Phenotypic and Molecular Detection of *IMP* and *SPM* Metallo-Beta-Lactamases in Clinical Isolates of Carbapenem Resistant *Pseudomonas aeruginosa*

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Research note

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Abstract

Objectives: Metallo-beta-lactamases play a major role in the resistance of *Pseudomonas aeruginosa* to carbapenems. The aim of this study was the phenotypic and molecular detection of *IMP* and *SPM* carbapenemase genes in 100 carbapenem-resistant clinical isolates of *P. aeruginosa*. The isolates identified using standard microbiological tests, and their antibiotic susceptibility pattern determined by disk agar diffusion (Kirby Bauer) method. Phenotypic identification of Metallo-beta-lactamase-producing strains assessed by the combined disk test (CDT). Then, PCR was used to detect the presence of *IMP* and *SPM* genes.

Results: The highest and lowest levels of antibiotic resistance were observed against gentamicin (40%) and piperacillin-tazobactam (13%), respectively. Besides, 40 isolates (40%) had the Multi-drug Resistant (MDR) phenotype, while 5 (12.5%) MDR isolates were resistant to all antibiotics tested. The results of the CDT showed that among 43 carbapenem non-susceptible clinical isolates of *P. aeruginosa*, 33 (76.74%) isolates were Metallo-beta-lactamase-producing strains. Also, the frequency of the *IMP* gene was determined to be 9%, while none of these isolates carried the *SPM* gene. Due to the high prevalence of carbapenem-resistant and MDR *P. aeruginosa* in this study, routine antibiotic susceptibility testing and phenotypic identification of carbapenemase production by this bacterium are necessary for proper selection of antibiotics.

Introduction

Pseudomonas aeruginosa is an important opportunistic gram-negative pathogen causing almost 10% of nosocomial infections worldwide such as urinary tract infections, bacteremia, sepsis, and pneumonia in immunocompromised patients, especially in intensive care units (ICUs), as well as cystic fibrosis and burned patients (1–3). Today, one of the most important complications related to this bacterium in developing countries is the emergence of multidrug resistant strains (4). Carbapenems are the last line treatment option for the infections caused by this bacterium, but recently resistance to carbapenems has been increased resulting to high mortality, especially in immunocompromised patients (5). Decreased expression of outer membrane proteins, increased expression of efflux systems, and secretion of beta-lactamases are the most important mechanisms of antibiotic resistance in clinical isolates of *P. aeruginosa* (6). One of the most important mechanisms of carbapenem resistance in *P. aeruginosa* is the production of Metallo-beta-lactamases (MBLs). These enzymes belonged to the class B beta-lactamases requiring the zinc for activity and can hydrolyze a wide range of beta-lactamases (6, 7). MBL-producing *P. aeruginosa* first reported in 1991 in Japan (8). The MBLs can hydrolyze penicillins and cephalosporins, and their encoding genes are often propagated horizontally among Gram-negative bacteria due to the presence of mobile genetic elements such as transposons and integrons (6, 9). The *IMP* (Imipenemase) and *SPM* (Sao Paulo Metallo-beta-lactamase) genes are the most clinically common MBLs (8). The *SPM* gene first identified in *P. aeruginosa* isolated from a blood culture sample of a 4-year-old girl with leukemia in Brazil (10), and the *IMP* gene first identified in Japan (6). Prompt diagnosis and accurate reporting of the presence of these genes in hospitals can lead to better and more effective control of carbapenem-resistant strains and eradication of nosocomial infections. Therefore, due to the importance of the presence of Metallo-beta-lactamases in *P. aeruginosa*, this study aimed to phenotypic identify MBLs and molecular assessment of the presence of *IMP* and *SPM* genes in the clinical isolates of carbapenem-resistant *P. aeruginosa* in teaching and treatment hospitals of Mazandaran province, north of Iran.

Materials And Methods

Patients and bacterial isolates

In this descriptive-analytical study, 100 non-repetitive *Pseudomonas aeruginosa* isolated from different clinical samples collected from hospitalized and outpatients from May 2018 to June 2019. The clinical isolates identified using standard microbiological methods and biochemical tests (3) and confirmed by the API (Analytical Profile Index) kit (France, BioMérieux, Lyon).

Antibiotic susceptibility testing

The antibiotic susceptibility pattern of the isolates was determined by the disk agar diffusion method (Kirby-Bauer) according to the instructions of the Clinical and Laboratory Standards Institute (CLSI) (11). In this test, we used the *Pseudomonas aeruginosa* ATCC 27853 as the control strain. The antibiotics studied included imipenem (10 µg), meropenem (10 µg), doripenem (10 µg), ceftazidime (30 µg), Aztreonam (30 µg), piperacillin-tazobactam (100 – 10 µg), ciprofloxacin (5 µg), and gentamicin (10 µg) (MAST Diagnostic Co., UK). Also, the agar dilution test used to investigate the susceptibility pattern of the clinical isolates against colistin according to the instructions of the Clinical and Laboratory Standards Institute (CLSI) (11). *Pseudomonas aeruginosa* ATCC 27853 chosen as the control strain in this test.

Phenotypic identification of Metallo-beta-lactamase producing strains

Combined Disk Test (CDT), using imipenem (10 µg) alone and imipenem-EDTA (10–750 µg), was used to phenotypic detection of the presence of MBLs in clinical isolates of *P. aeruginosa*. An increase in the diameter of the growth inhibition zone greater than or equal to 7 mm around the imipenem-EDTA combined disk compared to the imipenem disk alone indicates the production of MBLs in this test (12).

Extraction of the bacterial genome and amplification of the *IMP* and *SPM* genes by PCR

The genomic DNAs of *P. aeruginosa* isolates extracted by DNA extraction kit (Bioneer, South Korea). Amplification of the *IMP* and *SPM* genes using specific primers as *IMP*-F:GAAGGCGTTTATGTTTCATAC, *IMP*-R:GTATGTTTCAAGAGTGATGC (13), and *SPM*-F:AAAATCTGGGTACGCAAACG, *SPM*-R:ACATTATCCGCTGGAACAGG (14) done by thermocycler (Bio-Rad, USA). The condition of the PCR tests was as follows: initial denaturation at 94 °C for 2 minutes, and 30 cycles including denaturation at 94 °C for 30 seconds, annealing of the primers at 57 °C for 30 seconds, and extension of the DNA fragments at 72 °C for 45 seconds, and a final extension of the fragments at 72 °C for 10 minutes.

Results

Patients and bacterial isolates

Among 100 *Pseudomonas aeruginosa* clinical isolates in this study, 61 of them collected from men. The age of the patients varied from a few days old to 91 years old, and the mean age range of the patients was 48.65 ± 13.13 . Also, the ICU with 40 cases had the highest frequency of the isolates (Table 1), while 29, 26, 20, and 11 isolates collected from urine, sputum, wound, and other clinical samples, respectively. Besides, 14 isolates were related to outpatients, while the highest positive culture (71.4%) among outpatients belonged to the urine samples (Table 1).

Table 1

Frequency distribution of collected *Pseudomonas aeruginosa* isolates in terms of hospital ward and type of clinical sample

Hospital wards	No. (%) of positive culture for <i>Pseudomonas aeruginosa</i>								
	Stool	Plantar secretion	Eye secretion	Trachea	Catheter	Blood	Sputum	Urine	Wound
ICU (n = 40)	1 (2.5)	-	-	1 (2.5)	3 (7.5)	-	22 (55)	9 (22.5)	4 (10)
PICU (n = 2)	-	-	-	1 (50)	-	-	-	-	1 (50)
NICU (n = 1)	-	-	-	-	-	1 (100)	-	-	-
BICU (n = 3)	-	-	-	-	-	1 (33.3)	-	-	2 (66.6)
CCU (n = 6)	-	-	-	-	-	-	-	2 (33.3)	4 (66.6)
Internal medicine (n = 4)	-	-	-	-	1 (25)	1 (25)	-	2 (50)	-
Pediatrics (n = 6)	-	-	2 (33.33)	1 (16.6)	-	-	-	3 (50)	-
Women (n = 2)	-	-	-	1 (50)	-	-	1 (50)	-	-
Men (n = 4)	-	1 (25)	-	-	1 (25)	2 (50)	-	-	-
Surgery (n = 7)	-	-	-	-	4 (57.1)	-	-	1 (14.2)	2 (28.5)
Neurology (n = 4)	-	-	-	-	-	-	2 (50)	2 (50)	-
Burn (n = 6)	-	-	-	-	-	1 (16.6)	-	-	5 (83.3)
Oncology (n = 1)	-	-	-	-	-	-	1 (100)	-	-

Abbreviations: ICU; Intensive Care Unit, CCU; Cardiac care unit, NICU; Neonatal Intensive Care Unit (NICU), PICU; Pediatric Intensive Care Unit, BICU; Burnt Intensive Care Unit

Hospital wards	No. (%) of positive culture for <i>Pseudomonas aeruginosa</i>								
	Stool	Plantar secretion	Eye secretion	Trachea	Catheter	Blood	Sputum	Urine	Wound
Outpatient (n = 14)	1 (7.1)	-	-	-	-	1 (7.1)	-	10 (71.4)	2 (14.2)
Total	2	1	2	4	9	7	26	29	20
Abbreviations: ICU; Intensive Care Unit, CCU; Cardiac care unit, NICU; Neonatal Intensive Care Unit (NICU), PICU; Pediatric Intensive Care Unit, BICU; Burnt Intensive Care Unit									

Antimicrobial susceptibility testing

The highest resistance rate (40%) in the present study related to gentamicin and the lowest resistance (13%) observed against piperacillin-tazobactam (Table 2). Also, according to the results of the agar dilution test, 26 (26%) isolates were resistant to colistin, of which 22 (84.6%), 1 (3.8), 1 (3.8), 1 (3.8), and 1 (3.8) isolates had a MIC = 4, 8, 16, 32, and 256 µg/ml, respectively. However, 40 isolates (40%) shown the Multi-drug Resistance (MDR) phenotype, from which 26 (65%) isolates collected from male patients. Also, 5 (12.5%) MDR isolates were resistant to all antibiotics tested in this study. Regarding the type of clinical samples, the highest prevalence of MDR strains (27.5%) observed in bacteria isolated from sputum. In addition, the highest rate of antibiotic resistance among MDR isolates observed against gentamicin, while 38 (95%) isolates were resistant to this antibiotic. On the other hand, the highest antibiotic resistance observed in wound and catheter isolates, while *P. aeruginosa* isolated from feces and plantar secretions were resistant to all tested antibiotics. Also, only resistance to carbapenems was observed among the tracheal isolates of *P. aeruginosa* (Table 5).

Table 2
Antibiotic susceptibility pattern of 100 *Pseudomonas aeruginosa* clinical isolates

Antibiotics	Resistant %	Intermediate Resistant %	Susceptible %
Imipenem	28	8	64
Meropenem	34	7	59
Doripenem	28	13	59
Piperacillin-tazobactam	13	10	77
Cefotaxime	24	2	74
Aztreonam	39	23	38
Gentamicin	40	2	58
Ciprofloxacin	38	4	58

Table 3
Antibiotic resistance pattern of *Pseudomonas aeruginosa* clinical isolates based on sample type

Antibiotics	No. (%) of antibiotic resistant <i>Pseudomonas aeruginosa</i> isolated from								
	Stool (n = 2)	Plantar secretion (n = 1)	Eye secretion (n = 1)	Trachea (n = 4)	Catheter (n = 9)	Blood (n = 7)	Sputum (n = 26)	Urine (n = 29)	Wound (n = 20)
Imipenem	-	-	1 (100)	3 (75)	4 (44.4)	2 (28.5)	6 (23.07)	4 (13.7)	8 (40)
Meropenem	-	-	1 (100)	2 (50)	6 (66.6)	2 (28.5)	10 (38.4)	3 (10.3)	10 (50)
Doripenem	-	-	1 (100)	2 (50)	5 (55.5)	2 (28.5)	5 (19.2)	4 (13.7)	9 (45)
Piperacillin-tazobactam	-	-		-	3 (33.3)	1 (14.2)	3 (11.5)	3 (10.3)	3 (15)
Cefotaxime	-	-	1 (100)	-	4 (44.4)	-	7 (26.9)	7 (24.1)	5 (25)
Aztreonam	-	-	1 (100)	-	4 (44.4)	3 (42.8)	10 (38.4)	9 (31.03)	12 (60)
Gentamicin	-	-	1 (100)	-	6 (66.6)	3 (42.8)	10 (38.4)	10 (34.4)	10 (50)
Ciprofloxacin	-	-	1 (100)	-	5 (55.5)	3 (42.8)	8 (30.7)	9 (31.03)	12 (60)

Combined Disk Test and PCR

Using the phenotypic method (CDT), among 43 non-susceptible *P. aeruginosa* isolates (resistant or intermediate resistant) to at least one of the carbapenems (imipenem, meropenem or doripenem), 33 (76.74%) isolates detected as MBL-producer. Also, regarding sample types, 1/2 (50%), 2/4 (50%), 5/9 (55.5%), 2/7 (28.5%), 9/26 (34.6%), 3/29 (10.3%), and 11/20 (55%) of the *P. aeruginosa* isolated from eye secretion, trachea, catheters, blood, sputum, urine, and wounds were CDT positive, respectively. Moreover, out of 14 outpatients, 2 (14.2%) isolates showed a CDT positive result. Based on the hospital wards, 14 (35%), 1 (100%), and 2 (66.6%) isolates collected from ICU, NICU, and BICU had a positive combined disk test. However, none of the PICU, CCU, and oncology isolates were CDT positive, while 2/4 (50%), 2/6 (33.3%), 1/2 (50%), 3/7 (42.8%), 2/4 (50%), 1/4 (25%), and 3/6 (50%) of the *P. aeruginosa* isolated from Internal Medicine, Pediatrics, Women, Surgery, Neurology, Men, and Burn wards detected as CDT positive isolates, respectively.

On the other hand, the results of the PCR test showed that among 43 carbapenem non-susceptible isolates, only 3 (6.97%) isolates contained the *IMP* gene (Figure S1), while none of the isolates carried *SPM* gene (Figure S2).

All three isolates carrying the *IMP* gene were resistant to meropenem and doripenem, while 2 (66.6%) isolates carrying the gene were resistant towards imipenem. On the other hand, 1 (33.3%) *IMP* positive isolate detected as resistant against aztreonam as well as piperacillin/tazobactam. Interestingly, all three isolates carrying this gene showed resistance to ceftazidime and gentamicin, while resistance against ciprofloxacin was observed in 2 (66.6%) *IMP* containing isolates.

Discussion

The production of carbapenem hydrolyzing enzymes as well as producing a polysaccharide matrix in respiratory infections is one of the most significant mechanisms for resistance to carbapenems in *P. aeruginosa* strains (14, 15). However, not only the sputum isolates of this study were resistant to all antibiotics tested, but also 11 (42.3%) of them were detected as MDR. On the other hand, our clinical isolates had a relatively low resistance rate (< 40%) against all antibiotics tested, except gentamicin, as well as other Iranian studies (3, 16). However, the studies conducted by Radan et al. in 2016, and Mirsalehian et al. in 2017, reported the higher antibiotic resistance rate (5, 13), may be due to the type of samples, as all isolates collected from burn sections. Since patients with burns may be exposed to broad-spectrum antibiotics pressure, high antibiotic resistance rate observes in these wards (13). In another study conducted in Hamedan, as in our study, the lowest antibiotic resistance of the *P. aeruginosa* clinical isolates was reported against piperacillin-tazobactam (17). It seems that this antibiotic is still one of the effective drugs against this bacterium in Iran.

In the present study, the resistance rate against imipenem (28%) was similar to other studies in Iran (6, 18). On the other hand, the resistance towards imipenem and doripenem in the present study was less than meropenem. According to our assessment, the use of meropenem for the treatment of infections caused by this bacterium in our investigated hospitals was more than imipenem, while the doripenem is not widely used in the treatment of these infections. In another Iranian study conducted in 2017, the resistance to imipenem (8.4%) and meropenem (9.5%) was higher than that of penicillin (7.4%) (3). Also, in some studies, colistin has reported as an effective drug against multidrug-resistant as well as carbapenem-resistant *P. aeruginosa* (13), while 26% of our isolates detected as colistin-resistant. However, 8 (20%) MDR isolates in our study were resistant to colistin. The rate of colistin-resistance in clinical isolates of *P. aeruginosa* in other studies conducted in Iran and Iraq have reported as 11% and 18.2%, respectively (17, 19).

In the present study, out of 43 carbapenem-resistant isolates, 33 (76.74%) isolates were CDT positive consistent with some studies conducted in Iran and other countries (12, 20). According to the recent studies, we are witnessing an increasing prevalence of MBLs in clinical isolates of *P. aeruginosa*. The rate of MBLs in Kerman was negative in 2008 (21), while in 2015, it was 48% in Isfahan (7), however, in another study conducted in India, 37.5% of the *P. aeruginosa* clinical isolates have reported as MBL-producer (22). IMP is one of the most important Metallo-beta-lactamases causing resistance to beta-lactams and carbapenems (23), whereas in this study, all three isolates carrying the *IMP* gene were resistant to the carbapenems. In an Iranian study conducted in 2019, 4.7% of the isolates contained the *IMP* gene (6), while in another study conducted in India, out of all CDT positive isolates, 3% of them were carrying the *IMP* gene (20). Studies in Asian, African, and North American countries such as South Korea, India, Egypt, and Canada (9, 20, 24, 25) have reported an *IMP* gene prevalence of between 2 and 8%, indicating that the prevalence of this gene is low as well as our results.

On the other hand, some studies in Iran and other countries reported no *SPM* positive *P. aeruginosa* (7, 8, 26), however, Azimi et al. in 2018 reported a prevalence of 5.6% and 15.6% of the *SPM* and *IMP* gene, respectively, while all of their isolates collected from children admitted to the burn ward (27). The absence of the *SPM* gene and the low prevalence of the *IMP* gene in this study indicate that other mechanisms such as increased expression of efflux pumps, decreased expression of outer membrane proteins, and production of other carbapenemases may have involved in the development of carbapenem-resistant strains in this region. Considering the importance of carbapenem-resistant, colistin-resistant, and MDR isolates of *Pseudomonas aeruginosa*, routine antibiotic susceptibility testing and using more modern phenotypic tests such as Modified Hodge test and CarbaNP test are essential for the initial identification of carbapenemase-producing *P. aeruginosa*.

Limitations

The present study had some limitations. Lack of information about other carbapenemase encoding genes belonging to MBL group and genetic relationship between the resistant strains are not determined.

Abbreviations

IMP: Imipenemase; SPM: Sao Paulo Metallo-betalactamase; CDT: Combined Disk Test; MDR: Multi-drug Resistant; ICU: Intensive Care Unit; MBL: Metallo-beta-lactamase; TSI: Triple Sugar Iron; OF: Oxidation/Fermentation; MR: Methyl Red; VP: Voges Proskauer; API: Analytical Profile Index; CLSI: Clinical and Laboratory Standards Institute; ATCC: American Type Culture Collection; MIC: Minimum Inhibitory Concentration; EDTA: Ethylenediamine Tetraacetic Acid; PCR: Polymerase Chain Reaction; DNA: Deoxy Ribonucleic Acid; CCU; Cardiac care unit, NICU; Neonatal Intensive Care Unit (NICU), PICU; Pediatric Intensive Care Unit, BICU; Burnt Intensive Care Unit

Declarations

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Ethical approval and Consent to Participate

This study directed in accordance with the Declaration of Helsinki, however printed informed consent form was provided by the patients or a close relative before hospitalization, and classifying information of each sample was kept secret. Also, this research approved by the ethics committee of Mazandaran University of Medical Sciences, Sari, Iran, with ethical code IR.MAZUMS.REC.1331.3065.

Consent to Publication

Not applicable.

Competing interests

The authors declared no conflict of interest.

Availability of data and materials

Data generated or analyzed during this study are included in this published article.

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Authors' Contribution:

HRG and MA: Study concept and design; **ZNB, RG and HRG:** Acquisition of data; **ZNB, MA and MBHS:** Analysis and interpretation of data; **ZNB:** Drafting of manuscript; **MA and HRG:** Review of the article.

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