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Evaluation of the Relative Frequency of Carbapenemase Genes by Phenotypic and Genotypic Methods in *Pseudomonas aeruginosa* Isolates from Patients with Open Heart Surgery in Iran

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ABSTRACT

Backgrounds: Carbapenem resistance among *Pseudomonas aeruginosa* strains is alarming. This study aimed to investigate the relative frequency of carbapenem-resistant *P. aeruginosa* strains by phenotypic and genotypic methods.

Materials & Methods: The antibiotic susceptibility pattern of 60 *P. aeruginosa* isolates was determined by disk diffusion method (Kirby-Bauer). BD Phoenix automated microbiology system was used to identify carbapenem-resistant isolates, and the minimum inhibitory concentration (MIC) was determined using E-Test. In addition, mCIM (modified carbapenem inactivation method) phenotypic test was performed to evaluate carbapenem resistance genes in *P. aeruginosa* isolates. The prevalence of metallo-beta-lactamase (MβL) genes in carbapenem-resistant *P. aeruginosa* isolates was determined using conventional polymerase chain reaction (PCR).

Findings: The frequency of carbapenem-resistant *P. aeruginosa* isolates was 36% (22 of 60). The highest resistance was observed to imipenem and meropenem (36.6%), and the highest sensitivity was observed to amikacin (75%). All carbapenem-resistant *P. aeruginosa* isolates were confirmed by the BD Phoenix automated system (MIC>8 µg/mL for imipenem and meropenem), E-test (MIC <32 µg/mL), and mCIM assay (the growth inhibition zone diameter was 6-8 mm). In carbapenem-resistant *P. aeruginosa* isolates, the frequency of bla_{VIM}, bla_{IMP}, and bla_{SPM} genes was 9.1% (2 of 22), 4.5% (1 of 22), and 4.5% (1 of 22), respectively. Bla_{KPC} and bla_{NDM} genes were not found in any of the isolates.

Conclusion: Based on the present study results, all phenotypic tests used to identify carbapenemase-producing isolates had the same sensitivity (100%) and specificity (100%).

Keywords: Carbapenemases, *Pseudomonas aeruginosa*, Phenotypic, Bacterial sensitivity tests.

CITATION LINKS

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Introduction

Pseudomonas aeruginosa (P. aeruginosa) is a Gram-negative opportunistic pathogen. This bacterium is the third most common Gram-negative bacterial cause of nosocomial infections and the second leading cause of burn wound infection [1]. In the United States, *P. aeruginosa* causes approxx imately 51,000 healthcare-associated infections (HAI) annually [2]. Infections caused by *P. aeruginosa* are associated with substantial morbidity and mortality rates; *P. aeruginosa* accounts for 17% of ventilator-associated pneumonia (VAP) cases, 9% of nosocomial pneumonia cases, 10% of catheter-dependent urinary tract infections, and 6% of surgical infections [3]. The bacterium annually causes six million hospitalizations and more than four million deaths [4]. A recent study showed that patients with *Pseudomonas* bloodstream infection had a higher mortality rate than patients with the same infection caused by non–lactose fermenting bacilli such as *Enterobacteriaceae* [2].

Treatment of *P. aeruginosa* infections is often challenging due to its high resistance to many common antibiotics. Approximately 13% of *P. aeruginosa* strains causing HAI are multidrug resistant (MDR) [5]. This problem is more important in Iran due to the high use of antibiotics, especially carbapenems. Resistance to imipenem, as the second-line treatment for infections caused by MDR *P. aeruginosa* strains, appears to be increasing. Carbapenems are important antimicrobial drugs for the clinical management of serious *P. aeruginosa* infections.

Carbapenem-resistant *P. aeruginosa* (CRPA) has been reported to be as a concern; in 2014, 19.1% of *P. aeruginosa* isolates associated with selected HAIs and reported to the NHSN (National Healthcare Safety Network) were not susceptible to carbapenems [6]. In recent years, some studies have reported an increase in the frequency of CRPA [7]. CRPA is mainly mediated through chromosomal mutations that modify porins, efflux pump activity, and production of metallo- β-lactamases (MBLs) (VIM, IMP, NDM, SPM, GIM, AIM, DIM, KHM, SIM, TMB, SMB, and FIM). However, carbapenemase genes, which are usually encoded by mobile genetic elements, have the potential for quick spread [8, 9]. In Iran and other countries, the prevalence of CRPA is increasing; in burn centers, the prevalence of CRPA increased from 41 to 57.4% from 2008 to 2016 [10], and in 14 European countries, the frequency of CRPA increased from 12.3% in 2010 to 30.6% in 2011 [9].

In the present study, BD Phoenix automated identification and susceptibility testing system was used to identify carbapenem-resistant isolates; also, the only CLSI (Clinical and Laboratory Standards Institute)-approved phenotypic method for the identification of carbapenemases, namely mCIM (modified carbapenemase inactivated method) test, with 97% sensitivity and 100% specificity for the detection of SIM, IMI, IMP, VIM, NDM, KPC, and OXA was used to identify *P. aeruginosa* carbapenemases [11, 12].

Rapid detection and accurate reporting of the presence of carbapenemases in hospitals could help more efficiently control carbapenem-resistant strains and eradicate hospital infections [13, 14].

Objectives: Considering the importance of the presence of metallo-beta-lactamases in *P. aeruginosa* strains, this study was conducted with the aim of phenotypic and genotypic evaluation of MBLs in carbapenem-resistant *P. aeruginosa* clinical isolates isolated from hospitalized patients undergoing open-heart surgery in Shahid Rajaei Heart Hospital in Tehran, Iran during 2020-2021.

Materials and Methods

Bacterial isolates: In the present study, clinical samples, including blood (BACTEC), respiratory tract secretion (via suction), sputum, tissue (aortic valve), and wound specimens, were collected from 1186 hospitalized patients undergoing open-heart surgery in Shahid Rajaei Heart Hospital in Tehran, Iran from February 2020 to May 2021. Inclusion criteria included open-heart surgery and hospitalization in Shahid Rajaei Heart Hospital in Tehran, especially in the intensive care unit (ICU). The exclusion criterion was hospitalization time of less than 48 hours. Clinical isolates were identified using microbiological and biochemical tests and finally confirmed by the API (Analytical Profile Index) kit (France, BioMérieux, Lyon) [15, 16].

Antibiotic susceptibility pattern: The antibiotic sensitivity pattern of clinical isolates was determined using disk diffusion method (Kirby-Bauer) [17]. The antibiotics studied included gentamicin

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(10 µg), amikacin (30 µg), ciprofloxacin (5 µg), imipenem (10 µg), meropenem (10 µg), piperacillin (75 µg)-tazobactam (10 µg), and ceftazidime (30 µg), (MAST Diagnostic Co., UK). In this test, *P. aeruginosa* ATCC 27853 and *Escherichia coli* ATCC 25922 were used as the control strains.

Identification of isolates and determination of MIC using BD Phoenix system: In this study, the confirmation of identified carbapenem-resistant *P. aeruginosa* isolates and the evaluation of the minimum inhibitory concentration (MIC) were performed by the BD Phoenix automated microbiology system (Becton, Dickinson and Company, Sparks, MD, USA) according to the method described by Addis and colleagues (2021) [18].

Determination of MIC using E-test method: The MIC values of imipenem and meropenem against carbapenem-resistant *P. aeruginosa* isolates were determined by E-test (BioMerieux, CAT No. 412302). *P. aeruginosa* ATCC®27853 was used as a quality control strain. The obtained results were interpreted according to the CLSI guidelines [19].

mCIM (modified carbapenem inactivation method) test: In addition to the above-mentioned phenotypic methods, the mCIM phenotypic test was performed to evaluate the production of carbapenemase enzymes in *P. aeruginosa* isolates that were resistant to imipenem and meropenem based on the three methods of disk diffusion, Phoenix (broth microdilution method), and E-test. This test was performed according to the instructions described by Bakhat et al. (2019) [20]. A growth inhibition zone diameter of 6-15 mm is considered as positive, 15-19 mm as intermediate, and >19 mm as negative (i.e., no carbapenemase is detected). Also, according to the CLSI, the presence of small colonies within an area of 16-18 mm is considered as mCIM positive and indicates the presence of carbapenemase activity (heterogeneous strains) [21].

DNA extraction and PCR: Whole DNA of carbapenemase-producing isolates was extracted using a DNA extraction kit (Roche, Germany). The frequency of bla_{IMP}, bla_{VIM}, bla_{SPM}, bla_{KPC}, and bla_{NDM} genes was investigated by PCR test using specific primers listed in Table 1. The PCR reaction was carried out in a final volume of 20 µL including 10 µL of master mix (Amplicon, USA), 1 µL of each primer (forward and reverse) (10 pmol/µL), 3 µL of bacterial DNA (5 ng/µL), and 5 µL of sterile distilled water. A *P. aeruginosa* isolate obtained from Pasteur Institute of Iran was used as a positive control. The PCR program included an initial denaturation step at 95 °C for 5 min, followed by 33 cycles of denaturation at 95 °C for 1 min, annealing (at temperatures listed in Table 1) for 45 s, and extension at 72 °C for 1 min and a final extension step at 72 °C for 5 min.

Table 1) Primers used in the present study to detect resistance genes in *P. aeruginosa* isolates

Genes	Primer Sequence (5' → 3')	Product Size (bp)	Annealing Temperature	References
bla_{KPC}	F: CGTCTAGTTCTGCTGTCTTG R:CTTGTCATCCTTGTTAGGCG	798	57.2	[22]
bla_{NDM}	F:GGTTTGGCGATCTGGTTTTC R:CGGAATGGCTCATCACGATC	621	59	[22]
bla_{IMP}	F:GGAATAGAGTGGCTTAAYTCTC R:GGTTTAAYAAAACAACCACC	232	56	[23]
bla_{VIM}	F:GATGGTGTTTGGTCGCATA R:CGAATGCGCAGCACCAG	390	55.2	[23]
bla_{SPM}	F:AAAATCTGGGTACGCAAACG R:ACATTATCCGCTGGAACAGG	271	55	[23]

F: Forward; R: Reverse

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Table 2) Antibiotic resistance pattern of 60 isolated *P. aeruginosa* strains from patients with open heart surgery in the present study based on disk diffusion method

Antibiotic Name (Code)	R%	I%	S%
Imipenem (IMP)	36.6	0	63.3
Meropenem (MEM)	36.6	0	63.3
Piperacillin-Tazobactam (PTZ)	30	5	65
Amikacin (AN)	20	5	75
Gentamicin(GM)	33.3	3.3	63.3
Ciprofloxacin (CP)	28.3	0	71.6
Ceftazidime (CAZ)	23.3	3.3	73.3

R: Resistant; S: Sensitive, I: Intermediate

Table 3) MIC results for 22 isolated carbapenem-resistant *P. aeruginosa* strains (CRPA) from patients with open heart surgery in the present study by Phoenix system

Resistance Pattern Antibiotic Name (Code)	Sensitive (%)	Intermediate (%)	Resistant (%)
Imipenem (IMP) (10 µg)	-	-	100
Meropenem (MEM) (10 µg)	-	-	100
Amikacin (AN) (30 µg)	31.8	13.6	54.5
Ciprofloxacin (CP) (5 µg)	22.7	-	77.3
Gentamicin(GM) (10 µg)	22.7	4.5	72.7
Ceftazidime (CAZ)(30 µg)	45.5	9.1	45.5
Piperacillin-Tazobactam (PTZ) (75 µg/10 µg)	18.2	4.5	77.3

Findings

Patients and bacterial isolates: From a total of 1186 hospitalized patients undergoing open-heart surgery in Shahid Rajaei Heart Hospital in Tehran, 522 Gram-negative bacilli were isolated, including 60 *P. aeruginosa* isolates. The prevalence of carbapenem-resistant *P. aeruginosa* isolates was 36% (22 of 60). The carbapenem-resistant *P. aeruginosa* isolates were collected from different specimens, including respiratory tract secretions (via suction) (n= 8, 36.6%), sputum (n= 6, 27.2%), tissue (aortic valve) (n= 4, 18.1%), blood (n= 2, 9.0%), and wound secretions (n=2, 9.0%). Also, 17 (77.3%) isolates were collected from the ICU ward, and the rest of the isolates were recovered from other wards.

Determination of antibiotic resistance: *P. aeruginosa* isolates demonstrated the highest resistance level (n= 22, 36.6%) to imipenem and meropenem. Amikacin was the most effective antibiotic with 75% sensitivity (n= 45). The frequency of resistance to gentamicin, piperacillin-tazobactam, ciprofloxacin, and ceftazidime was 33.3% (n=20), 30% (n=18), 28.3% (n=17), and 23.3% (n=14), respectively (Table 2). It should be noted that according to the results of antibiogram, imipenem- and meropenem-resistant isolates were considered as carbapenem-resistant isolates.

Identification of isolates and determination of MIC using BD Phoenix system: Carbapenem-resistant *P. aeruginosa* isolates and the MIC values of the studied antibiotics were determined using the BD Phoenix automated system.

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Among 60 *P. aeruginosa* isolates, 22 isolates were identified as imipenem and/or meropenem resistant using disk diffusion method and selected as candidates for carbapenemase enzyme production testing. All the candidates were detected as carbapenemase producers by the BD Phoenix automated system (MIC>8 µg/mL for imipenem and meropenem), and the highest sensitivity was observed to ceftazidime (45.5%) (Table 3).

Determination of MIC using E-test method: The MIC value of imipenem and meropenem against carbapenem-resistant *P. aeruginosa* isolates was <32 µg/mL using E-test. The MIC values obtained using E-test and Phoenix methods against carbapenem-resistant *P. aeruginosa* isolates were consistent.

mCIM (modified carbapenem inactivation method) test: In this test, the growth inhibition zone diameter was between 6-8 mm, indicating that all carbapenem-resistant *P. aeruginosa* isolates phenotypically produced carbapenemase enzymes and hydrolyzed the antibiotic meropenem.

Prevalence of resistance genes by PCR method: In carbapenem-resistant *P. aeruginosa* isolates, the prevalence of $bla_{VIM,}$ $bla_{IMP,}$ and bla_{SPM} genes was 9.1% (2 of 22), 4.5% (1 of 22), and 4.5% (1 of 22), respectively. Bla_{KPC} and bla_{NDM} genes were not detected in any of the isolates. The highest frequency of metallobetalactamase genes was observed in *P. aeruginosa* isolates recovered from blood, sternal tissue, and respiratory secretion samples (Table 4).

Discussion

P. aeruginosa is a significant human pathogen that causes acute and chronic infections, especially in immunocompromised patients. Over the last decades, overuse of antibiotics has prompted the emergence of antibiotic-resistant *P. aeruginosa* strains. In addition, the increasing prevalence of MDR and XDR (extensively drug-resistant) *P. aeruginosa* isolates in recent years has significantly limited therapeutic options. One of the main reasons for this augmentation is the high intrinsic resistance of *P. aeruginosa* strains to a wide spectrum of antimicrobial agents and also the development of resistance during the course of treatment through the acquisition of resistance genes [24]. Carbapenem antibiotics (imipenem, meropenem, ortapenem, and doripenem) are often the last line of effective treatment for serious infections caused by multidrug-resistant bacteria [25].

In the current study, 36.6% of the isolates were resistant to imipenem and meropenem, which were considered as carbapenem-resistant isolates. Amikacin was the most effective antibiotic against *P. aeruginosa* isolates, and 54.5% of the isolates were identified as MDR. In a study conducted by Ma and An (2018) [24], *P. aeruginosa* isolates showed %100 resistance to cefazolin and cefuroxime, and the lowest resistance was observed to ceftazidime (%15.0). In another study performed by Beig and Arabestani (2019) [26], 50.51% of *P. aeruginosa* clinical isolates were carbapenem-resistant, and the highest and lowest resistance was observed to cefoxitin (93.8%) and piperacillin/tazobactam (39.2%), respectively. This difference observed in antibiotic resistance patterns may be due to the differences in the sample size of the studied strains and geographical areas. The current study also demonstrated that the distribution of carbapenem-resistant *P. aeruginosa* isolates varied based on infection sites. The respiratory tract was the most common site of *P. aeruginosa* infection (36.4%). This result is consistent with the finding of a systematic study conducted by Cai et al. (2017) [27]. Diversity in the distribution of carbapenem-resistant infections based on infection sites has also been reported in other studies [28, 29].

P. aeruginosa is inherently resistant to several antibiotics due to low outer membrane permeability, overexpression of efflux pumps, and enzyme-mediated resistance via the acquisition of carbapenemase genes [30]. In the present study, all the isolates were subjected to phenotypic carbapenemase screening prior to genotypic assessment. All carbapenem-resistant *P. aeruginosa* isolates were confirmed by phenotypic tests such as E-test, BD Phoenix system, and mCIM. These tests had 100% sensitivity and specificity for the detection of carbapenem-resistant isolates, which is in agreement with other studies results [31]. Evaluation of carbapenemase genes using PCR technique showed a very low frequency of these genes in the isolates. This suggests that some carbapenemases may be beyond the detectable range of current genotypic assays and emphasizes the role of other carbapenem resistance-mediating factors such as porine and efflux

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Table 4) Profile of isolated carbapenem-resistant *P. aeruginosa* strains from patients with open heart surgery in the present study

	mCIM	MIC (IMP/ MEM)	Genes	Ward	Sex/Age	Specimen
1	Positive	>8	Non	ICU	Male/25Y	Respiratory secretions by suction
2	Positive	>8	Non	ICU	Male/60Y	Respiratory secretions by suction
3	Positive	>8	Non	Surgery room	Female/49Y	Tissue
4	Positive	>8	Non	ICUC	Female/70	Sputum
5	Positive	>8	Non	ICUC	Female/59y	Respiratory secretions by suction
6	Positive	>8	Non	ICUC	Male/53Y	Sputum
7	Positive	>8	Non	ICUP	Female/6Y	Respiratory secretions by suction
8	Positive	>8	Non	ICU	Male/80Y	Respiratory secretions by suction
9	Positive	>8	Non	ICU	Male/61Y	Sputum
10	Positive	>8	Non	ICUC	Female/81	Blood
11	Positive	>8	bla_{VIM}	ICUP	Male/1Y	Blood
12	Positive	>8	Non	ICUC	Female/89Y	Respiratory secretions by suction
13	Positive	>8	Non	Men's interior	Male/61Y	Sputum
14	Positive	>8	Non	ICUP	Male/1Y	Blood
15	Positive	>8	Non	Women's interior	Female/69Y	Sputum
16	Positive	>8	Non	ICUC	Male/52Y	Respiratory secretions by suction
17	Positive	>8	bla_{VIM}+ bla_{SPM}	Surgery room	Male/63Y	Tissue
18	Positive	>8	Non	ICUC	Male/58Y	Tissue
19	Positive	>8	Non	ICUP	Male/1Y	Respiratory secretions by suction
20	Positive	>8	Non	ICU	Female/79Y	Tissue
21	Positive	>8	Non	ICU	Male/90Y	Blood
22	Positive	>8	bla_{IMP}	ICU	Male/50Y	Respiratory secretions by suction

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pumps [30]. Similar to previous studies [32, 33], VIM was the most prevalent carbapenemase. Bla_{IMP} and bla_{SPM} were the second most common genes identified in this study, and clinical concerns regarding these genes are increasing. *P. aeruginosa* strains carrying bla_{IMP} are considered endemic in Southeast Asia [34], but research conducted by Gill et al. (2021) [33] confirmed the global spread of IMP-harboring isolates.

The prevalence of carbapenem-resistant *P. aeruginosa* strains is alarming and highlights the need to implement strict infection control measures to prevent the spread of carbapenemase-encoding genes to other clones or other bacterial species. This represents the therapeutic challenges facing clinicians and highlights the need for more effective antibiotics and faster and more accurate information on antibiotic susceptibility.

Conclusion

According to the current study results, all phenotypic tests used to identify carbapenemase-producing isolates had the same sensitivity (100%) and specificity (100%). Considering the importance of carbapenem-resistant *P. aeruginosa* strains in hospitals, rapid identification and evaluation of antibiotic resistance in these bacteria could be considered as a fundamental step in the treatment and control of *Pseudomonas* infections.

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