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Distribution of *Pseudomonas*-Derived Cephalosporinase and Metallo-β-Lactamases in Carbapenem-Resistant *Pseudomonas aeruginosa* Isolates from Korea

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The emergence of carbapenem resistance among *Pseudomonas aeruginosa* is an increasing problem in many parts of the world. In particular, metallo-β-lactamases (MBLs) and AmpC β-lactamases are responsible for high-level resistance to carbapenem and cephalosporin. We studied the diversity and frequency of β-lactamases and characterized chromosomal AmpC β-lactamase from carbapenem-resistant *P. aeruginosa* isolates. Sixty-one carbapenem-resistant *P. aeruginosa* isolates were collected from patients in a tertiary hospital in Daejeon, Korea, from January 2011 to June 2014. Minimum inhibitory concentrations (MICs) of four antimicrobial agents were determined using the agar-dilution method. Polymerase chain reaction and sequencing were used to identify the various β-lactamase genes, class 1 integrons, and chromosomally encoded and plasmid-mediated *ampC* genes. In addition, the epidemiological relationship was investigated by multilocus sequence typing. Among 61 carbapenem-resistant *P. aeruginosa* isolates, 25 isolates (41.0%) were MBL producers. Additionally, 30 isolates producing PDC (*Pseudomonas*-derived cephalosporinase)-2 were highly resistant to ceftazidime (MIC_{50} = 256 μg/ml) and cefepime (MIC_{50} = 256 μg/ml). Of all the PDC variants, 25 isolates harboring MBL genes showed high levels of cephalosporin and carbapenem resistance, whereas 36 isolates that did not harbor MBL genes revealed relatively low-level resistance (ceftazidime, $p < 0.001$; cefepime, $p < 0.001$; imipenem, $p = 0.003$; meropenem, $p < 0.001$). The coexistence of MBLs and AmpC β-lactamases suggests that these may be important contributing factors for cephalosporin and carbapenem resistance. Therefore, efficient detection and intervention to control drug resistance are necessary to prevent the emergence of *P. aeruginosa* possessing this combination of β-lactamases.

Keywords: AmpC β-lactamase, metallo-β-lactamase

Introduction

Pseudomonas aeruginosa is a major opportunistic pathogen responsible for hospital-acquired infections and is notorious for its capacity to develop resistance to multiple classes of β-lactams. Recently, carbapenems have been shown to be the most important and effective therapeutic options against serious infections caused by these pathogens, but resistance to these agents is increasingly reported worldwide [1, 2, 32].

Carbapenem resistance in *P. aeruginosa* is mainly due to a combination of different factors: low outer membrane permeability, overexpression of the efflux pump MexAB-OprM, hyperproduction of derepressed AmpC chromosomal β-lactamase, and the presence of transferable resistance determinants, in particular, carbapenem-hydrolyzing enzymes [6, 9, 19, 20]. Carbapenem-hydrolyzing enzymes are divided into two types based on molecular classification: serine enzymes, which are derivatives of class A or D enzymes, and metallo enzymes, which belong to class B [25].

Serine carbapenemases of the *Klebsiella pneumoniae* carbapenemase (KPC), Guiana extended-spectrum (GES), and oxacilinase (OXA) families have been occasionally reported in this species in certain parts of the world, whereas metallo-β-lactamases (MBLs), particularly Verona imipenemase (VIM), and IMP (active against imipenem) types, are the most widespread and have been reported globally [4, 12, 26, 29].

In addition to carbapenem-hydrolyzing enzymes, another important mechanism of resistance to β-lactams in *P. aeruginosa* is the production of chromosomal AmpC β-lactamases, which can be induced or derepressed to confer high-level penicillin and cephalosporin resistance [27]. Inducible AmpC can be upregulated by subinhibitory concentrations of certain β-lactams. Furthermore, mutations can occur in the regulatory components of AmpC, leading to stable hyperproduction of AmpC with concomitant high-level resistance to many classes of β-lactams [21, 24]. Several chromosomally mediated *Pseudomonas*-derived cephalosporinases (PDCs) with extended-spectrum cephalosporinase activities have been reported among *P. aeruginosa* [23]. There are several reports on the prevalence of MBL genes and molecular epidemiology in carbapenem-resistant *P. aeruginosa* isolates from Korea, but the contribution of other mechanisms to carbapenem resistance such as class A and D β-lactamase and PDC genes is unknown.

The aim of this study was to determine the diversity and frequency of β-lactamases and characterize chromosomal AmpC β-lactamase in carbapenem-resistant *P. aeruginosa* isolates obtained from a tertiary hospital in Korea during a 4-year period. In addition, we investigated the epidemiological relationship and potential correlations between genetic characteristics and resistance to carbapenems.

Materials and Methods

Bacterial Isolation and Identification

A total of 61 consecutive and non-duplicated carbapenem-resistant *P. aeruginosa* isolates were collected from patients in a tertiary hospital in Daejeon, Korea, from January 2011 to June 2014. The isolates were identified with the Vitek 2 automated ID system (BioMérieux, Hazelwood, MO, USA), and carbapenem-resistant *P. aeruginosa* isolates were selected based on resistance to imipenem and meropenem.

Antimicrobial Susceptibility Testing

In the antimicrobial susceptibility tests, the minimum inhibitory concentration (MIC) was determined by using the agar dilution method according to the Clinical and Laboratory Standards Institute (CLSI) guidelines [3]. Four antimicrobial agents were tested, including imipenem, meropenem, ceftazidime, and cefepime (Sigma-Aldrich, St. Louis, MO, USA). The interpretation of susceptibility was performed according to the CLSI breakpoints. *E. coli* ATCC 25922 and *P. aeruginosa* ATCC 27853 were used as quality control strains.

Multilocus Sequence Typing

Multilocus sequence typing (MLST) was performed according to the methods described on the *P. aeruginosa* MLST database website (http://pubmlst.org/paeruginosa/). PCR and sequencing were performed for seven housekeeping genes (*acsA*, *aroE*, *guaA*, *mutL*, *nuoD*, *ppsA*, and *trpE*). The nucleotide sequences of these genes were compared with the sequences submitted to the MLST database to determine the allelic numbers and sequence types (STs).

Identification and Analysis of β-Lactamase Genes and Integrons

PCR assays were performed to amplify the sequence of MBLs, including the bla_{IMP}, bla_{VIM}, bla_{GIM}, bla_{SPM}, bla_{SIM}, bla_{NDM}, bla_{AIM}, bla_{DIM}, and bla_{FIM} genes, as described previously (Table 1) [17, 18]. PCR detection of class A and D β-lactamase genes (bla_{TEM}, bla_{SHV}, bla_{GES}, bla_{VEB}, bla_{KPC}, bla_{PSE}, bla_{PER}, $bla_{CTX\text{-}M\text{-}1,2,9\ group}$, $bla_{OXA\text{-}I,II,III\ group}$, $bla_{OXA\text{-}23}$, $bla_{OXA\text{-}24}$, $bla_{OXA\text{-}48}$, $bla_{OXA\text{-}51}$, and $bla_{OXA\text{-}58}$) and class 1, 2, and 3 integrons was also performed as previously described [7, 11, 20, 31]. Sequence analyses were confirmed with the BLAST program at the National Center for Biotechnology Information server (http://www.ncbi.nlm.nih.gov/). The structure of variable regions of integrons was determined by PCR mapping and sequencing.

Genotypic Detection and Sequencing of Chromosomally Encoded and Plasmid-Mediated *ampC* Gene

The genotypes of all 61 carbapenem-resistant *P. aeruginosa* isolates were analyzed for the presence of chromosomal *PDC* genes and for different families of plasmid-mediated *ampC* genes by multiplex PCR as described previously [15, 23]. Amplified PCR products were purified and sequenced; the results of DNA sequencing were compared with known β-lactamase gene sequences using the BLAST program.

Statistical Analysis

The data were analyzed using SPSS ver. 21.0 (SPSS, Chicago, IL, USA) with one-way analysis of variance. The differences were considered statistically significant at $p < 0.05$.

Results

MLST Analysis of Carbapenem-Resistant *P. aeruginosa*

Among the 61 carbapenem-resistant *P. aeruginosa* isolates, the sites of isolation were sputum (29 isolates, 47.5%), urine (22 isolates, 36.1%), blood (4 isolates, 6.6%), wounds (3 isolates, 4.9%), bile (2 isolates, 3.3%), and pus (1 isolate, 1.6%) (Table 2). A total of 61 carbapenem-resistant *P. aeruginosa* isolates were identified as 17 different STs by MLST

Table 1. Oligonucleotides used as primers for amplication and sequencing in this study.

Gene target	Primer sequence (5′-3′)	Product size (bp)	Reference
bla_{IMP}	GGAATAGAGTGGCTTAAYTCTC	232	[17]
	GGTTTAAYAAAACAACCACC		
bla_{VIM}	GATGGTGTTTGGTCGCATA	390	[17]
	CGAATGCGCAGCACCAG		
bla_{GIM}	TCGACACACCTTGGTCTGAA	477	[17]
	AACTTCCAACTTTGCCATGC		
bla_{SPM}	AAAATCTGGGTACGCAAACG	271	[17]
	ACATTATCCGCTGGAACAGG		
bla_{SIM}	TACAAGGGATTCGGCATCG	570	[17]
	TAATGGCCTGTTCCCATGTG		
bla_{NDM}	GGTTTGGCGATCTGGTTTTC	621	[17]
	CGGAATGGCTCATCACGATC		
bla_{AIM}	CTGAAGGTGTACGGAAACAC	322	[17]
	GTTCGGCCACCTCGAATTG		
bla_{DIM}	GCTTGTCTTCGCTTGCTAACG	699	[17]
	CGTTCGGCTGGATTGATTTG		
bla_{FIM}	GAAGCACATGGAAAACTGGG	435	[18]
	GATGGGCGAATGAGACAGC		
bla_{TEM}	CTTCCTGTTTTTGCTCACC	636	[11]
	AGCAATAAACCAGCCAGC		
bla_{SHV}	TCAGCGAAAAACACCTTG	472	[11]
	TCCCGCAGATAAATCACC		
bla_{GES}	ATGCGCTTCATTCACGCAC	844	[11]
	CTATTTGTCCGTGCTCAGG		
bla_{VEB}	CATTTCCCGATGCAAAGCGT	648	[20]
	CGAAGTTTCTTTGGACTCTG		
bla_{KPC}	CGTCTAGTTCTGCTGTCTTG	798 and 232	[17]
	CTTGTCATCCTTGTTAGGCG		
bla_{PSE}	AATGGCAATCAGCGCTTC	698	[11]
	GCGCGACTGTGATGTATA		
bla_{PER}	GGGACARTCSKATGAATGTCA	926	[11]
	GGYSGCTTAGATAGTGCTGAT		
$bla_{CTX\text{-}M\text{-}1\ group}$	TTAGGAARTGTGCCGCTGYA	688	[20]
	CGATATCGTTGGTGGTRCCAT		
$bla_{CTX\text{-}M\text{-}2\ group}$	CGTTAACGGCACGATGAC	404	[20]
	CGATATCGTTGGTGGTRCCAT		
$bla_{CTX\text{-}M\text{-}9\ group}$	TCAAGCCTGCCGATCTGGT	561	[20]
	TGATTCTCGCCGCTGAAG		
$bla_{OXA\text{-}I\ group}$	TCAACAAATCGCCAGAGAAG	276	[20]
	TCCCACACCAGAAAAACCAG		
$bla_{OXA\text{-}II\ group}$	AAGAAACGCTACTCGCCTGC	478	[20]
	CCACTCAACCCATCCTACCC		
$bla_{OXA\text{-}III\ group}$	TTTTCTGTTGTTTGGGTTTT	427	[20]
	TTTCTTGGCTTTTATGCTTG		

Table 1. Continued.

Gene target	Primer sequence (5′-3′)	Product size (bp)	Reference
bla_{OXA-23}	GATCGGATTGGAGAACCAGA	501	[31]
	ATTTCTGACCGCATTTCCAT		
bla_{OXA-24}	GGTTAGTTGGCCCCCTTAAA	246	[31]
	AGTTGAGCGAAAAGGGGATT		
bla_{OXA-48}	GCGTGGTTAAGGATGAACAC	438	[17]
	CATCAAGTTCAACCCAACCG		
bla_{OXA-51}	TAATGCTTTGATCGGCCTTG	353	[31]
	TGGATTGCACTTCATCTTGG		
bla_{OXA-58}	AAGTATTGGGGCTTGTGCTG	599	[31]
	CCCCTCTGCGCTCTACATAC		
Class 1 integron	AAGCAGACTTGACCTGA	-	[7]
	GGCATCCAAGCAGCAAG		
Class 2 integron	CGGGATCCCGGACGGCATGCAC	-	[7]
	GATGCCATCGCAAGTACGAG		
Class 3 integron	TGTTCTTGTATCGGCAGGTG	-	[7]
	AGTGGGTGGCGAATGAGTG		

Table 2. MLST analysis of 61 carbapenem-resistant *Pseudomonas aeruginosa* isolates collected during a 4-year period.

Source (no. of isolates)	ST	Allelic profile							No. of isolates (%)
		acsA	*aroE*	*guaA*	*mutL*	*nuoD*	*ppsA*	*trpE*	
Sputum (29)	235	38	11	3	13	1	2	4	5 (8.2)
	654	17	5	26	3	4	4	26	5 (8.2)
	245	39	6	12	11	3	15	2	4 (6.6)
	357	2	4	5	3	1	6	11	4 (6.6)
	111	17	5	5	4	4	4	3	2 (3.3)
	257	35	24	36	11	4	15	14	2 (3.3)
	179	36	27	28	3	4	13	7	1 (1.6)
	195	89	30	64	26	48	24	32	1 (1.6)
	244	17	5	12	3	14	4	7	1 (1.6)
	274	23	5	11	7	1	12	7	1 (1.6)
	589	15	5	11	3	4	12	7	1 (1.6)
	1455	15	5	11	3	58	42	9	1 (1.6)
	1663	17	18	17	5	4	4	9	1 (1.6)
Urine (22)	235	38	11	3	13	1	2	4	20 (32.8)
	245	39	6	12	11	3	15	2	1 (1.6)
	257	35	24	36	11	4	15	14	1 (1.6)
Blood (4)	235	38	11	3	13	1	2	4	2 (3.3)
	267	19	5	12	11	11	4	14	1 (1.6)
	1062	22	5	91	33	4	4	1	1 (1.6)
Wound (3)	235	38	11	3	13	1	2	4	3 (4.9)
Bile (2)	111	17	5	5	4	4	4	3	1 (1.6)
	708	11	3	11	3	1	4	60	1 (1.6)
Pus (1)	645	6	5	5	3	3	13	1	1 (1.6)

Abbreviations: ST, sequence type.

Table 3. Prevalence of Ambler class A, B, and D β-lactamases in 61 carbapenem-resistant *Pseudomonas aeruginosa* isolates.

Class	Type of β-lactamase	No. of isolates (%)	Sequence type
None	-	23 (37.7)	111(3), 179, 195, 235, 245(4), 257(3), 267, 274, 357, 589, 645, 654, 708, 1062, 1455, 1663
Class A	PSE-1	2 (3.3)	654
Class D	OXA-1	2 (3.3)	244, 245
	OXA-2	1 (1.6)	654
	OXA-10	7 (11.5)	235
Class A+D	PSE-1 + OXA-2	1 (1.6)	654
Class B+D	VIM-2 + OXA-1	3 (4.9)	357
	IMP-6 + OXA-1	10 (16.4)	235
	IMP-6 + OXA-1 + OXA-10	12 (19.7)	235

experiments. ST235 (30 isolates, 49.2%) was the most frequently detected clone. According to frequency, five other detected STs were ST245 (5 isolates, 8.2%), ST654 (5 isolates, 8.2%), ST357 (4 isolates, 6.6%), ST111 (3 isolates, 4.9%), and ST257 (3 isolates, 4.9%). The remaining 11 STs (ST179, ST195, ST244, ST267, ST274, ST589, ST645, ST708, ST1062, ST1455, and ST1663) were each represented by one isolate (1.6%).

Prevalence of β-Lactamases

Of the 61 isolates, 10 isolates (16.4%) harbored OXA-type and 2 (3.3%) harbored *Pseudomonas*-specific enzyme (PSE)-type enzyme (Table 3). Of the OXA β-lactamases, OXA-10 was the most prevalent, followed by OXA-1 and OXA-2. Fourteen isolates (23.0%) harbored two different β-lactamases, and 12 isolates (19.7%) harbored three enzymes. Of these, MBL genes were identified in 25 isolates (41.0%) harboring $bla_{\text{OXA-1}}$. Two MBL genes, $bla_{\text{IMP-6}}$ and $bla_{\text{VIM-2}}$, were identified in 22 (36.1%) and 3 isolates (4.9%), respectively. All 22 isolates carrying the $bla_{\text{IMP-6}}$ gene belonged to ST235 and three isolates carrying the $bla_{\text{VIM-2}}$ gene belonged to ST357.

Structure of Class 1 Integrons

Class 1 integrons were detected in 36 (59.0%) of the 61 carbapenem-resistant *P. aeruginosa* isolates and no class 2 or 3 integrons were found. The gene cassettes found in this study were divided into six types (Type A, B, C, D, E, and F) according to the cassette composition (Table 4). Type A (4.0 kb), obtained in 18 isolates, carried the *aadB-cmlA-*$bla_{\text{OXA-10}}$*-aadA1* gene cassette. Eleven isolates of type B

Table 4. Schematic representation of gene cassette structures located in the class 1 integron isolated from 61 carbapenem-resistant *Pseudomonas aeruginosa* isolates.

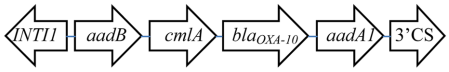
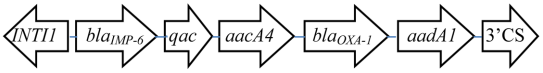
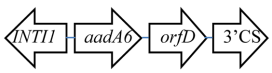
Type	Genetic environment	No. of isolates	Sequence type
A		18	235(10), 357(2), 111(1), 244(1) 267(1), 274(1), 654(1), 1455(1)
B		11	235(11)
C		2	235(1), 654(1)
D		2	235(1), 179(1)
E		2	589(1), 654(1)
F		1	195(1)

Table 5. Distribution of AmpC-type variants (PDC) and MIC of β-lactam drugs for 61 carbapenem-resistant *Pseudomonas aeruginosa* isolates.

PDC variant	N (%)	CAZ			CFP			IPM			MEM		
		Range	MIC_{50}	MIC_{90}	Range	MIC_{50}	MIC_{90}	Range	MIC_{50}	MIC_{90}	Range	MIC_{50}	MIC_{90}
PDC-1	4 (6.6)	4 to >256	8	>256	4 to >256	32	>256	32 to >256	>256	>256	16 to >256	64	>256
PDC-2	30 (49.2)	2 to >256	256	>256	4 to >256	256	>256	8 to >256	>256	>256	16 to >256	>256	>256
PDC-3	10 (16.4)	2 to >256	16	128	2 to >256	16	256	32 to >256	>256	>256	8 to >256	128	>256
PDC-5	9 (14.8)	2 to >256	16	>256	2 to >256	32	>256	8 to >256	>256	>256	8 to >256	64	>256
PDC-7	6 (9.8)	4 to >256	8	>256	4 to >256	8	>256	8 to >256	256	>256	8 to >256	128	>256
PDC-8	2 (3.3)	16 to 128	16	128	64 to >256	64	>256	>256	>256	>256	16 to >256	16	>256

Abbreviations: N, number of isolates; CAZ, ceftazidime; CFP, cefepime; IPM, imipenem; MEM, meropenem.

(5.5 kb) belonged to ST235 and carried bla_{IMP-6}-*qac-aacA4*-bla_{OXA-10}- *aadA2*. Type C (1.8 kb), found in two isolates, carried *aacA4*-bla_{OXA-2}-*orfD*, and type D (1.2 kb) had *aadA6*-*orfD* (2 isolates). Two isolates contained type E (1.0 kb) carrying the *aadA4* gene cassette. Type F (2.5 kb) was detected in only one isolate and carried *aadA4*-bla_{OXA-10}-*aadA2*.

Identification of AmpC Variants

On performing PCR for the presence of chromosomal *ampC* gene, all 61 isolates of *P. aeruginosa* harbored PDC gene while the plasmid-mediated *ampC* gene was not present. Sequencing analysis of the PCR product of the chromosomal *ampC* gene revealed that 61 isolates obtained six variants (PDC-1, PDC-2, PDC-3, PDC-5, PDC-7, and PDC-8) (Table 5).

The most frequent variant was PDC-2 (30 isolates, 49.2%) and PDC-3 was the second most frequently detected variant (10 isolates, 16.4%). Thirty isolates producing PDC-2 were highly resistant to ceftazidime (MIC_{50} = 256 μg/ml) and cefepime (MIC_{50} = 256 μg/ml). Meanwhile, the remaining 31 isolates showed full or intermediate susceptibility, with the ceftazidime MIC_{50} ranging from 8 to 16 μg/ml. Additionally, 25 isolates harboring MBL genes were highly resistant to ceftazidime (MIC range 64 to >256 μg/ml), cefepime (MIC range 64 to >256 μg/ml), imipenem (MIC range >256 μg/ml), and meropenem (MIC range >256 μg/ml) (Table 6). In contrast, 36 isolates that did not contain MBL genes showed relatively low resistance, with MICs ranging from 2 to 128 μg/ml for ceftazidime, 2 to >256 μg/ml for cefepime, 8 to >256 μg/ml for imipenem, and 8 to >256 μg/ml for meropenem.

Discussion

Resistance to β-lactams (particularly carbapenem and cephalosporin) in *P. aeruginosa* has been increasingly reported worldwide, and this is also the case in Korea. According to previous Korean National Surveillance Antimicrobial Resistance (KONSAR) studies, from 2005 to 2011, resistance rates of *P. aeruginosa* to imipenem increased from 19% to 26%, and to ceftazidime increased from 19% to 23% [33].

Table 6. AmpC-type variants (PDC) and β-lactam MIC according to expression of the MBL gene in 61 carbapenem-resistant *Pseudomonas aeruginosa* isolates.

PDC variant	MBL-negative isolates					MBL-positive isolates				
	N	MIC (μg/ml)				N	MIC (μg/ml)			
		CAZ	CFP	IPM	MEM		CAZ	CFP	IPM	MEM
PDC-1	3	4 to 32	4 to >256	32 to >256	16 to >256	1	>256	>256	>256	>256
PDC-2	12	2 to 128	4 to 256	8 to >256	16 to >256	18	64 to >256	64 to >256	>256	>256
PDC-3	9	2 to 128	2 to 256	32 to >256	8 to >256	1	>256	>256	>256	>256
PDC-5	6	2 to 64	2 to 32	8 to >256	8 to >256	3	64 to >256	>256	>256	>256
PDC-7	4	4 to 32	4 to 16	8 to >256	8 to 256	2	>256	>256	>256	>256
PDC-8	2	16 to 128	64 to >256	>256	16 to >256	0				
Total	36	2 to 128	2 to >256	8 to >256	8 to >256	25	64 to >256	64 to >256	>256	>256

Abbreviations: N, number of isolates; CAZ, ceftazidime; CFP, cefepime; IPM, imipenem; MEM, meropenem

This study analyzed various genes of *P. aeruginosa* isolates that are responsible for resistance to carbapenems. Class D OXA β-lactamases were more frequently detected than class A in *P. aeruginosa* (16.4% versus 3.3%). In particular, OXA-10 was only observed in ST235 isolates (19 isolates), and was accompanied by bla_{IMP-6} in 12 isolates (63.2%). Similarly, a previous study found that 35 (60.3%) of 58 OXA-10-producing isolates harbored bla_{IMP-6} and/or bla_{VIM-2}, and belonged to only ST235 [1]. In addition, 25 (41.0%) carbapenem-resistant *P. aeruginosa* isolates were MBL producers.

Compared with a previous study, the rates of MBL production showed a 2.5-fold increase from 16.2% in 2008–2012 to 41.0% in 2011–2014 among carbapenem-resistant *P. aeruginosa* isolates [2]. Among the 22 ST235 IMP-6-producing isolates, 11 isolates (50.0%) shared an identical class 1 integron with a gene cassette array (bla_{IMP-6}-*qac-aacA4*-bla_{OXA-1}-*aadA1*) between the 5′ and 3′ conserved sequence. The bla_{VIM-2} gene was identified in three isolates of ST357.

Mechanisms of drug resistance in AmpC β-lactamase can either be chromosomally or plasmid-mediated. The majority of AmpC β-lactamases are chromosomally mediated and are found in *Serratia*, *Pseudomonas*, *Acinetobacter*, *Citrobacter*, and *Enterbacter* spp. Chromosomally mediated resistance is due to mutation(s) in the bacterial DNA, and such genes are not easily transferable to other bacterial species [5, 8]. In the present study, six variants of the chromosomally mediated *ampC* enzyme PDC were identified in all 61 carbapenem-resistant *P. aeruginosa* isolates. The most frequent AmpC-type variant was PDC-2, containing the substitutions G27D, A97V, T105A, and V205L. Substitutions in this region have been previously linked to the broadening of the enzyme's hydrolytic spectrum, facilitating the degradation of compounds such as ceftazidime [10, 22]. In our study, 30 isolates harboring PDC-2 exhibited high levels of resistance to ceftazidime (MIC_{50} = 256 μg/ml) and cefepime (MIC_{50} = 256 μg/ml). Additionally, of the 30 isolates, 20 (66.7%) belonged to ST235 and most of the ST235 isolates were recovered from urine (14 isolates, 70%). Another study from France reported 10 variants of a PDC (PDC 1-10) gene, in which several variants showed reduced susceptibility to ceftazidime, cefepime, and imipenem [23, 28].

Plasmid-mediated AmpC β-lactamases can spread laterally, making them transferable to other bacteria. Therefore, they are frequently seen in many bacterial species such as *E. coli*, *K. pneumonia*, *Salmonella* spp., *Citrobacter freundii*, *Enterobacter aerogenes*, and *Proteus mirabilis* [5, 16]. In this study, we were unable to detect plasmid-mediated *ampC* genes, which is consistent with a previous study by Wang *et al*. [30], who reported that no plasmid-mediated *ampC* genes were detected among 258 carbapenem-resistant *P. aeruginosa* isolates.

Of all the PDC variants, 25 isolates harboring MBL genes showed high levels of cephalosporin (MIC range for ceftazidime and cefepime, 64 to >256 μg/ml) and carbapenem (MIC range for imipenem and meropenem, >256 μg/ml) resistance, whereas 36 isolates that did not harbor MBL genes revealed relatively low-level resistance (MIC range for ceftazidime, 2–128 μg/ml; cefepime, 2 to >256 μg/ml; imipenem and meropenem, 8 to >256 μg/ml). These results highlight the importance of MBL genes in cephalosporin and carbapenem resistance in *P. aeruginosa* (ceftazidime, cefepime, and meropenem, $p < 0.001$; imipenem, $p = 0.003$).

Similarly, Bae *et al*. [1] reported that production of IMP-6 and VIM-2 MBLs is the main mechanism for acquiring resistance to ceftazidime and carbapenems in *P. aeruginosa* isolates. In addition, Neyestanaki *et al*. [11] found that the production of MBLs and AmpC β-lactamases was the major emerging mechanism of resistance to carbapenem among *P. aeruginosa* isolates in Teharan, Iran. Similarly, combinations of various β-lactamases have recently been reported in studies from India, Brazil, Italy, and Argentina [13, 14].

In conclusion, the coexistence of MBLs and AmpC β-lactamases suggests that these may be important contributing factors for cephalosporin and carbapenem resistance. Therefore, efficient detection and intervention to control drug resistance are necessary to prevent the emergence of *P. aeruginosa* possessing this combination of β-lactamases.

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