Prevalence and Antibiotic Resistance Patterns of Metallo-Beta-Lactamase-Producing Pseudomonas aeruginosa Isolated from Patients in a Hospital in Zabol, Southeast of Iran

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ABSTRACT

Background: Treatment of infections caused by metallo-beta-lactamase (MBL)-producing *Pseudomonas aeruginosa* (*P. aeruginosa*) is a major healthcare-associated concern. Therefore, the purpose of this study was to ascertain antibiotic resistance patterns and prevalence of MBL genes in clinical isolates of *P. aeruginosa*.
Materials & Methods: In total, 90 non-repetitive clinical isolates of P. aeruginosa were collected from clinical specimens of patients who referred to Amir Al-Momenin hospital in Zabol, southeast of Iran, from January 2019 to November 2022. Antibiotic susceptibility patterns were determined according to CLSI guidelines. Combined disk test (CDT) was used to detect MBL-producing P. aeruginosa isolates. MBL genes (*blaIMP, blaVIM, blaNDM, and blaSPM*) were detected by PCR (polymerase chain reaction) method.
Findings: The isolates were mostly resistant to ceftriaxone (51.1%, 46 of 90) and gentamicin (43.3%, 39 of 90). Based on CDT results, 89.4% (17 of 19) of carbapenem-resistant isolates were MBL positive. In addition, MBL genes including *bla*VIM, *bla*IMP, and *bla*NDM were detected in 20% (18 of 90), 8.9% (8 of 90), and 5.6% (5 of 90) of the isolates, respectively.
Conclusion: Based on this study findings, the use of ceftriaxone and gentamicin should be restricted. In addition, MBL genes (*blaVIM and blaIMP*) seem to play a crucial role in the spread of carbapenem-resistant infections and the emergence of multidrug-resistant isolates, leading to antibiotic treatment failure.

Keywords: *P. aeruginosa,* Metallo-beta-lactamase genes, Carbapenem, Imipenem .

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Introduction

Pseudomonas aeruginosa (*P. aeruginosa*), a non-fermentative Gram-negative bacterium, is usually responsible for nosocomial- and community-acquired infections [1]. Wound infection, especially among burn patients, urinary tract infection (UTI), pneumonia, and blood stream infection (BSI) are the most prevalent infections caused by this bacterium [2]. Carbapenems are widely used to treat *P. aeruginosa* infections; however, due to different resistance mechanisms, including mutations in outer membrane porins and the production of Class A, B, and D extended-spectrum beta-lactamases (ESBLs), their effectiveness has been significantly reduced [3, 4]. Class B enzymes, also known as metallo-beta-lactamases (MBLs), are predominantly produced by *P. aeruginosa* and require zinc in their active site to catalyze the hydrolysis of *β*-lactam antibiotics. Different types of MBL genes including *blaVIM*, *blaIMP, blaNDM,* and *blaSPM* have been reported so far [5]. Owing to their capacity to hydrolyze and inactivate different types of beta-lactam antibiotics, MBL-producing isolates are of concern to physicians. In fact, due to its significant impact on patient outcomes, MBL-producing *P. aeruginosa* is a worldwide concern. These isolates are usually resistant to multiple antibiotics, especially carbapenems [5-7].

It has been reported that other resistance determinants such as aminoglycoside and fluoroquinolone resistance genes along with MBL genes are usually harbored by mobile genetic elements such as IncP-type plasmids and integrons, which enhance the ability of bacteria to disseminate resistance genes among multiple species [8, 9].

Objectives: The purpose of the present study was to ascertain antibiotic resistance patterns and prevalence of MBL genes among clinical isolates of *P. aeruginosa.*

Materials and Methods

Specimen collection: In this cross-sectional descriptive study, a total of 90 non repetitive clinical isolates of *P. aeruginosa* were obtained from patients who referred to Amir Al-Momenin hospital in Zabol province, southeast of Iran, from January 2019 to November 2022. Isolates were collected from urine, wound, sputum, blood, and stool specimens. In order to identify *P. aeruginosa* isolates, standard laboratory microbiology tests including growth on *Pseudomonas* agar medium (HiMedia, India) at 42 °C, pigment production, Gram staining, TSI (triple sugar iron agar; HiMedia, India) test, and oxidase and catalase test were used [10]. The collected isolates were stored at -20 °C using cryovials containing brain-heart infusion (BHI) broth (HiMedia, India) with 20% glycerol until used for antibiotic susceptibility testing, genome extraction, and PCR (polymerase chain reaction) assay.

Antibiotic susceptibility patterns: Antibiotic susceptibility patterns were determined based on CLSI (Clinical and Laboratory Standards Institute) guidelines [11]. Following antibiotics (Padtan Teb, Iran) were applied, ofloxacin (5 µg), imipenem (10 µg), ceftazidime (30 µg), ceftriaxone (30 µg), cefotaxime (30 µg), meropenem (10 µg), gentamicin (10 µg), streptomycin (10 µg), amikacin (30 µg), piperacillin (100 µg), tobramycin (10 µg), ciprofloxacin (5 µg), cefepime (30 µg), and levofloxacin (5 µg). Multidrug-resistant (MDR) isolates were resistant to at least one antibiotic in three different antibiotic groups [12]. *P. aeruginosa* ATCC 27853 and *Escherichia coli* 25922 were used as the quality controls.

Screening of MBL by combined disk test: Combined disk test (CDT) was used to detect MBL-producing *P. aeruginosa* isolates. In brief, two disks of imipenem and imipenem plus 0.5 M EDTA (ethylenediaminetetraacetic acid, Merck, Germany) were placed on

culture medium and incubated at 35 °C for 16-18 hrs, a difference equal to or more than 7 mm in inhibition zone diameter between imipenem-EDTA and imipenem disks was considered as a positive result [13]. In addition, known clinical MBL-positive isolates of *Klebsiella pneumoniae* and *P. aeruginosa* were used as the quality strains. **Genomic DNA extraction and detection of MBL genes by PCR**: Bacterial genomic DNA was extracted using boiling method [14]. For this purpose, three colonies of fresh culture of *P. aeruginosa* were completely dissolved in 250 µL of distilled sterile water. The microtube was heated at 98 °C for 9 min. Supernatant (after centrifugation at 12500 g for 18 min) was applied for PCR assay. Table 1 shows specific primers used to detect MBL genes (*blaIMP, blaVIM, blaNDM,* and *blaSPM*) [15]. Each PCR reaction (Eppendorf thermal cycler, Hamburg, Germany) was performed in a final volume of 20 µL of ready-to-use Ampliqon (Denmark) master mix containing 15 µL of ready-to-use master mix, 2 µL of DNA template, and 1.5 µL of forward and reverse primers (10 µM). PCR program used to amplify MBL genes was as follows: denaturation at 95 °C for 6 min, followed by 35 cycles of denaturation at 95 °C for 50 s, annealing (at 52, 53, 54, and 52 °C for *IMP, VIM, SPM,* and *NDM*, respectively)

for 50 s, and extension at 72 °C for 60 s as well as a final extension step at 72 °C for 10 min. PCR products were separated by agarose gel electrophoresis and visualized. **Statistical analysis:** Statistical analysis was carried out using SPSS software (Ver.18, Chicago). Chi-square test was applied to analyze data. A *p* value of < .05 was considered statistically significant.

Findings

In this study, 90 *P. aeruginosa* strains were obtained from clinical specimens, including urine (n=40, 44.4%), sputum (n=34, 37.8%), stool (n=7, 7.8%), blood (n=5, 5.6%), and wound (n=4, 4.4%). Out of 90 patients, 53 (58.9%) patients were female. The isolates were mostly resistant to ceftriaxone (51.1%, 46 of 90) and gentamicin (43.3%, 39 of 90) (Table 2). Out of 90 evaluated isolates, 31 (34.4%) cases were found to be MDR. Based on CDT results, 17 out of 19 (89.4%) carbapenem-resistant isolates were found to be MBL-producing strains. In addition, MBL genes including *blaVIM, blaIMP,* and *blaNDM* were detected in 20% (18 of 90), 8.9% (8 of 90), and 5.6% (5 of 90) of the isolates, respectively (Fig. 1, 2, and 3).

Table 1) PCR primer sets used to detect MBL genes in this study

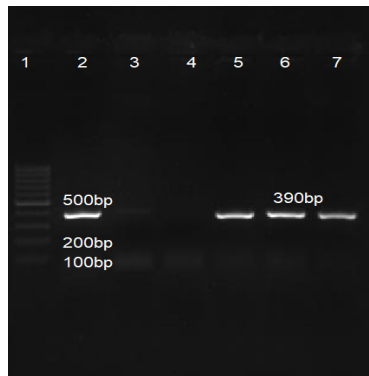| Genes	Sequence	Amplicon	Reference
*bla*NDM	F5- GGTTTGGCGATCTGGTTTTC-3	621	15
	R5- CGGAATGGCTCATCACGATC-3		
*bla*VIM	F5- GATGGTGTTTGGTCGCATA-3	390	
	R5- CGAATGCGCAGCACCAG-3		
blaIMP	F5- GGAATAGAGTGGCTTAAYTCTC-3	232	
	R5- GGTTTAAYAAAACAACCACC-3		
*bla*SPM	F5- AAAATCTGGGTACGCAAACG-3	271	
	R5- ACATTATCCGCTGGAACAGG-3		

Figure 1) *blaVIM* PCR products: Lane 1: 100 bp DNA ladder, lane 2: positive control, lane 3: negative control, lane 4: negative clinical sample, and lanes 5-7: positive clinical isolates
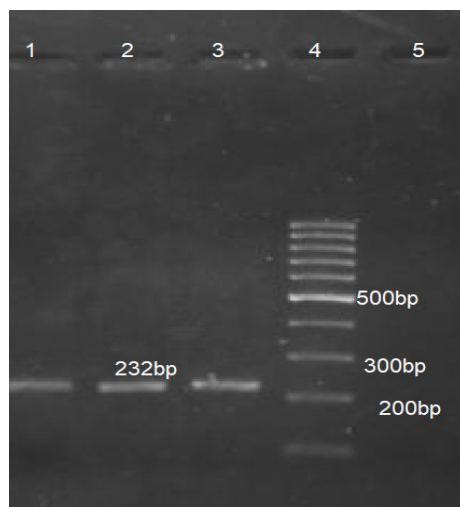

Figure 2) *blaIMP* PCR products: Lane 1: positive control, lanes 2 and 3: positive clinical isolates, lane 4: 100 bp DNA ladder, and lane 5: negative control
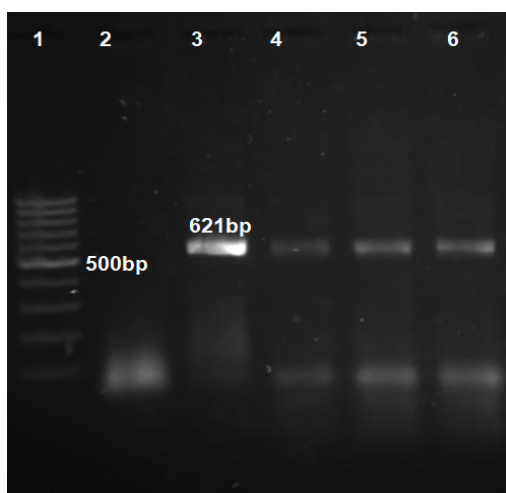

Figure 3) *blaNDM* PCR products: Lane 1: 100 bp DNA ladder, lane 2: negative control, lane 3: positive control, and lanes 4-6: positive clinical isolates

Other MBL genes (*blaSPM*) were not detected. The prevalence of resistance to other antibiotics among MBL-positive isolates was significantly higher than among MBL-negative isolates (Table 2).

Discussion

The high prevalence of antibiotic resistance in *P. aeruginosa* strains, especially in developing countries such as Iran, is one of the most important health care concerns [16]. In addition, in order to achieve appropriate antibiotic treatment and launch successful antibiotic stewardship programs in each region, awareness of antibiotic resistance patterns and antibiotic resistance mechanisms involved is necessary [17]. *P. aeruginosa* is a common etiological agent of bacterial infections and considered as a public health concern worldwide owing to the presence of multiple virulence genes and various antibiotic resistance determinants [18]. Therefore, in this study, antibiotic resistance profiles against different antibiotic classes, including aminoglycosides, beta-lactams, and fluoroquinolones, and the prevalence of MBL genes were evaluated in *P. aeruginosa* isolates collected from different clinical samples.

This study results revealed that the investigated isolates were mostly resistant to ceftriaxone (51.1%), gentamicin (43.3%), ofloxacin (40%), ciprofloxacin (34%), and ceftazidime (30%). These results are similar to those of other studies conducted in Iran [8]. For instance, in a comprehensive meta-analysis study carried out in Iran, the majority of investigated isolates in different provinces of Iran were reported to be resistant against ceftazidime (50.4%), gentamicin (46.9%), and ciprofloxacin (47%) [6].

Carbapenems (imipenem and meropenem) are considered as last-line antibiotics, usually prescribed to treat infections that are resistant against other antibiotics. In

Table 2) Antibiotic resistance patterns of MBL-positive and MBL-negative *P. aeruginosa* isolate.

Antibiotics	MBL-Positive (n=18)		MBL-Negative (n=72)		Total Resistance	*P* Value
	Resistance N (%)	Susceptible N (%)	Resistance N (%)	Susceptible N (%)	N (%)	
Ofloxacin	18 (100)	0 (5.6)	18 (25)	54 (75)	36 (40)	≤ .001
Imipenem	17 (94.4)	1 (5.6)	0 (0)	72 (100)	17 (18.9)	≤ .001
Ceftazidime	14 (77.8)	4 (22.2)	13 (18.1)	59 (77.8)	27 (30)	≤ .001
Ceftriaxone	18 (100)	0 (0)	28 (38.9)	44 (61.1)	46 (51.1)	≤ .001
Cefotaxime	18 (100)	0 (0)	13 (18.1)	59 (77.8)	31 (34.4)	≤ .001
Meropenem	18 (100)	0 (0)	1 (1.4)	71 (98.6)	19 (21.1)	≤ .001
Gentamicin	13 (72.2)	5 (27.8)	26 (36.1)	46 (63.9)	39 (43.3)	≤ .001
Streptomycin	12 (66.7)	6 (33.7)	18 (25)	54 (75)	30 (33.3)	≤ .001
Amikacin	13 (72.2)	5 (27.8)	5 (6.9)	67 (93.1)	18 (20)	≤ .001
Piperacillin	18 (100)	0 (0)	13 (18.1)	59 (81.9)	31 (34.4)	≤ .001
Tobramycin	13 (72.2)	5 (27.8)	14 (19.4)	58 (80.6)	27 (30)	≤ .001
Ciprofloxacin	18 (100)	0 (0)	13 (18.1)	59 (81.9)	31 (34.4)	≤ .001
Cefepime	13 (72.2)	5 (27.8)	15 (20.8)	57 (79.2)	28 (31.1)	≤ .001
Levofloxacin	18 (100)	0 (5.6)	9 (12.5)	63 (87.5)	27 (30)	≤ .001

this study, isolates were mostly susceptible to imipenem (81%) and meropenem (79%) (Table 2). These findings are in agreement with the results of other studies conducted in other provinces of Iran, such as Guilan and Zahedan, indicating 23.3 and 17.2% resistance against imipenem, respectively [19, 20]. However, these findings are significantly lower than those reported in Isfahan (96%) and Tehran (88%) [21, 22].

In a comprehensive antibiotic resistance surveillance in European countries, the prevalence of resistance to carbapenems in many countries such as Malta, Sweden, Norway, and Finland was reported to be less than 5%, while in other countries

such as Spain (18.6%) and Lithuania (21.8%), resistance to carbapenems was in agreement with the present study findings [23]. Also, resistance to ceftazidime and aminoglycosides was documented to vary from 0% (Iceland) to 46.7% (Romania) and 0% (Iceland) to 50.7% (Romania), respectively [23].

The prevalence of resistance to carbapenems in different Asian countries has been reported to be as follows: Japan 28.5%, Philippines 31.1%, Singapore 23.3%, Thailand 28.7%, and Korea 22%, which are similar to this study findings [13].

Different factors influence the frequency of antibiotic resistance. Some of the contributing factors are as follows; indiscriminate and arbitrary use of antibiotics in veterinary medicine, unlimited access to antibiotics, and non-adherence to the recommendations of antibiotic stewardship and the principles and guidelines of infection prevention and control programs [20- 26].

In the current study, MBL genes including *blaVIM, blaIMP,* and *blaNDM* were detected in 20% (18 of 90), 8.9% (8 of 90), and 5.6% (5 of 90) of the isolates, respectively. Likewise, the results of a meta-analysis study performed in Iran revealed that the most prevalent MBL genes in Iran were *blaVIM* (%19) and *blaIMP* (%11) [8]. Also, the highest prevalence rates of *blaVIM* and *blaIMP* have been reported in Mashhad (58%) and Isfahan (31.3%), respectively [24, 25]. Likewise, in China, Taiwan, Canada, German, Uganda, and the United Arab Emirates, *blaVIM* has been reported to be the most prevalent resistance gene [26].

In this study, one MBL-negative isolate was found to be carbapenem resistant. This finding may indicate the presence of other resistance mechanisms. For instance, enzyme-mediated (Ambler Class C and Class D beta-lactamases and KPC genes) and non-enzyme-mediated (porin loss or efflux pumps) resistance mechanisms are among other carbapenem resistance mechanisms that could confer resistance against imipenem and meropenem [27-32].

Limitations of the current study were as follows: the minimum inhibitory concentration of carbapenems was not determined; also, other carbapenem resistance genes and mechanisms, including OXA-like genes, KPC genes, and efflux pump overexpression, as well as other MBL genes (*SIM, GIM, AIM, FIM,* and *KHM*) were not evaluated.

Conclusion
This study findings are alarming since they demonstrate the high level of resistance to different antibiotics, especially ceftriaxone and gentamicin. Therefore, restricted prescription of ceftriaxone and gentamicin is recommended. In addition, MBL genes, especially *blaVIM* and *blaIMP,* seem to play a significant role in the spread of resistance to carbapenems and the emergence of MDR isolates, leading to antibiotic treatment failure.

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