

# Detection Rate of Metallo- $\beta$ -Lactamase-Expressing Genes; *blaVIM-1*, *blaVIM-2* and *blaSPM-1* in *Pseudomonas aeruginosa* Isolates

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## Abstract

**Introduction:** Imipenem-resistant *Pseudomonas aeruginosa* is an organism expressing metallo- $\beta$ -lactamase (MBL) enzyme, and is a serious agent of hospital infection holding a serious universal therapeutic challenge. Carbapenems are potent options for the treatment of *P. aeruginosa* infections. The rate of MBLs expression has been variable among imipenem-resistant *P. aeruginosa* isolates. In the present study, we investigated the presence of MBL in the clinical isolates of *P. aeruginosa*.

**Methods:** A total of 60 *P. aeruginosa* isolates were obtained from Kerman hospitals during 2014-2015. The antibiotics susceptibility was assessed using disk diffusion test. MBL positivity in *P. aeruginosa* was investigated using double disk synergy test (DDST) and polymerase chain reaction (PCR) with amplification of *blaVIM-2*, *blaVIM-1* and *blaSPM-1*.

**Results:** From 60 *P. aeruginosa* isolates, 28 (46.6%) were imipenem-resistant. Among these, 17 (60.7%) were identified as MBL-producing *P. aeruginosa* isolates using DDST. Results of PCR test demonstrated the existence of 8 (28.5%) *P. aeruginosa*, producing *blaSPM-1*.

**Conclusion:** The frequency of *blaSPM-1*-producing *P. aeruginosa* isolates from Kerman Hospitals was relatively high. Therefore, it is recommended that the distribution of MBL-mediated resistances be managed.

**Keywords:** *Pseudomonas aeruginosa*, Metallo- $\beta$ -lactamases, Antibiotic resistance, *blaSPM-1*.

## Introduction

*Pseudomonas aeruginosa* is broadly recognized as an opportunistic aggressor rather than a cause of early infection in healthy persons.<sup>1</sup> It constitutes around 10%-20% of hospital-acquired infections in intensive care units (ICUs), cystic fibrosis, respiratory and renal infections, and body surface infections.<sup>2</sup> Multidrug-resistant mechanisms of *P. aeruginosa* include expression of efflux pumps, production of biofilms, and secretion of resistance-inducing enzymes such as  $\beta$ -lactamases and aminoglycosidases.<sup>3</sup> This bacterium is a concerning issue considering antimicrobial chemotherapy.<sup>4</sup>

Enzyme production is a prominent  $\beta$ -lactam resistance mechanism in *P. aeruginosa*. Penicilloyl-serine transferases separate the amide bond of the  $\beta$ -lactamase ring

removing the antibacterial activity (5).  $\beta$ -lactamases have been recently classified in 2 ways: the molecular and the functional. Regarding the molecular classification, several categories of  $\beta$ -lactamases have been identified; these are known as class A, B, C and D. Enzymes belonging to the classes A, C and D use serine amino acid for their activity, while enzymes of class B are dependent on divalent cations for their activity.<sup>1</sup> On the basis of molecular features, metallo- $\beta$ -lactamases (MBLs) are comprised of the following 6 groups: IMP, VIM, SIM, SPM, GIM, and AIM.<sup>6</sup> Carbapenems resistances may result from decreased outer membrane permeability, exclusion from the cell by efflux pumps, changes of penicillin-binding protein, and production of  $\beta$ -lactamase.<sup>7</sup>

Carbapenems are currently used for the

treatment of *Pseudomonas* infections. Resistance genes such as MBLs can easily be transmitted by genomic compartments such as plasmids and class I integrons, and this may be a source of dissemination of antibiotic resistance throughout the clinics.<sup>8,9</sup> MBLs can intensely hydrolyze all beta-lactam antibiotics except aztreonam. These enzymes need zinc as cofactor.<sup>10</sup> Sulbactam, tazobactam, and clavulanic acid which are often used to inhibit beta-lactamase enzymes are not useful against MBLs.<sup>11,12</sup> Due to our limitation in providing efficient antibiotic alternatives in cases of resistant organisms, the presence of MBL in clinical isolates of *P. aeruginosa* were investigated. In addition, prolonged hospitalization and high fatality rates have been associated with MBL positive *P. aeruginosa* infections. The aim of this study was to investigate the antibiotic resistance profile and the prevalence of *bla*VIM-1, *bla*VIM-2 and *bla*SPM-1 genes encoding MBLs among clinical isolates of *P. aeruginosa* obtained from Afzalipour and Bahaonar hospitals of Kerman city, by double disk synergy test (DDST) and multiplex polymerase chain reaction (PCR).

### Methods

In the present cross-sectional study, 60 suspected isolates of *Pseudomonas* were collected out of 234 examined isolates obtained from hospitalized patients in Kerman, Iran, during 2014-2015. These bacteria had been recovered from several nosocomial samples such as urine, wound, blood, trachea and other clinical samples. The colonies were again cultured in MacConkey agar medium and pure colonies were recognized as *P. aeruginosa* according to Gram staining and bacteriological tests such as oxidase, catalase, oxidative-fermentative test, growth on media such as TSI, SIM, cefrimide agar (Merk, Germany) and growth at 42°C. Isolates were conserved in Trypticase soy broth media (TSB) including 20% glycerol and stored at -70°C until use.<sup>13</sup>

### Antibiotic Susceptibility Tests

Disk diffusion assay (Kirby-Bauer) on Muller-Hinton agar plates (Merck, Germany) was accomplished with the antibiotic susceptibility test according to the Clinical & Laboratory Standards Institute (CLSI).<sup>14</sup> The antimicrobial disks used (Mast Co, UK) are shown in Table 1. *Pseudomonas aeruginosa* ATCC27853 was applied as a control strain for the susceptibility testing. DDST was applied for phenotypic examination of MBLs. Therefore, 0.5 M EDTA solution was prepared using 186.1 g of disodium EDTA. H<sub>2</sub>O<sub>2</sub> was dissolved in 1000 mL distilled water (pH 8.0 which was adjusted by addition of NaOH). Then, 930 µg of this solution was applied on imipenem disk and incubated to be dried. These disks were then placed on a plate of Muller-Hinton agar with cultured *P. aeruginosa*. After an incubation period of 16–18 hours at 37°C, a positive result for MBL expression was considered as a diameter equal or greater than 7 mm of IMP-EDTA disk respective to the imipenem disk.<sup>12</sup>

**Table 1.** Results of Antimicrobial Susceptibility Testing of 60 *Pseudomonas aeruginosa* Clinical Isolates

Antibiotic (µg)	Resistant Strains No. (%)
Imipenem (10 µg)	28 (46.6)
Meropenem (10 µg)	26 (43.3)
Ceftazidime (30 µg)	41 (68.3)
Carbencillin (100 µg)	44 (73.3)
Tobramycin (10 µg)	47 (78.3)
Amikacin (30 µg)	32 (53.3)
Ticarcillin (75 µg)	35 (58.3)
Gentamicin (10 µg)	36 (60)
Cefotaxime (30 µg)	39 (65)
Ceftizoxime (30 µg)	45 (75)

### DNA Extraction and PCR Reaction

For DNA extraction, boiling assay was used. Colonies were extracted from cefrimide agar and were incubated at 37°C for 16 hours. The bacterial precipitate was dissolved in 500 µL distilled water, and the bacteria in the mixture were heated at 100°C for 10 minutes, and then centrifuged at 13 000×g (10 minutes, room temperature). The supernatant containing DNA was collected into a new micro tube.<sup>12</sup> PCR reaction for diagnosis of MBL genes was accomplished in Mastercycler Eppendorf (Eppendorf, Germany) and in final volume of 25 µL including PCR Buffer (10x, 2.5µL) (CinnaGen, Iran), MgCl<sub>2</sub> (50mM, 1 µL) (CinnaGen, Iran), dNTPs (10mM, 1 µL) (CinnaGen, Iran), forward and reverse primers (10 pmol/µL, 1 µL each), Taq DNA polymerase (50 U/µL, 1 µL) (CinnaGen, Iran), DNA (2 µL), and distilled water (15.5 µL). *P. aeruginosa* PO510 (Pasargad Research Laboratory) producing *bla*VIM-1 and *P. aeruginosa* 16 producing *bla*SPM-1 were applied as the positive controls, while *P. aeruginosa* ATCC 27853 served as the negative control. The amplicons were analyzed by 1% (w/v) agarose gel electrophoresis and stained with ethidium bromide and visualized on gel documentation (Bio Rad, USA) (Figure 1). Primer sequences were designed using the NCBI Primer BLAST database. Primer sequences used for detection of MBL genes and PCR temperature profile for amplification of MBL genes are shown in Tables 2 and 3, respectively. For the statistical analyses, SPSS software version 19.0 was applied.

### Results

The samples were taken from urine (n=24, 40%), blood (n=4, 6.6%), wound (n=9, 15%), tracheal aspirate (n=12, 20%), and other sources (n=11, 18.3%). Thirty-three (66.66%) out of 60 isolates were multidrug resistant (MDR). PCR analysis was performed for all the *P. aeruginosa* isolates. The isolates demonstrated major resistance to carbencillin (73.3%), tobramycin (78.3%), and ceftizoxime (75%) (Table 1). Of 28 imipenem-resistant strains, 17 (60.7%) were positive for MBL as displayed with DDST. *bla*VIM-1 and *bla*VIM-2 genes were not de-

**Table 2.** Specific Primers Used for Detection of *bla*SPM-1, *bla*VIM-1, and *bla*VIM-2 and Lengths of the PCR Products

Target Gene	Primer Sequences	Amplicon Size (bp)	Tm
<i>bla</i> SPM-1	F: 5 CCTACAATCTAACGGCGACC 3 R: 5 TCGCCGTGTCCAGGTATAAC 3	786	58.15 59.54
<i>bla</i> VIM-1	F: 5 TTATGGAGCAGCAACCGATGT 3 R: 5 CAAAAGTCCCGCTCCAACGA 3	920	60.07 60.88
<i>bla</i> VIM-2	F: 5 AAAGTTATGCCGCACTCACC 3 R: 5 TGCAACTTCATGTTATGCCG 3	865	58.83 57.08

**Table 3.** PCR Programs for Amplification of MBL Genes

Target Gene	First Denaturation	Denaturation	Annealing	Extension	Numebr of Cycles	Final Extension
<i>bla</i> SPM-1	95°C 5 min	95°C 1min	40°C 1min	68°C 1min	30	68°C 5min
<i>bla</i> VIM-1 and <i>bla</i> VIM-2	94°C 3 min	94°C 1min	55°C 1min	72°C 2min	35	72°C 7min

tected, while *bla*SPM gene was positive in 8 (28.5%) of the imipenem-resistant isolates. Table 1 displays the data for susceptibility testing of 60 *P. aeruginosa* clinical isolates.

### Discussion

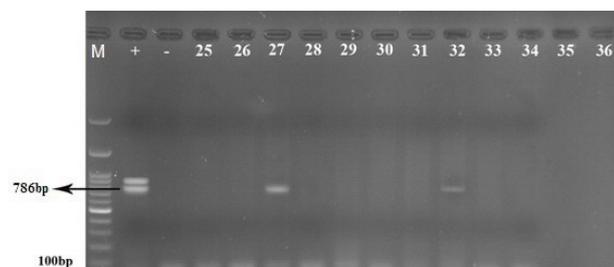
Carbapenems are effective  $\beta$ -lactam antibiotics for drastic cure of infections caused by Gram-negative bacteria.<sup>15</sup> Among  $\beta$ -lactamases, imipenem is the chosen antibiotic for fighting against this bacterium, however *P. aeruginosa* can disintegrate this antibiotic through MBL enzyme.<sup>16</sup> The rate of imipenem-resistant isolates (46.4%) in our study indicated a menace regarding therapy alternatives in our clinics. Imipenem and meropenem are commonly used antibiotics for the treatment of hospital-acquired infections; nevertheless, enhanced resistance against these agents has restricted their success.<sup>17</sup>

In our results, among 28 imipenem-resistant strains, 17 (60.7%) were positive for production of MBL as shown by DDST. Kalantar et al<sup>18</sup> exhibited that 22% of the imipenem-resistant *P. aeruginosa* isolates were MBL-positive and 8 strains (8%) were positive for *bla*VIM1 as proved by PCR. Sarhangi et al<sup>19</sup> assessed 240 *P. aeruginosa* clinical isolates; 19 (23.17%) of imipenem-resistant isolates were MBL positive as were investigated by DDST. Aghamiri et

al<sup>20</sup> detected that 70 (70%) of imipenem-resistant *P. aeruginosa* expressed MBLs. PCR methods revealed that 70 (33%) of these strains contained the *bla*VIM gene.<sup>20</sup> Based on DDST test, MBL was detected in 36 (87.8%) of the *P. aeruginosa* isolates as studied by Doosti et al. Among 41 imipenem-resistant isolates investigated by PCR, 23 (56%) isolates harbored *bla*VIM gene.<sup>21</sup> The variation seen between the results of our study and those of the previous reports may be due to the diversity in geographical areas, diversity in sorts of the diseases, the increased consumption of antibiotics, or diversity in antibiotic treatment abstinence.

Some MBL encoding genes have been mapped as mobile genes with possibility of transmission between organisms.<sup>16</sup> The most prevalent, introduced families are IMP, VIM, GIM, SPM, and SIM.<sup>1</sup> In our study, 60.7% of the total 60 *P. aeruginosa* isolates were MBL producer, which were more than the outbreak of MBL producers in Egyptian studies (27% and 32.3%) and Indian study (28.57%).<sup>1</sup> VIM enzymes were also most prevalent in Korea (88%) and Greece (85%).<sup>22</sup> Previous studies demonstrated that IMP and VIM producer genes of MBLs were also common in Asian studies.<sup>23-25</sup> VIM type was also highly prevalent in Turkey.<sup>26</sup> In previous studies in Egypt and Taiwan, VIM-2 demonstrated the highest outbreak among imipenem-resistant *P. aeruginosa* strains.<sup>1,27</sup> Since most of the integrones harbored VIM-1 gene code for aminoglycosides destructing enzymes, the matter is of clinical importance.<sup>28</sup>

In the present study, SPM was the commonest (28.5%) gene detected among imipenem-resistant *P. aeruginosa* isolates. The other MBL genes were not identified. MBL producer imipenem resistant isolates that were negative for the MBL genes may benefit from other resistant genes like IMP, SIM, NDM GIM or other resistance mechanisms to carbapenem. Identifying the MBL non-producer isolates with DDST that expressed the MBL genes may indicate low sensitivity of our phenotypic assay. The imi-

**Figure 1.** Gel Electrophoresis of PCR Products Following Amplification With Specific Primers for *bla*VIM-1, *bla*VIM-2, and *bla*SPM-1 Genes.

Lanes: M, 100 bp DNA ladder; +, positive control; -, negative control; 25-36, clinical isolates.

penem resistant isolates negative for MBL genes may also have other mechanisms associated with carbapenem resistance (such as AmpC  $\beta$ -lactamase expression, or using membrane efflux pathways).

SPM is a widespread MBL gene among the *P. aeruginosa* strains; nevertheless, other low frequency enzymes have also been detected.<sup>29-32</sup> SPM carbapenemase represents a major mechanism responsible for resistance to ceftazidime on ceftazidime-resistant *P. aeruginosa* isolates.<sup>30</sup> Zavascki et al identified 86 MBL producing strains, 16.27% of which expressed the *bla*SPM gene,<sup>33</sup> and Graf et al also discovered the *bla*SPM gene in 13.51% strains that were only susceptible to polymyxin B.<sup>34</sup> Our data are in accordance with these studies regarding high prevalence of SPM among *P. aeruginosa* isolates. The results of Moosavian et al study on 122 imipenem resistant *P. aeruginosa* isolates showed that only 2 isolates (1.6%) contained *bla*VIM-2 gene, whereas none of them were positive for *bla*SPM-1.<sup>13</sup> Sarhangi et al<sup>19</sup> discovered that 12.1% of imipenem-resistant *P. aeruginosa* isolates expressed *bla*VIM-2 gene, while no occurrence of *bla*SPM1 was found. In the study of Ghamgosha et al,<sup>35</sup> 77.7% of MBL producing *P. aeruginosa* had VIM-1 gene, with no SPM-1 gene detected. Concurrent resistance to imipenem and meropenem may propose carbapenem resistance mechanisms other than the enzymatic pathway (such as porin loss and/or upregulation of efflux pumps).<sup>30</sup> High resistance to carbapenems and ceftazidime has been conferred to MBL production.<sup>36</sup> Current studies corroborate this propensity.<sup>29,30,37,38</sup> Resistance to other antimicrobial agents has decreased the alternative treatments for managing multidrug-resistant *P. aeruginosa*.<sup>38,39</sup> Widespread strains of the drug-resistant *P. aeruginosa* strains express either IMP or VIM-type genes that encode MBL. These are significant general health issues menacing the success of antimicrobial chemotherapy.

The limitation of this study was low number of the isolated *P. aeruginosa* strains compared to some other studies, and the strength was the use of DDST and molecular detection of the isolates.

### Conclusion

We propose applying powerful superintendence and in-transient infection controlling strategies to control infectious diseases. A controlled antibiotic usage should be considered to prevent the distribution of antibiotic resistance genes among hospital pathogens.

### Ethical Approval

The informed consent was obtained from the patients before using their samples, and the data were kept confidential.

### Competing Interests

Authors declare that they have no competing interests.

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