes can replicate rapidly in different types of bacteria (13). It is critical to discover novel treatments/therapies for patients with MBLs-producing *P. aeruginosa* infections due to rapidly increasing rate of resistance against traditional antibiotics. The *blaSPM-1* is one of the MBL gene families, which induces resistance to the carbapenem class of antibiotics including imipenem as one of the new antibiotics. Studies done in Iran have paid less attention to the detection and frequency of the mentioned gene among other metallo-β-lactamase genes; this gene has been shown to have a high prevalence in other countries.

2. Objectives

The aim of the current study was to examine the prevalence of MBL-producing *P. aeruginosa* and to detect MBL genes *blaSPM-1* among imipenem-resistant isolates obtained from various hospitals of Isfahan.

3. Patients and Methods

3.1. Bacterial Strains

In this cross-sectional descriptive study, 180 isolated samples were collected from nine hospitals of Isfahan (Al-Zahra, Imam Musa Kazim, Seyedolshohada, Amin, Isa benne Maeyam, Feyz, Ayatollah Kashani and Khanevade clinic) between August 2012 and March 2013. The specimens included urine (n = 37, 38.54%), blood (n = 24, 25%), burn (n = 17, 17.7%), wound (n = 9, 9.37%), sputum (n = 4, 4.16%), peritoneum (n = 3, 3.12%) and eye (n = 2, 2.08%). Specimens were collected from patients admitted to different hospital wards including ICU, urology, respiratory and surgery. Replicated specimens from the same patients were excluded from the study. *Pseudomonas aeruginosa* isolates were identified using various phenotypic and bacteriological tests including Gram staining, colony morphologies, MacConkey's agar, triple sugar iron (TSI) test, oxidase reaction, and growth at 42°C. The existence of antibiotic resistant infection among the hospitalized patients was considered as inclusion criteria. Also, improved and treated infections were excluded.

3.2. Antibiotic Susceptibility Testing

Antibiotic susceptibility testing was performed as recommended by the Clinical and Laboratory Standards Institute (CLSI) rules, using disks containing ceftazidime (CAZ: 30 μg), cefotaxime (CTX: 30 μg), gentamicin (GM: 10 μg), cefepime (CP: 30 μg), amikacin (AN: 30 μg), imipenem (IMP:10 μg), ciprofloxacin (CIP:5 μg), meropenem (MEM:10 μg) and aztreonam (ATM: 30 μg) (MAST, Merseyside, U.K). *Pseudomonas aeruginosa* ATCC 27853 was used as a control for antibiotic resistance (7).

3.3. Minimum Inhibitory Concentration (MIC)

Minimum inhibitory concentration test was performed by the E-test method for imipenem resistant isolates on a Mueller-Hinton agar medium. Plates consisting of E-test-imipenem strips were incubated at 35°C for 16 to 20 hours (14). Non-susceptible *P. aeruginosa* strains with MIC $\geq$ 32 μg/mL were categorized as imipenem-resistant strains (15).

3.4. Phenotypic Detection of Metallo-Beta-Lactamases-Producing Isolates

Phenotypic detection of MBL production (EDTA-IMP method) was carried out for imipenem-resistant strains. Initially, 186.1 g of disodium EDTA was dissolved in 1000 mL of distilled H_2O until EDTA solution was reached. A 0.64 M EDTA solution adjusted to pH 8 was prepared. Next, 750 μg of EDTA solution was added to the imipenem disk, and dried in an incubator. The disks were placed on a plate containing Muller-Hinton agar and incubated at 35°C for 18 - 24 hours. Post incubation, if there was a 7 mm increase in inhibition zone diameter or more towards the IMP plus EDTA in comparison to IMP disk alone, the organism was considered MBL positive (16).

3.5. Molecular Analysis

For molecular diagnosis, the total DNA from MBL positive *P. aeruginosa* isolates was extracted by a boiling method, which caused the cell walls to lyse (17). A PCR assay was performed to detect the *blaSPM-1*gene. A strain

of *P. aeruginosa* that contained this gene was used as a positive control (12).

3.6. Polymerase Chain Reaction Tests Were Done Under the Following Conditions

The *blaSPM-1* gene was detected using PCR on the total DNA using SPM-1 primers (forward: 5'-*CCTACAATCTAACGGCGACC-3*' and reverse: 5'-*TCGCCGTGTCCAGGTATAAC-3*'). The size of the amplification product was 831 bp. Cycling parameters for the *blaSPM-1* gene were as follows: initially the samples were denatured at 95°C for five minutes, followed by 30 cycles of denaturation at 95°C for one minute, annealing at 40°C for one minute, with an extension at 68°C for one minute and a final extension at 68°C for five minutes. The PCR products were detected using a 2.0% agarose gel consisting of 1% ethidium bromide. The gel was electrophoresed. The positive control visualized under UV light was the MBL-producing *P. aeruginosa* clinical strain that includes the *blaSPM-1* gene (17).

3.7. Sample Size and Statistical Analysis

The sample size was calculated using the one sample proportion to ensure type one error = 0.05, P = 0.64, and desired precision (d) as 0.07. Convenience sampling method was used in this study to collect isolated samples from different hospitals, which were affiliated to Isfahan University of Medical Sciences. Among these hospitals, three of them were referral to ensure more representativeness of samples. Descriptive statistics were used in this study with the help of the SPSS software for Windows (version 16, SPSS Inc., Chicago, IL, USA).

3.8. Ethical Considerations

Patients were included in the study after completing the informed consent in the presence of hospital nurses. The study was also approved by the ethical committee of Isfahan University of Medical sciences.

4. Results

Among the 96 collected strains, the highest proportion of samples were isolated from urine (38.54%) and the lowest from the eye (2.08%) (Figure 1). The disk diffusion method showed that 34 isolates (35.41%) were resistant to imipenem and meropenem. Resistance to other antibiotics is shown in Figure 2. The MIC test illustrated that MIC in all of the imipenem-resistant *P. aeruginosa* (IRPA) strains was ≥ 32 μg/mL (Figure 3). As indicated by the phenotypic method, thirteen (38.23%) of the imipenem-resistant isolates produced the MBL enzyme; demonstrated by an increase of ≥ 7 mm in zone diameter of EDTA-IMP disk compared to the imipenem disk (Figure 4). The molecular assay method (PCR) did not detect the *blaSPM-1* gene in MBL positive isolates of this study (Figure 5).

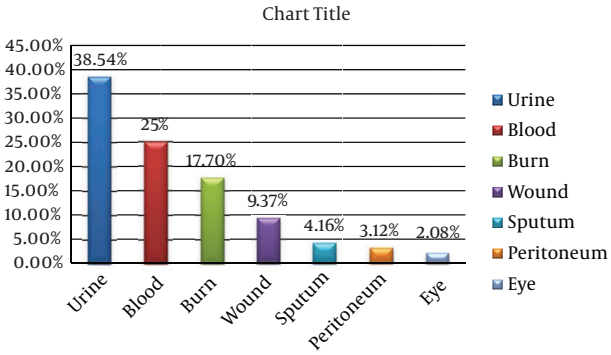


Figure 1. The Percentage of Isolation of *Pseudomonas aeruginosa* Isolated From Clinical Specimens

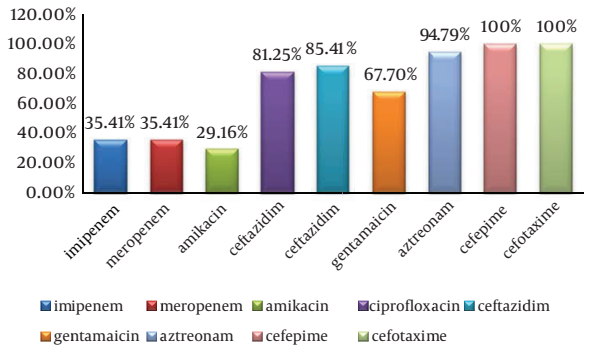


Figure 2. Resistance Pattern of *Pseudomonas aeruginosa* Isolates to Different Antimicrobial Agents

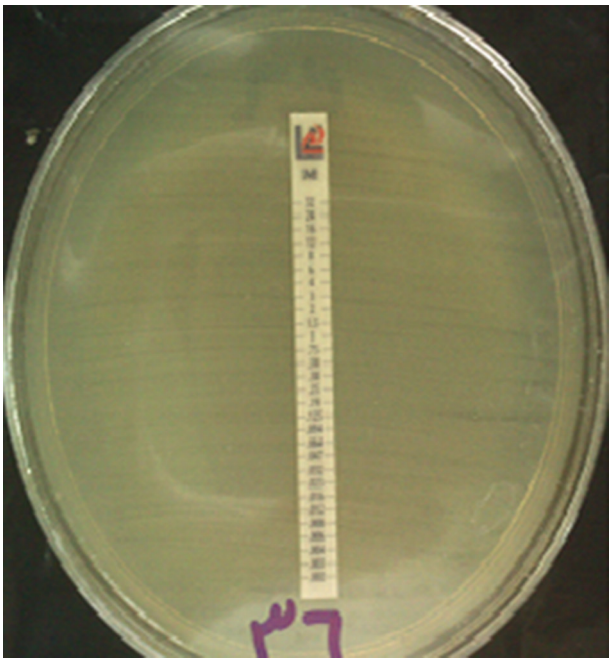


Figure 3. Measurement of the Minimum Inhibitory Concentration of Imipenem-Resistant Isolates by the E-test Method

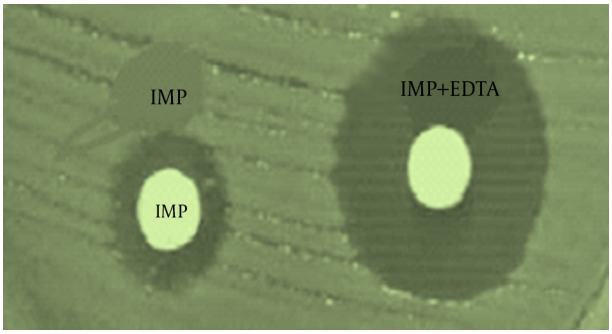
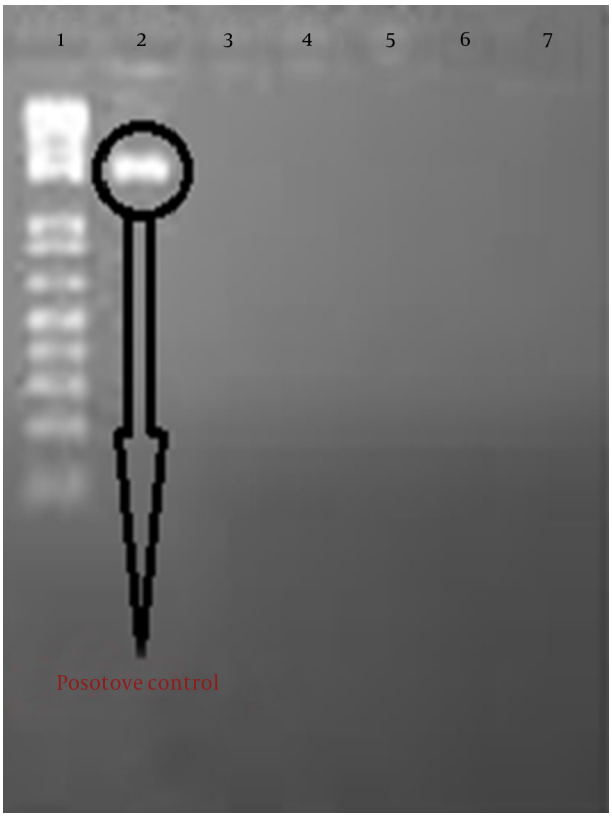


Figure 4. Phenotypic Detection of Metallo-β-Lactamase by Combined disks (EDTA + IMP) Among *Pseudomonas aeruginosa* Isolates

Figure 5. Polymerase Chain Reaction Assay for the Detection of *blaSPM-1* Gene (product size: 831 bp)



Lane 1, DNA ladder 100 bp; lane 2, positive control; lanes 4 - 7, amplified products; lane 7, negative control.

5. Discussion

Worldwide there is a rapid increase in MBLs among gram-negative pathogens, in particular *P. aeruginosa* (18, 19). There is great concern about *P. aeruginosa* as one of several opportunistic pathogens affecting inpatients with immune deficiency (19, 20). In addition, the presence of limited therapeutic strategies for imipenem-resistant *P. aeruginosa is alarming*. Metallo-β-lactamase enzymes may play a vital role in IRPA; however, this is dependent on the probability of spreading of carbapenemases amongst nosocomial isolates. Overtime, there has been an increase in the prevalence of MBLs; currently 40% of IRPA cases worldwide are associated with MBLs enzyme, with types varying in different regions (21).

In this study, 96 *P. aeruginosa* strains were obtained independently from hospitalized patients in different hospitals of Isfahan. An antibiotic susceptibility pattern was determined in these strains and imipenem-resistant strains were subjected to testing for MIC, EDTA-IMP, and molecular analysis. Metallo-beta-lactamases are a group of β-lactamase enzymes that need one or two zinc (Zn) atoms 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 (22). Several studies have reported significant results regarding the *blaSPM-1*gene. In a study conducted by Magalhaes et al. from Brazil (23), 48 *P. aeruginosa* samples were collected and among these isolates, 24 strains were resistant to imipenem. Among resistant strains, 15 isolates were MBL-producing and all isolates were *blaSPM-1* carriers (24).

In a study by Franco and colleagues (17), conducted on the blood isolates of *P. aeruginosa*, the prevalence of imipenem resistant strains was reported as 34%. Seventy-seven percent of strains were MBL-producing and amongst these isolates, 81% were positive for *blaSPM-1*. Sadeghi et al. (12) conducted a study on 108 isolates of *P. aeruginosa* in the central province of Iran, and reported that the *blaSPM-1* gene was not present in these strains. In a study by Yousefi et al. (25) in the northwest of Iran, from 104 isolates of *Pseudomonas aeruginosa*, 39 isolates were MBL-producing by the phenotypic test, yet none of them had the *blaSPM-1* gene by the PCR test. In a study by Shahcheraghi et al. (7) at Imam Khomeini Hospital of Tehran that tested 243 samples of *P. aeruginosa*, 28 samples were resistant to imipenem and 22 samples were MBL enzyme producers, yet none of these strains showed the *blaSPM-1* by PCR tests. However, in this study among 13 strains, of those producing MBLs, none of them had the *blaSPM-1* gene. Thus, while in Iran resistance to imipenem is high and positive MBL strains have increased, strains with the *blaSPM-1* gene have not yet appeared in this country. These results indicate that another MBL gene such as IMP, SPM, GIM, SIM or AIM is probably involved in resistant strains that were MBL positive by the phenotypic test (combined disk). A high prevalence of MBL among *P. aeruginosa* strains is a critical problem representing a practical therapeutic challenge. Although routine laboratory testing may result in early recognition of MBL, keen attention is essential for interpretation of phenotypic tests. In order to confirm results from phenotypic tests, PCR can be performed.

Various local factors associated with MBL screening include, type of bacteria, prevalence of MBL enzyme and the technical skills and capabilities available in facilities. Further genetic investigations for genes responsible for

carbapenems resistance are recommended and strict infection control procedures should be followed. All of our MBL-producing *P. aeruginosa* isolates exhibited cross-resistance to many anti-pseudomonal drugs including imipenem. Combination therapy can be useful to prevent resistance during therapy. Regarding 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 involved patients must be regularly done in hospitals.

One of the limitations of the present study was that, other MBL family genes were not assessed, which include five genes with activities similar to the *blaSPM-1* gene. The resistance to imipenem in *Pseudomonas aeruginosa* isolates could be attributed to the mentioned genes. Also, other antibiotic resistance mechanisms were not assessed due to the financial constraints of molecular and gene tests.

Acknowledgements

We would like to thank the members of Al-Zahra, Shariati, khanevade clinic, Ayatolla Kashani and Fayez Hospitals of Isfahan city for their technical assistance and help with the English language version of this paper.

Funding/Support

This study was funded by the Isfahan University of Medical Sciences.

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