

Multilocus sequence typing and variations in the *oprD* gene of *Pseudomonas aeruginosa* isolated from a hospital in China

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Objectives: To provide information about the genetic relationships and mechanism underlying carbapenem resistance in *Pseudomonas aeruginosa* clinical isolates of a hospital in China.

Materials and methods: One hundred and sixty *P. aeruginosa* strains were isolated from a hospital in China. Susceptibility to 14 antimicrobial agents was determined by antimicrobial susceptibility testing. Multilocus sequence typing was used to characterize the genetic backgrounds of these clinical isolates. Forty-five strains were randomly selected for further evaluation of their carbapenem resistance mechanism. Their *oprD* gene was compared with the PAO1 sequence.

Results: Multilocus sequence typing analysis demonstrated that these isolates were highly diverse; 68 sequence types were identified, of which 28 were novel sequence types. Polygenic and eBURST analysis demonstrated genetically similar clones with dissimilar resistance profiles. Among the 45 randomly selected strains associated with carbapenem resistance, 2 were metallo- β -lactamase producers; all the 45 strains were not AmpC overproducers. Sequence analysis revealed a high diversity in the *oprD* sequences among isolates. Strains susceptible to imipenem and meropenem with shortened L7 and L8 loops in *oprD* were the major strain types observed in this hospital.

Conclusion: This study indicated that *oprD* provided the main mechanism for carbapenem resistance. The shortened L7 and L8 loops are responsible for carbapenem susceptibility.

Keywords: *Pseudomonas aeruginosa*, multilocus sequence typing, carbapenem resistance, L7 and L8 loops in OprD

Introduction

The continued high rates of antibiotics usage in hospitals, farms, and agriculture have contributed to antibiotic resistance, forcing a rapid dissemination of multidrug-resistant (MDR) clones worldwide.¹ Globally, infectious diseases have become an increasingly important cause of human morbidity and mortality.² *Pseudomonas aeruginosa* is one of the most common hospital-acquired pathogens that cause numerous acute and chronic human infections, such as burn wound, urinary tract, and respiratory tract infections; it especially causes infections in intensive care units.³ According to the American Center for Disease Control and Prevention, *P. aeruginosa* causes about 10% of hospital-acquired infections.⁴ In recent years, the increasing resistance of *P. aeruginosa* strains to different antibiotics has led to an increase in the emergence of MDR *P. aeruginosa*. The treatment of infectious diseases caused by MDR *P. aeruginosa* is becoming more challenging with each passing year.⁵

Carbapenems are a class of β -lactam antibiotics that are highly effective for treating infections caused by MDR *P. aeruginosa*.⁶ However, resistance of *P. aeruginosa*

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to carbapenems is already common in many countries.^{7,8} Previous studies have shown that the decreased expression of the outer membrane protein OprD, *oprD* mutations, loss of OprD, and overproduction of metallo β -lactamases (MBLs) and AmpC, and overexpression of the major resistance-nodulation-division efflux pump systems (MexAB-OprM, MexCD-OprJ, MexEF-OprN, and MexXY-OprN) mainly contribute to carbapenem resistance.^{8–12} OprD is an outer membrane porin protein facilitating the permeation of basic amino acids, small peptides, and carbapenem antibiotics.^{13,14} A topological model predicts that OprD comprises 16 antiparallel strands connected by eight longer loop regions (L1–L8) on its external surface.¹⁵

P. aeruginosa is a ubiquitous microorganism present in many diverse environmental conditions; it can be transmitted by nursing staff, medical equipment, or utensils in hospitals.^{3,16} Typing of *P. aeruginosa* isolated from hospital settings is especially important for effective management of hospital-acquired infections and regulation of drug therapy. In this study, 160 *P. aeruginosa* strains were isolated from a hospital in China. Susceptibility to 14 antimicrobial agents was determined by antimicrobial susceptibility testing. Multilocus sequence typing (MLST) was used to characterize the genetic backgrounds of the clinical isolates. Among the 160 isolates, some carbapenem-resistant strains were selected for further evaluation of their carbapenem resistance mechanism.

The aims of this study were to investigate the drug resistance and genetic background of *P. aeruginosa*. It is useful to recognize their different mechanisms of resistance to carbapenems and devise a proper infection control strategy.

Materials and methods

Bacterial strains

A total of 160 clinical isolates of *P. aeruginosa* were collected at Shaanxi Provincial People's Hospital between July 2016 and January 2017. The clinical isolates were isolated from sputum (n=134), urine (n=8), blood (n=6), endotracheal intubation discharge (n=3), bronchoalveolar lavage fluid (n=5), and skin secretions (n=4). These isolates were identified using the VITEK 2 system (bioMérieux, Marcy l'Etoile, France) and 16S rRNA gene sequencing. *P. aeruginosa* PAO1 and *P. aeruginosa* ATCC27853 (kindly donated by The Children's Hospital of Xi'an City) were used as reference strains.

Antimicrobial susceptibility testing

Antibiotic susceptibility tests of selected strains were performed using the disk diffusion method; the results were interpreted according to the Clinical and Laboratory

Standards Institute guidelines.¹⁷ The antibiotics used included amikacin, gentamicin, tobramycin, ciprofloxacin, levofloxacin, polymyxin, aztreonam, ceftazidime, cefepime, imipenem (IPM), meropenem (MEM), piperacillin, piperacillin/tazobactam, and cefoperazone/sulbactam.

Multilocus sequence typing

MLST was performed as per previously published protocols.¹⁸ Seven housekeeping genes (*acsA*, *aroE*, *guaA*, *mutL*, *nuoD*, *ppsA*, and *trpE*) were amplified in all isolates using the primers described by Curran et al.¹⁸ The polymerase chain reaction products were sequenced and submitted to the *P. aeruginosa* MLST database (<https://pubmlst.org/paeruginosa/>) for assignment of allelic numbers. Each isolate was then assigned a sequence type (ST) based on the combination of seven allelic numbers. Any STs or allelic profiles that did not match with those in the existing database were designated as "new". New allele sequences and the allelic profiles of novel STs were submitted to the *P. aeruginosa* MLST database for assignment of the correct allelic numbers and STs.

Phylogenetic analysis

DnaSP v5 was used to analyze the genetic variability at MLST loci for the 160 isolated strains.¹⁹ The mean G+C content, polymorphic sites, difference in number of nucleotides, and the nonsynonymous/synonymous ratio (dN/dS) were calculated.

MLST allelic profiles were analyzed to examine the relationship within the STs of the 160 clinical isolates using eBURST v3, available from <http://eburst.mlst.net/>. Isolates with five or more identical alleles were considered as part of the same clonal complexes (CCs).

For sequence-based phylogenetic tree analysis, the seven MLST gene fragment sequences of each ST strain were concatenated into a single 2800 bp sequence. The concatenated sequences were aligned and subjected to maximum parsimony analysis using the MEGA 5 software program.²⁰ Rooted neighbor-joining trees based on the concatenated seven-gene sequence data were generated using the Kimura two-parameter model for distance calculations and 1000 bootstrap replicates.^{21,22}

Sequence analysis of the *oprD* gene and the *ampC* gene

Primers OprD-F1 (CGCCGACAAGAAGAACTAGC) and OprD-R1 (GTCGATTACAGGATCGACAG) were used for *oprD* amplification and sequencing, as described previously.²³ The sequences were compared with the PAO1 sequence

(<http://www.pseudomonas.com/>) and analyzed with DNA-MAN program, version 5.2.2.

Screening for AmpC and MBL production

MBL production was detected by the double-disk synergy test, performed using 10 µg IPM and 2.5 µM EDTA.²⁴ AmpC production was screened using the cloxacillin double-disk synergy test, as previously described.²⁵

Results

Antimicrobial susceptibility analysis

Using the disk diffusion method as described in the “Materials and methods” section, antimicrobial susceptibility testing was carried out for the collected clinical isolates. Carbapenems are one of the antibiotics considered the last resort against *P. aeruginosa* infections.⁶ In the collected 160 isolates, the results indicated that 93 (60%) and 86 (53.7%) strains displayed resistance to IPM and MEM, respectively (Table 1). Rates of resistance to aztreonam, piperacillin, levofloxacin, piperacillin/tazobactam, cefoperazone sodium/sulbactam, cefepime, and ceftazidime were 44.4%, 43.1%, 40.0%, 34.4%, 33.1%, 32.5%, and 30.0%, respectively. Lower rates of resistance were observed against tobramycin (5%), amikacin (8.8%), and gentamicin (11.9%).

Multilocus sequence typing

MLST was used to distinguish isolated strains.¹⁸ According to the *P. aeruginosa* MLST database, typing of all isolated strains was successful. Three novel alleles were found among the 160 isolates and were submitted to the MLST database. A new allele found for the *acsA* gene was assigned a new allelic number, 164. Two new alleles were found for the *nuoD* gene and were assigned the corresponding allelic numbers, 104 and 105. A total of 66 different STs were assigned to the 160 isolates investigated; 132 isolates belonged to 38 known STs and 28 isolates demonstrated 28 new STs (Table 2). *P. aeruginosa* ATCC27853 and PAO1 were typed as ST155 and ST549, respectively. The ratio of the number of STs to the number of isolates was 0.41. The three predominant STs were ST1639 (22 isolates), ST485 (11 isolates), and ST261 (9 isolates). It was thus demonstrated that the STs of the isolates collected from the hospital were diverse.

Allelic variation in *P. aeruginosa*

The characteristics of seven housekeeping genes are summarized in Table 3. Among the 160 strains, the number of distinct alleles for each gene varied from 14 (*ppsA*) to 24 (*acsA*), and the polymorphic sites for each gene ranged from 13 (*nuoD*) to 23 (*trpE*). The ratio of dN/dS was calculated for

Table 1 The activity of 14 antimicrobial agents against 160 clinically isolated strains

Antibiotic	Number (%) of 160 strains		
	Sensitive	Intermediary	Resistant
Aminoglycoside			
Amikacin	146 (91.2)	0 (0)	14 (8.8)
Gentamicin	132 (82.5)	9 (5.6)	19 (11.9)
Tobramycin	151 (94.4)	1 (0.6)	8 (5)
Quinolones			
Ciprofloxacin	99 (61.9)	9 (5.6)	52 (32.5)
Levofloxacin	79 (49.4)	17 (10.6)	64 (40.0)
Polypeptides			
Ploymixin	160 (100)	0 (0)	0 (0)
Monolactams			
Aztreonam	52 (32.5)	37 (23.1)	71 (44.4)
Cephalosporins			
Ceftazidime	103 (64.4)	9 (5.6)	48 (30)
Cefepime	84 (52.5)	24 (15.0)	52 (32.5)
Carbapenems			
Imipenem	63 (39.4)	4 (2.5)	93 (58.1)
Meropenem	67 (41.9)	7 (4.4)	86 (53.7)
Ureidopenicillins			
Piperacillin	73 (45.6)	18 (11.3)	69 (43.1)
β-Lactam/inhibitor complex			
Piperacillin/tazobactam	85 (53.1)	20 (12.5)	55 (34.4)
Cefoperazone sodium/sulbactam	78 (48.8)	29 (18.1)	53 (33.1)

Table 2 The correspondent ST, allelic profile, and frequency of STs among 160 isolated strains, PAOI, and *Pseudomonas aeruginosa* ATCC27853

ST	Number	<i>acsA</i>	<i>aroE</i>	<i>guaA</i>	<i>mutL</i>	<i>nuoD</i>	<i>ppsA</i>	<i>trpE</i>
ST1639	22	11	5	6	61	3	7	1
ST485	11	11	76	5	3	61	14	3
ST261	9	105	5	30	3	3	4	14
ST639	7	11	19	19	3	4	4	7
ST2375 (new)	6	28	5	11	2	4	38	9
ST882	6	17	5	11	3	4	6	37
ST209	5	125	5	11	7	1	12	7
ST357	5	2	4	5	3	1	6	11
ST277	5	39	5	9	11	27	5	2
ST291	5	6	5	5	3	52	4	2
ST16	5	28	5	12	11	27	1	44
ST2389 (new)	4	164 (new)	3	7	5	2	13	3
ST244	3	17	5	12	3	14	4	7
ST836	3	6	5	1	5	27	15	7
ST1743	3	15	181	1	63	4	15	10
ST2374 (new)	3	17	5	26	3	4	4	7
ST274	2	23	5	11	7	1	12	7
ST1203	2	33	1	25	6	6	7	5
ST381	2	11	20	1	65	4	4	10
ST2363 (new)	2	6	5	11	11	4	4	1
ST2380 (new)	2	28	5	11	2	4	38	7
ST2381 (new)	2	17	3	6	3	4	1	18
ST226	2	11	5	36	3	1	29	7
ST2372 (new)	2	125	5	11	7	73	12	7
ST830	1	5	13	109	5	1	1	47
ST597	1	17	5	12	3	4	4	7
ST1453	1	28	61	3	6	1	33	41
ST2100	1	17	8	3	11	1	15	1
ST270	1	22	3	17	5	2	10	7
ST1950	1	40	22	11	14	4	4	7
ST1091	1	6	5	11	5	3	6	7
ST273	1	104	4	36	71	4	4	53
ST108	1	39	5	20	5	1	6	31
ST621	1	15	5	20	5	1	4	25
ST1226	1	11	66	11	3	29	4	9
ST527	1	16	52	11	85	59	15	10
ST408	1	17	5	37	3	1	4	2
ST664	1	9	5	11	3	4	40	18
ST676	1	28	5	11	77	3	4	92
ST612	1	28	5	58	11	4	15	44
ST845	1	11	5	1	7	4	4	7
ST554	1	16	5	12	3	2	15	12
ST792	1	6	5	11	3	2	15	1
ST207	1	47	4	5	33	1	6	40
ST1157	1	1	74	11	5	4	12	7
ST2362 (new)	1	6	5	1	29	92	4	68
ST2364 (new)	1	5	5	20	5	1	4	25
ST2365 (new)	1	11	5	9	11	27	5	2
ST2366 (new)	1	28	5	6	11	4	4	7
ST2367 (new)	1	15	5	1	63	4	15	10
ST2368 (new)	1	11	5	11	3	4	6	37
ST2369 (new)	1	2	4	5	5	1	6	11
ST2370 (new)	1	125	5	11	61	1	12	7
ST2371 (new)	1	17	5	7	3	4	4	7
ST2373 (new)	1	125	5	11	7	1	5	7

(Continued)

Table 2 (Continued)

ST	Frequency (%)	<i>acsA</i>	<i>aroE</i>	<i>guaA</i>	<i>mutL</i>	<i>nuoD</i>	<i>ppsA</i>	<i>trpE</i>
ST2376 (new)	1	36	27	28	3	52	13	7
ST2377 (new)	1	4	1	59	6	1	33	42
ST2378 (new)	1	17	5	11	7	1	12	7
ST2379 (new)	1	11	5	6	61	3	7	37
ST2382 (new)	1	28	5	58	61	4	15	44
ST2383 (new)	1	23	5	11	7	1	4	7
ST2388 (new)	1	11	5	6	61	104 (new)	7	1
ST2392 (new)	1	164 (new)	3	7	3	2	13	3
ST2393 (new)	1	164 (new)	3	7	5	4	13	3
ST2405 (new)	1	125	5	11	7	105 (new)	12	7
ST2406 (new)	1	29	8	36	67	1	6	3
ST549 (PAO1)	1	7	5	12	3	4	1	7
ST155 (ATCC27853)	1	28	5	36	3	3	13	7

Notes: The number assigned to each gene corresponds to the allelic type available in the MLST database. Alleles that did not match with the existing database were designated "new". Types for strains having seven known alleles that did not match with any known strain type in the database were also designated "new".

Abbreviations: MLST, multilocus sequence typing; ST, sequence type.

all seven genes and found to be equal to 0 for *acsA*, 0.1621 for *nuoD*, 0.1507 for *guaA*, 0.5799 for *mutL*, 1.6741 for *aroE*, and 2.3272 for *trpE*. The *acsA* gene showed synonymous substitutions, while the *ppsA* gene showed nonsynonymous substitution. *guaA*, *mutL*, *nuoD*, *aroE*, and *trpE* exhibited both synonymous and nonsynonymous substitutions (Table 3).

eBURST analysis

In order to determine the clonal relationship between isolates, 68 STs, including PAO1 and ATCC27853, were clustered by eBURST analysis (<http://eburst.mlst.net>).²⁶ Figure 1 shows that 68 STs were segregated into 11 CCs because of the sequence identity among five or more alleles. The largest CC consisted of eight STs (ST2378, ST2372, ST274, ST2383, ST2370, ST2373, ST2405, and ST209, with ST209 being the primary founder). The second CC consisted of four STs (ST244, ST2374, ST2371, and ST597, with ST597 being the primary founder). The nine other groups contained two or three STs. Twenty-six STs were classified as singletons (Figure 1).

Phylogenetic analysis of the MLST sequences

To estimate the phylogenetic relationships among the isolates, the concatenated seven-gene sequence of each strain was further analyzed to construct rooted trees using the neighbor-joining method. Sixty-six concatenated sequences from 66 STs and the corresponding sequences from PAO1 and ATCC27853 were used (<https://www.ncbi.nlm.nih.gov/>). The MLST tree revealed a high genetic diversity in those isolates. The analysis revealed a weak bootstrapping value, especially in major branches. The phylogenetic tree shows

that ST2378, ST2372, ST274, ST2383, ST2370, ST2373, ST2405, and ST209 were clustered together (Figure 2). Several close clusters were identified; these were also previously obtained by the eBURST algorithm.

Sequence analysis of the *oprD* gene in carbapenem-resistant isolates

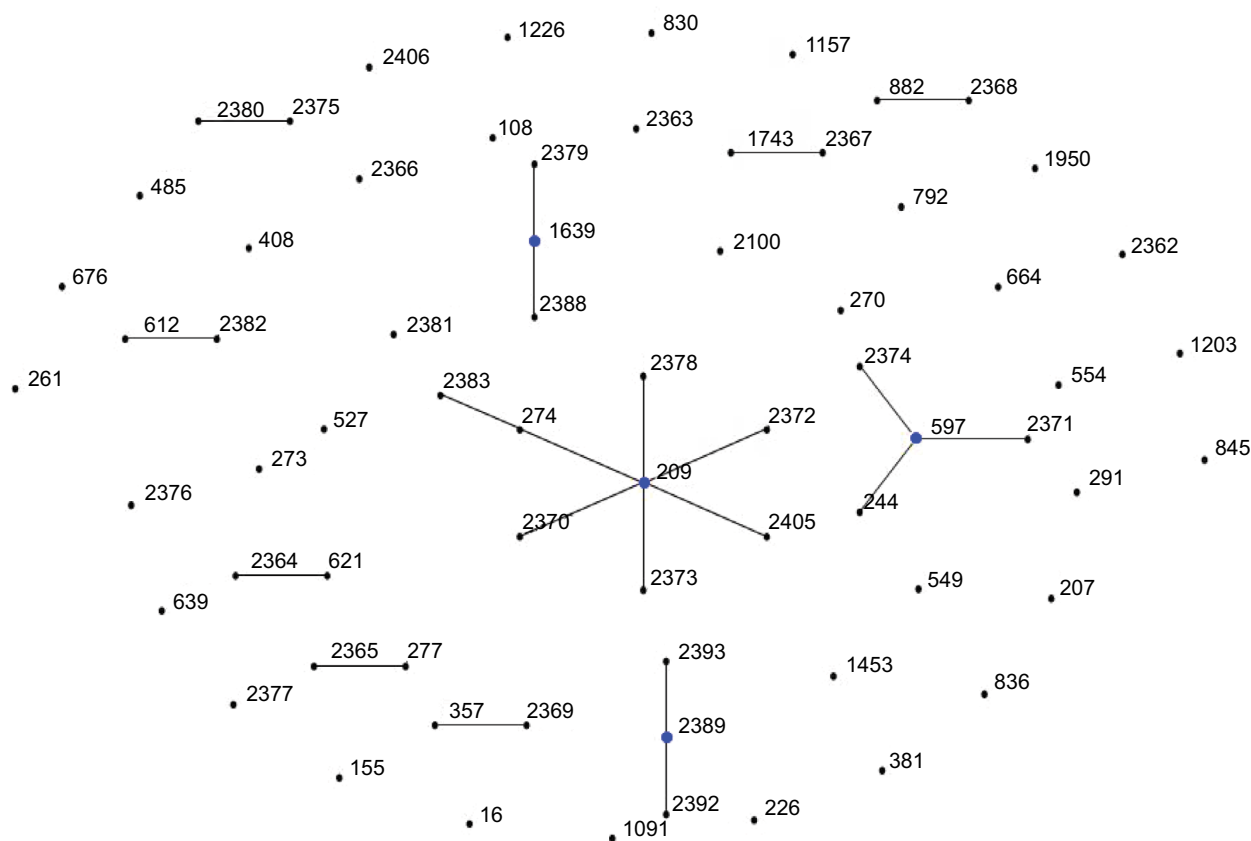
To assess the variations in *oprD* gene in carbapenem-resistant strains isolated from this hospital, *oprD* gene amplification and sequencing were done in 30 carbapenem-resistant and 15 carbapenem-susceptible isolates. These 45 strains were randomly selected. Of the 30 carbapenem-resistant isolates, there were 8 IPM-resistant and MEM-susceptible strains, 5 IPM-susceptible and MEM-resistant strains, and 17 IPM- and MEM-resistant strains. Fifteen strains with susceptibility to carbapenem were used as controls. The sequences were compared with the wild-type PAO1 sequence. As expected, the results showed a high diversity in the *oprD* sequences among isolates, especially in IPM- and MEM-resistant strains. The IPM-resistant and MEM-susceptible strains had relatively fewer mutations.

A comparative analysis between IPM-resistant and IPM-susceptible strains strongly suggested that alterations in L7 and L8 loops were responsible for their IPM susceptibility. In 17 of the IPM- and MEM-resistant strains, 14 strains encoded incomplete OprD proteins due to the presence of a premature stop codon at nt 150 (1 isolate), 624 (4 isolates), 714 (1 isolate), 912 (1 isolate), 951 (1 isolate), 1035 (3 isolates), and 1302 (3 isolates), caused by either a frameshift, nonsense mutation or large deletion (Table 4). Five of these 14 strains also carried large deletions at nt 52–61 (1 isolate), 561–624

Table 3 Characteristics and polymorphism of loci gene of *Pseudomonas aeruginosa*

Allele	Length	Haplotype	Polymorphic sites	π	θ	G+C	dN	dS	dN/dS
<i>acsA</i>	390	24	18	0.01258	0.01236	0.6871	0	0.05066	0
<i>aroE</i>	498	16	21	0.01466	0.01271	0.7044	0.02188	0.01307	1.6741
<i>guaA</i>	373	20	14	0.00889	0.01058	0.6568	0.0036	0.02389	0.1507
<i>mutL</i>	442	16	18	0.00792	0.0123	0.6715	0.00769	0.01326	0.5799
<i>nuoD</i>	366	15	13	0.00775	0.01092	0.6324	0.00471	0.02904	0.1621
<i>ppsA</i>	370	14	14	0.0087	0.0119	0.6676	0.01221	0	n.a
<i>trpE</i>	443	21	23	0.01403	0.01506	0.6616	0.01771	0.00761	2.3272
Concatenate	2882	68	120	0.00748	0.00877	0.6709	0.01318	0.00548	2.4051

Notes: The G+C content was calculated by Geneious 9.1. Haplotype, polymorphic sites, average number of synonymous (dS) and nonsynonymous sites (dN), average nonsynonymous/synonymous ratio (dN/dS), the nucleotide diversity per site (π), and the average number of nucleotide differences per site (θ) were found using DnaSP 5.0.

**Figure 1** Analysis of *Pseudomonas aeruginosa* STs using eBURST.

Notes: eBURST v3 was used to analyze the 68 unique STs. Each circle corresponds to an ST.

Abbreviation: ST, sequence type.

(3 isolates), and 816–855 (1 isolate), as shown in Table 4. The remaining three strains did not have a stop codon at the end of the *oprD* sequence. This result demonstrated that the loss or alteration of the original *oprD* confers resistance to carbapenem in *P. aeruginosa*.

On comparing the PAO1 strain with 15 IPM- and MEM-susceptible strains, 8 strains had 3 bp deletions at

nt 1116–1118 and 1147–1149 in L7 and 5 bp deletions at nt 1288–1292 in L8; 1 strain had 3 bp deletions at nt 1147–1149 in L7 and 5 bp deletions at nt 1288–1292 in L8; 1 strain had 3 bp deletions at nt 348–350 in L2, and 1116–1118 and 1147–1149 in L7. The remaining five IPM- and MEM-susceptible isolates had a premature stop codon at nt 162 (2 isolates), 219 (1 isolate), 693 (1 isolate), and

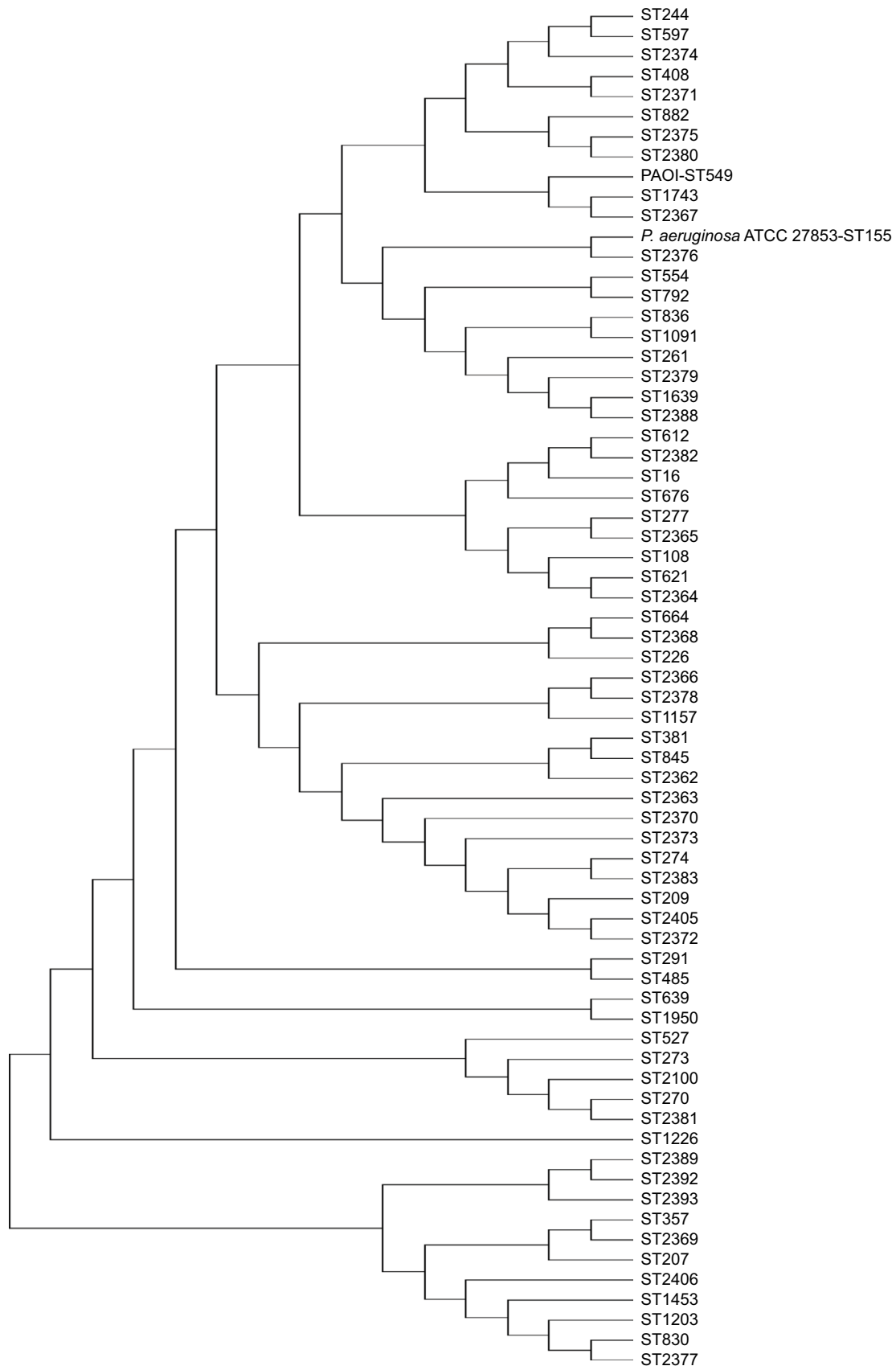


Figure 2 Rooted tree of the concatenated sequences (2800–2882 bp) of seven housekeeping genes of *Pseudomonas aeruginosa* using the NJ method based on Kimura 2 correction algorithm.

Abbreviations: NJ, neighbor joining; ST, sequence type.

Table 4 *OprD* genotype classification of 45 strains

Resistance phenotype Number of strains	Number of strains	Stop codon site	Large deletion site (nt)
IPM(R) MEM(R) 17	1	150	—
	3	624	561–624
	1	624	—
	1	714	—
	1	912	—
	1	951	816–855
	3	1035	—
	3	1302	—
	1	—	561–624
	2	—	—
IPM(R) MEM(S) 8	4	281	—
	1	906	862–866
	1	1119	—
	2	1302	—
IPM(S) MEM(R) 5	2	219	—
	1	381	—
	1	354	—
	1	—	1116–1118, 1147–1149
IPM(S) MEM(S) 15	2	162	—
	1	219	—
	1	693	—
	1	699	—
	8	1296	1116–1118, 1147–1149, 1288–1292
	1	1302	348–350, 1147–1149, 1288–1292
1	1302	—	

Notes: Forty-five strains were randomly selected: 17 IPM- and MEM-resistant strains, 8 IPM-resistant and MEM-susceptible strains, 5 IPM-susceptible and MEM-resistant strains, and 15 strains with susceptibility to carbapenem used as controls. The sequences were compared with the wild-type PAO1 sequence. “—” Represents “no”.

Abbreviations: IPM, imipenem; MEM, meropenem.

699 (1 isolate) due to various point, insertion, and deletion mutations (Table 4).

Screening for AmpC β -lactamase and MBL production

To characterize the clinical isolates further, all randomly selected *P. aeruginosa* isolates were tested for MBL and AmpC production. Among the 45 clinical isolates, two MBL-positive strains were identified: one of them was an IPM- and MEM-resistant strain and the other one was an IPM-resistant and MEM-susceptible strain. All 45 isolates were confirmed as AmpC-producing isolates, but not AmpC-overproducing isolates.

Discussion

P. aeruginosa infection in hospitalized patients remains an important issue; it is associated with high rates of mortality and morbidity in immunocompromised patients.²⁷ From a review of the rate of resistance to 14 antibiotics determined in this study, it was concluded that the treatment of *P. aeruginosa* infection is a challenge. In this study, 58.1% and 53.7% of the clinical isolates were resistant to IPM and MEM, respectively. IPM and MEM resistance rates reported from the surgical intensive care unit of the Peking University First Hospital were 84% and 31%, respectively.²⁸ Data from an earlier study of 65 hospitals from 22 regions in China in 2010 demonstrated that the rates of resistance of isolates to IPM and MEM were 23.1% and 18.1%, respectively.²⁹ There was no obvious association between the antibiotic resistance and geographic origin of the isolates. However, previous studies indicate an increased worldwide prevalence of carbapenem resistance.

MLST is an important tool in the study of the population structure and genetic diversity of *P. aeruginosa*.¹⁸ In this study, 68 STs were distinguished among 160 isolates; 28 of these were new STs. The three predominant STs in these isolates were ST1639, ST485, and ST261. Evidently, these isolates were extraordinarily rich in diversity and contained numerous novel genotypes. We found that the MLST STs and antimicrobial resistance profiles were not correlated in this study. Isolates with the same ST did not show a unique resistance profile pattern. This demonstrated that there is no definitive link between the ST of isolates and their resistance to these 14 antimicrobial agents. Genetic adaptation plays a major role in the successful establishment of a *P. aeruginosa* infection.³⁰ Analysis of dN/dS ratios showed that the seven housekeeping genes, except *aroE* and *trpE*, appeared under purifying selection. *aroE* and *trpE* are evolving predominantly by positive selection.

By eBURST and phylogenetic tree analysis of all the clinical isolates in this study, we demonstrated multiple genetically distinct clones in this hospital. Here, we confirm the non-clonal epidemic structure of the population.³¹ The CCs cluster together in the phylogenetic tree and belong to the same CC in a relaxed eBURST analysis. Mapping of resistance profile data onto the eBURST analysis data and the phylogenetic tree revealed the following: 1) the most similar resistance profiles did not cluster together and 2) isolates with the same STs did not share similar resistance phenotypes. Taken together, this shows that these strains displayed a relatively high degree of genetic variability, demonstrating that antibiotic resistance was most likely determined by individual genetic combinations.

Multiple studies have demonstrated that decreased expression or loss of OprD is the important mechanism of resistance to carbapenem in *P. aeruginosa*.³² In this study, 14 of 17 IPM- and MEM-resistant strains had a premature stop codon; it demonstrated that the deficient OprD plays an important role in resistance to carbapenem. On the other hand, strains susceptible to IPM and MEM with shortened loops in L7 and L8 were the major strain types in this hospital. This suggested that shortened L7 and L8 loops are responsible for IPM and MEM susceptibility in our clinical isolates. It has been suggested that L5, L7 and L8 may contribute to the constriction of the OprD channel opening; the shortened loop of L7 seems to open the OprD porin channel sufficiently to increase the penetration of MEM in the clinical strains.^{12,15} In this study, 9 of 15 IPM- and MEM-susceptible strains had modifications in L8 of OprD, but the role of L8 loop on carbapenem susceptibility has not been reported. On the other hand, five carbapenem-susceptible strains carry premature stop codons in the upstream direction (at the following positions: 162, 219, 693, and 699), which indicates that the loss of OprD is not only restricted to carbapenem-resistant clinical isolates but also found in carbapenem-susceptible clinical isolates. It seemed that the issue of carbapenem-susceptible clinical isolates with deficient OprD is not fully explained by the aforementioned carbapenem resistance mechanisms. El Amin et al also found that carbapenem-susceptible clinical isolates had a significant reduction of *oprD* mRNA and the presence of *oprD* mutations causing a frameshift or translational stop.³³ Ocampo-Sosa et al assessed the impact of the mutations in *oprD* on carbapenem resistance phenotype in a group of clinical isolates of *P. aeruginosa*. They believe that the function of OprD has not been completely defined.³⁴ In our clinical isolates, no *ampC* gene mutations were detected. MBL and AmpC production was also investigated in this study; however, it was demonstrated that only two isolates were MBL overproducers. As a result, MBL and AmpC may not significantly alter carbapenem susceptibility in our clinical strains.

In conclusion, *P. aeruginosa* isolates from the hospital in this study exhibited multidrug resistance to a variety of drugs and a higher rate of resistance against carbapenem. MLST analysis further revealed the genetic diversity of these clinical isolates of *P. aeruginosa*. *oprD* polymorphisms, particularly those resulting in L7 and L8 shortening, are the major cause of carbapenem sensitivity. Moreover, our results also show a high incidence of carbapenem-resistant strains with a loss of OprD, indicating that intact OprD is necessary for resistance to carbapenem. However, the resistance patterns observed

in this hospital cannot be fully explained by the currently described carbapenem resistance mechanisms. The role of OprD in carbapenem resistance needs to be intensively studied. These findings will help us to understand the mechanism of carbapenem resistance in *P. aeruginosa* isolates better.

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Disclosure

The authors report no conflicts of interest in this work.

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