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Original Article

Role of efflux pump and *OprD* porin expression in carbapenem resistance of *Pseudomonas aeruginosa* clinical isolates

Tuba Muderris[1], Rıza Durmaz[1,2], Birsen Ozdem[1], Tuba Dal[1,2], Ozlem Unaldı[3], Sibel Aydogan[1], Nevreste Celikbilek[1], Ziya C Acıkgoz[1,2]

[1] *Medical Microbiology Laboratory, Atatürk Training and Research Hospital, Ankara, Turkey*
[2] *Department of Medical Microbiology, Yıldırım Beyazıt University, Faculty of Medicine, Ankara, Turkey*
[3] *National Molecular Microbiology Reference Laboratory, Public Health Institution of Turkey, Ankara, Turkey*

Abstract

Introduction: In recent years, the prevalence of multidrug-resistant *P. aeruginosa* has remarkably increased. Thus, we wanted to investigate the carbapenem resistance mechanisms and clonal relationship among 80 carbapenem-resistant *P. aeruginosa* strains.

Methodology: Carbapenemase production was detected using the Modified Hodge Test (MHT), EDTA combined disc method (ECD), and PCR. Expression levels of efflux and porin genes were mesured by real-time reverse transcription PCR. Clonal relationship of the isolates was investigated by pulsed-field gel electrophoresis (PFGE).

Results: Carbapenemase production was detected in 7.5% of the isolates with MHT/ECD tests and in 11.3% of the isolates with PCR. Although the specificity of MHT/ECD was high, the sensitivitivity was low. *oprD* downregulation and *mexB*, *mexY*, and *mexD* overexpression were demonstrated in 55%, 16.3%, 2.5%, and 2.5% of the isolates, respectively. Multiple carbapenem resistance mechanisms were found in nearly a quarter of the isolates. PFGE typing of the 80 *P. aeruginosa* isolates yielded 61 different patterns. A total of 29 isolates (36.3%) were classified in 10 clusters, containing 2 to 7 strains. We could not find a strict relationship between PFGE profile and carbapenem resistance mechanisms.

Conclusions: Although *oprD* downregulation and MexAB-OprM overexpression were the most common mechanisms, carbapenem resistance was associated with multiple mechanisms in the study. MHT/ECD tests should not be used alone for investigation of carbapenemase production in *P. aeruginosa*. Rapid tests with high sensitivity and specificity should be developed for the detection of carbapenemase production in *P. aeruginosa*.

Key words: *Pseudomonas aeruginosa*; carbapenem resistance mechanisms; efflux pumps; porin; carbapenemase.

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Introduction

Pseudomonas aeruginosa is an opportunistic pathogen that causes a variety of infections in immunocompromised patients [1]. In recent years, because of increasing prevalence of multidrug-resistant (MDR) *P. aeruginosa* isolates, the selection of appropriate treatments has become difficult and associated morbidity and mortality rates have increased. Although carbapenems remain effective antibiotics for therapy of infections caused by MDR *P. aeruginosa* isolates, carbapenem resistance in *P. aeruginosa* isolates has been reported worldwide [2].

Carbapenems act primarily by inhibiting the peptidoglycan-assembling transpeptidases (penicillin-binding proteins [PBP]) located on the outer face of the cytoplasmic membrane. Carbapenem resistance of *P. aeruginosa* is mainly due to a combination of different factors including low outer membrane permeability, presence of the inducible AmpC chromosomal ß-lactamase, synergistic action of several efflux systems (MexAB–OprM, MexCD–OprJ, MexEF–OprN, MexXY–OprM), and the prevalence of transferable resistance determinants, in particular, carbapenem-hydrolyzing enzymes (mainly metallo-ß-lactamases [MBLs]) [3].

The outer membrane of Gram negative bacteria constitutes a semipermeable barrier that slows the penetration of antibiotics. The outer membrane plays an important physiological role in the transport of substances required for metabolism. The *P. aeruginosa* porin OprD is a substrate-specific porin that facilitates the diffusion of basic amino acids, small peptides, and carbapenems into the cell. OprD mediated resistance occurs as a result of decreased transcriptional expression of *oprD* and/or function mutations that disrupt protein activity [4,5].

The carbapenems, meropenem, ertapenem, and doripenem are substrates of the efflux pumps, whereas imipenem is not [6]. Therefore mutations leading to the upregulation of the MexAB-OprM active efflux system may increase the resistance to meropenem and doripenem, while imipenem is not affected by this route [3,7]. Determining the mechanisms involved in carbapenem resistance will contribute to the management of carbapenem-resistant *P. aeruginosa* infections. Hence, to investigate carbapenem resistance mechanisms including carbapenemase enzymes, we aimed to study OprD porin downregulation and MexAB-OprM, MexCD–OprJ, and MexXY-OprM efflux pumps upregulation, and to show the relationship between these mechanisms and minimal inhibitory concentrations (MIC) of carbapenems against carbapenem-resistant *P. aeruginosa* clinical isolates. Additionally, analyzing the clonal relationship among isolates was another aim of our study.

Methodology

This study was carried out at a training and education hospital with a 488-bed capacity, having ten intensive-care units with 67 beds, between April 2012 and January 2015. This is one of the biggest tertiary hospitals in the capital city of Turkey. The study was approved by the institutional review board (Date: 05/21/2014, Number: 77).

Pseudomonas aeruginosa isolates

A total of 951 *P. aeruginosa* isolates were included in this study. Of these isolates, 491 were meropenem and/or imipenem non-susceptible. Further studies were conducted on the non-repeated meropenem and/or imipenem-resistant 80 *P. aeruginosa* isolates selected according to stratified sampling (place, viability of the isolates, type of clinical samples, isolation periods). Only one isolate was obtained from each patient.

Identification of the isolates was done by conventional methods and by API 20NE (Bio-Meriéux, Marcy-l'Étoile, France).

Antibiotic resistance testing

Antibiotic resistance was detected by the Kirby Bauer disk diffusion method according to Clinical and Laboratory Standards Institute (CLSI) criteria [8]. Meropenem, imipenem, and ceftazidime resistance was confirmed by a gradient diffusion method (E test, Bio-Meriéux, Marcy-l'Étoile, France).

Modified Hodge Test (MHT)

In order to investigate carbapenemase production, MHT was performed according to the method of CLSI [8]. After an 18-24 hour incubation period, the plates were examined for enhanced growth around the microorganism at the intersection of the straight line and the zone of inhibition. Enhanced growth was evaluated as being positive for carbapenemase production [8].

Imipenem-EDTA combined disc method (ECD) / Meropenem-ECD Test

The Imipenem-EDTA combined disc method ECD was performed according to the method previously described for the detection of MBLs [9]. If the increase in the inhibition zone with the imipenem/meropenem and EDTA disc was ≥ 7 mm than the imipenem/meropenem disc alone, it was considered as MBL positive [9].

Detection of carbapenemase-encoding genes

Carbapenem-resistant *P. aeruginosa* isolates were tested for the carbapenemase genes KPC, IMP, VIM, OXA, and NDM by multiplex PCR as previously described [7,10-12]. In order to check the efficiency of the boiling methods for DNA isolation, each multiplex PCR tube also included the primers specific for the ribosomal *rpsL* housekeeping gene.

Molecular typing of the isolates

The clonal relatedness of the 80 *P. aeruginosa* isolates was evaluated by using pulsed-field gel electrophoresis (PFGE) analysis following the protocol described by Durmaz *et al.* [13]. Clonal relationship among isolates was evaluated by using the criteria of Tenover *et al.* [14].

Analyzing the transcription levels by real-time reverse transcription PCR (RT-PCR)

The transcription levels of the genes coding for the efflux proteins MexB, MexD, and MexY, as well as outer membrane protein OprD, were analyzed using real-time RT-PCR. Briefly, bacterial cells were cultured in 10 ml of LB broth (Merck, Darmstadt,Germany) at 37 °C and 180 rpm shaking to the late log phase (optical density at 600 nm [OD_{600}] = 0.8 to 1) and collected by centrifugation. Total RNA was extracted with a GeneJET RNA Purification Kit (Thermo Scientific, Lithuania), according to the manufacturer's recommendations. DNA was removed with RNase-Free DNase I treatment (Thermo Scientific, Vilnius, Lithuania). RNA concentrations were determined in a

UV-visible spectrophotometer (Nano-Drop 1000; Thermo Scientific, Wilmington, Delaware, USA). A gene coding for a ribosomal protein, *rpsL*, was used as a reference for normalizing the transcription levels of the target genes. Total RNA (20 ng) from all isolates was reverse-transcribed into single-stranded cDNA using the TaKaRa RNA PCR Kit (TaKaRa Bio Inc, Shiga, Japan). With a random primer, AMV reverse transcriptase synthesized cDNA at non-specific points along the RNA template, enabling amplification of the three efflux genes, *oprD* and *rpsL* from the same cDNA sample. Real-time PCR was performed using a Maxima SYBR Green qPCR master mix kit (Thermo Scientific, Waltham, Massachusetts, USA) following the manufacturer's recommendations. Primers specific for the genes of efflux proteins, *oprD* and *rpsL* were used in this study as described in the literature [7].

The Rotor-Gene Q software 1.7 was used to analyze expression levels. Isolates were considered to overexpress *mexB*, *mexY*, *mexD* when the expression levels of these genes were at least two, four and two fold higher than that of wild-type *P. aeruginosa* strain PAO1, respectively [7]. The expression levels of *mexB*, *mexY*, or *mexD* 1.8-1.9, 3-4 and 1.8-1.9 fold higher than those of *P. aeruginosa* PAO1 reference strain were considered as borderline results, respectively. Additionally, the expression levels of *mexB*, *mexY*, or *mexD* in clinic isolates higher than expression levels in *P. aeruginosa* PAO1 reference strains but less than borderline expression levels were considered as upregulation in this study. Downregulation of *oprD* expression was considered when it was equal or less than 30% of the expression level of the *P. aeruginosa* PAO1 reference strain [7]. Any expression level that was higher than 30% of the *P. aeruginosa* PAO1 expression level was considered as reduced *oprD* expression in this study.

Results

Characteristics of carbapenem-resistant P. aeruginosa isolates

Meropenem and/or imipenem resistance was determined in 491 of 951 *P. aeruginosa* isolates (51.6%). Overall, 33.3% (317/951), 46% (437/951), and 23.4% (223/951) of these isolates were resistant to imipenem, meropenem and ceftazidime, respectively. Among the 80 isolates further analyzed, the rates of resistance to these antibiotics were found to be 97.5% (78/80), 87.5% (70/80), and 32.5% (26/80), respectively. Of the 80 *P. aeruginosa* isolates, 41 (51.3%) were isolated from endotracheal aspirate/bronchoalveolar lavage, 16 (20%) were from wound/abscess, 13 (16.3%) were from urine, 6 (7.5%) were from blood, 2 (2.5%) were from sputum, and 2 (2.5%) were from pleura/peritoneal fluid. 81.3% (65/80) of the *P. aeruginosa* isolates were obtained from patients hospitalized in various intensive care units.

Production of carbapenemases

Six (7.5%) of the 80 *P. aeruginosa* isolates were both MHT and ECD positive. The carbapenemase genes were found in 9 (11.3%) isolates. VIM and KPC were detected in 6.3% (5/80) and 5% (4/80) of these isolates, respectively. MHT and ECD were positive in four of VIM positive isolates, though one VIM positive isolate was both MHT and ECD negative. All of the KPC positive isolates yielded negative results with MHT. The carbapenemase genes were not found in two MHT- and ECD-positive isolates. All of the isolates having the MBL gene showed high-level resistance (MIC ≥32 mg/L) to carbapenems (Table 1). The sensitivity and specificity for MHT/ECD were found to be 44% and 97%, respectively.

Table 1. Characterization of *P. aeruginosa* clinical isolates found positive by phenotypic and/or genotypic tests for carbapenemase.

Isolate	*mexB* exp.lev.	*mexD* exp.lev.	*mexY* exp.lev.	*OprD* exp.lev.	MHT	ECD	C	IMP	MEM	CAZ
86	1	1.1	1.2	**0.05**	+	+	-	≥ 32	≥ 32	256↑
17	1	1	**4.2**	**0.3**	+	+	VIM	≥ 32	≥ 32	16
31	**1.8***	1	1	**0.3**	-	-	KPC	≥ 32	≥ 32	1
59	1.4	1	1	1	-	-	VIM	≥ 32	≥ 32	0.75
32	1	1	1.5	0.5	-	-	KPC	≥ 32	≥ 32	0.5
157	**2.4**	1	1.3	**0.06**	+	+	VIM	≥ 32	≥ 32	32
50	**2.5**	1	1	**0.08**	-	-	KPC	≥ 32	4	0.5
64	1	1	1	**0.3**	-	-	KPC	≥ 32	8	16
152	1	1	1	**0.07**	+	+	VIM	≥ 32	≥ 32	256↑
153	1	1	2.1	**0.09**	+	+	VIM	≥ 32	≥ 32	12
137	**2.1**	**2.0**	2.2	1	+	+	-	≥ 32	≥ 32	256↑

C: Carbapenemase genes, IMP: Imipenem, MEM: Meropenem, CAZ: Ceftazidime. (-): Negative result, (+): Positive result, exp. lev.: Expression level; *Borderline expression; Bold digits: Indicated overexpression of efflux pump and downregulation of *oprD* porin.

Table 2. Prevalence of resistance mechanism combinations in the 80 *P. aeruginosa* clinical isolates and MIC range for IMP, MEM, CAZ of these isolates.

	No. (%) of isolates with:			
	Combinations of the resistance genes No. (%) of isolates	**MIC of IMP**	**MIC of MEM**	**MIC of CAZ**
↓*oprD*+↑*mexB*	4 (5)	16 - ≥ 32	24 - ≥ 32	0.75 - 48
↓*oprD*+↑*mexB*+↑*mexD*	1 (1.3)	≥ 32	6 - ≥ 32	0.5
↓*oprD*+↑*mexB*+ *mexY**	1 (1.3)	≥ 32	6 - ≥ 32	0.75
↓*oprD*+↑*mexB*+KPC	1 (1.3)	≥ 32	4 - ≥ 32	0.5
↓*oprD*+*mexB**+KPC	1 (1.3)	≥ 32	≥ 32	1
↓*oprD*+↑*mexB*+VIM	1 (1.3)	≥ 32	≥ 32	32
↓*oprD*+↑*mexY*+VIM	1 (1.3)	≥ 32	≥ 32	16
↓*oprD*+KPC	1 (1.3)	≥ 32	8 - ≥ 32	16
↓*oprD*+VIM	2 (2.5)	≥ 32	≥ 32	12 - ≥ 256
↑*mexB*+↑*mexD*	1 (1.3)	≥ 32	≥ 32	≥ 256
↑*mexB*+*mexY**	1 (1.3)	12 - ≥ 32	≥ 32	16

IMP: Imipenem, MEM: Meropenem, CAZ: Ceftazidime; *Borderline expression.

Expression levels of OprD porin and efflux pumps

The most common mechanism for carbapenem resistance was porin downregulation, observed in 55% of the isolates. The rate of the isolates showing reduced expression for *oprD* (but not downregulation) was 25%. MexAB-OprM was the predominant efflux system, with approximately 47.6% of the isolates showing increased expression. The rate of *mexB* upregulation, overexpression, and borderline expression levels were 28.8%, 16.3% and 2.5%, respectively. Increased expressions for *mexD* and *mexY* were seen in 17.5% and 28.8% of the isolates, respectively. The rate of the isolates showing overexpression for these two efflux pumps was 2.5%. The rate of the strains having both an overexpressed efflux pump and a downregulated *oprD* was very low, varying from 1.3% to 5%.

Almost all the isolates showing only downregulation of *oprD* had high levels of imipenem MIC (≥32 mg/L). The isolates having only overexpression of *mexB* had high levels of resistance to imipenem (≥32 mg/L) and meropenem MIC (≥32 mg/L). Overexpression for both *mexD* and *mexY* had high levels of imipenem MIC (≥32 mg/L). Detailed information on the relationship between the combinations of resistance mechanisms and antibiotic resistance is provided in Table 2. In our study, all isolates with the MBL gene showed high level resistance (MIC ≥32 mg/L) to the carbapenems. Additionally, most of these isolates had downregulated *oprD* (Table 1).

PFGE typing results

*Xba*I digestion of the 80 *P. aeruginosa* isolates yielded 61 different PFGE patterns. Ten of these PFGE patterns (pulsotypes) were clusters containing from 2 to 7 isolates, while the remaining 51 were unique, having only one isolate each. The largest pulsotype, 16, included 7 (8.8%) isolates, followed by pulsotype 11 with 4 isolates, pulsotypes 23 and 57 (3 isolates, 3.8%) and pulsotypes 9, 15, 21, 35, 46, 59 (2 isolates, 2.5%). A total of 29 isolates were in clusters, with a clustering ratio of 36.3%. When we consider a similarity coefficient higher than 85%, 13 major PFGE groups, designated as PFGE group I, II, III, IV, V, VI, VII, VIII, IX, X, XI, XII and XIII, were defined, and genetic relatedness was detected in 61.3% (49/80) of the strains tested. The remaining 31 strains were clonally unrelated.

There was no direct relationship between PFGE profiles and carbapenem resistance mechanism. Almost all *mexD* and *mexY* overexpressed isolates displayed unique pulsotypes (Table 3). The largest pulsotype (pulsotype 16) harbored seven isolates cultured from the patients hospitalized in two clinical settings in a

Table 3. Prevalence of resistance patterns according to PFGE profiles.

	Resistance gene(s)														
	mexB				***mexD***				***mexY***				***oprD***		
PFGE profiles	**U**	**O**	**B**	**N**	**U**	**O**	**B**	**N**	**U**	**O**	**B**	**N**	**RE**	**D**	**N**
Unique (n:31)	10	6	0	15	5	1	0	25	7	2	2	20	7	15	9
Group (n:49 strains in eight groups)	13	7	2	27	7	1	0	41	11	0	1	37	13	29	7
Total (n:80)	23	13	2	42	12	2	0	66	18	2	3	57	20	44	16

U: Upregulation, O: Overexpression, B: Borderline expression, N: Normal expression, RE: Reduce expression, D: Downregulation.

period of more than 16 months. The other large cluster (pulsotype 11) included four isolates recovered from patients in reanimation intensive care units between January 01, 2014 and June 02, 2014. Only two of the 13 *mexB* overexpressed *P. aeruginosa* isolates were in the same pulsotype, the remaining 11 were in different pulsotypes. The 44 isolates showing *oprD* downregulation were divided into 35 distinct pulsotypes.

Discussion

Carbapenem resistance is an increasingly serious problem worldwide, including in Turkey [15]. Usually, antibiotic use is high in developing countries, particularly in intensive care

units. This high consumption of antimicrobials results in a higher incidence of resistant bacteria [4]. The rate of carbapenem resistance differs in distinct geographic regions. In various studies from different countries, the prevalence rates of imipenem, meropenem, and ceftazidime resistance in *P. aeruginosa* isolates were reported as 21.9–44.9%, 15.4–38.4% and 22.4–29.4%, respectively [16-18]. These rates were found to be 30.1–51.0%, 37.2–45%, and 35.1–48% in Turkey [19,20]. The rates of resistance to imipenem (46%), meropenem, (33%), and ceftazidime (23.4%) observed in the current study were similar to the literature data.

In various studies, the prevalence of carbapenem resistance mechanisms of *P. aeruginosa* including carbapenemase enzymes, downregulation of OprD porin, overexpression of efflux pumps, and overproduction of AmpC were reported as 10–78.4%, 75–94.7%, 66.7–84.2%, and 47.4–65.6%, respectively. Additionally, in these studies, the prevalence rates of efflux pumps overexpression, including MexAB-OprM, MexEF-OprN, MexXY-OprM, and MexCD-OprJ were found to be 21.1–57.9%, 3.5–26%, 15.8–37.5%, and 2.6–31.3% respectively [7, 21-25]. In our study, the prevalence of carbapenem resistance mechanisms in *P. aeruginosa* was comparable with that in previous studies, except for the prevalence of overexpression of MexXY-OprM, which was lower.

Carbapenems are important drugs for the treatment of *P. aeruginosa* infections and MBLs are characterized by the ability to hydrolyze carbapenems. Genes encoding MBLs are transferred by mobile genetic elements [26]. MBL production is noted in massive outbreaks, including the international dissemination of resistant strains [27]. So, early detection of MBL producing *P. aeruginosa* is very important in terms of determining treatment and preventing the spread of infection with resistant isolates. Although MHT is a phenotypic method recommended for detecting carbapenemase production in *Enterobacteriaceae* isolates [8,24], there are many studies related to the use of MHT in *Pseudomonas* spp. isolates [24,25,28]. In the current study, phenotypic results were confirmed with PCR and four of the six MHT and ECD positive isolates were VIM-positive. Two isolates were found positive for MHT and ECD, although MBL genes were not detected in these isolates. In parallel to our results, previous studies also demonstrated false positive results with MHT in the presence of extended-spectrum β-lactamases (ESBLs) and AmpC β-lactamases [24]. Even though the sensitivity of MHT in KPC producing *P. aeruginosa* was reported to be 77% in a previous study [29], none of the isolates defined KPC-positive with PCR was found to be a carbapenemase producers, with MHT, in our study. Previous studies showed that the sensitivity and specificity of MHT for detection of metallo-β-lactamases ranged from 58 to 78% and 57% to 100%, respectively [28,30]. While the specificity rate was high (97%), sensitivity was slightly lower (44%) in our study. This may be due to the limited number of our isolates and/or the presence of other resistance mechanisms such as expression of AmpC, and/or the presence of other class C beta lactamases. A limitation of this study is that we did not test the expression of AmpC nor other class C beta-lactamases, this could be done in further studies. On the other hand our results and the published data, suggest that MHT and ECD can be used because they are easily accessible, cheap and easy to perform tests [24,25,28,31], but they should be confirmed by molecular tests.

Acquired carbapenem resistance due to the production of MBLs has been increasingly reported in *Pseudomonas* spp. The prevalence of *Pseudomonas* spp that produce MBLs can be markedly different in distinct geographical areas, even among different hospitals in the same area [32]. In Turkey, the prevalence of *P. aeruginosa* that produce MBLs were reported as between 10% and 56.8% in previous studies [21,32,33]. Additionally, studies indicate that VIM producing *Pseudomonas* spp. are endemic in various Mediterranean countries, including Turkey [34]. The lower rate of VIM positivity (6.3%) in our current study indicated that, despite the significant increase in carbapenem resistance in *P. aeruginosa* isolates in Turkey over the last several years, MBL-producing strains did not become a serious clinical and therapeutic problem in our hospital during the study period. This is positive news, because the risk of spread of the resistance mechanism is much lower.

Although downregulation of the OprD porin alone is a source of intermediate susceptibility or resistance to imipenem [35], it decreases the susceptibility to a lesser extent to meropenem in *P. aeruginosa* [36]. In our study, more than half of the isolates had OprD downregulation and in 70.5% of the isolates, OprD downregulation was the unique detected mechanism for carbapenem resistance. On the other hand, more than 90% of the isolates with OprD downregulation showed an imipenem MIC value equal or more than 32 mg/L, and 86.4% of the isolates had a meropenem MIC ≥2 mg/L. However, while our data was in agreement with the results of Livermore [35], it wasn't in agreement with the results of Rostami [26] and Ellappan [36]. In addition, reduced expression (but not downregulation) or normal expression for *oprD* of some *P. aeruginosa* isolates were found, though these isolates showed high level resistance to imipenem in our study. This may be caused by mutational changes contributing to a loss of OprD function, such as mutations in loops L2 and L3 of the OprD porin.

The MexAB-OprM system is one of the largest multidrug-resistant RND family efflux pumps with high levels of expression in *P. aeruginosa* [37,38]. This is the most significant one among the known efflux pump mechanisms [39]. Similarly, the MexAB-OprM system was the predominant efflux pump among the studied efflux systems in the current study. Although meropenem is an efflux pump substrate, imipenem is not a substrate due to the lack of a heterocyclic side chain [6]. In our study, almost all of the isolates with overexpression of MexAB-OprM alone and overexpression of MexXY-OprM alone showed high resistance for meropenem. Although other efflux pumps, except MexAB-OprM, do not affect ceftazidime susceptibility [39-42], the isolate that overexpressed MexXY-OprM was ceftazidime-resistant in our study. It may be caused by another resistance mechanism, such as the inducible AmpC chromosomal ß-lactamase, mutations in *oprD,* or efflux pumps regulators, and unknown mechanisms.

PFGE typing results demonstrated that most of the carbapenem-resistant *P. aeruginosa* isolates belonged to distinct pulsotypes. Similar to our results, high genetic heterogeneity was also reported among carbapenem-resistant and -susceptible strains of *P. aeruginosa* in previous studies [3,43,44]. These findings indicated that carbapenem resistance in our hospital is not the result of cross-transmission within the hospital. In parallel to previous observations [45], we found that many isolates with common antibiotic resistance patterns showed distinct pulsotypes. These observations support previous data indicating that the outbreak strains can exhibit the same pulsotype and antibiotype [43-45]; however, the strains collected during a long time period such as ours may display different typing profiles.

Lack of data about the expression of AmpC and other class C beta lactamases and also the mutation in the *oprD* gene are limitations of this study.

Conclusion

Many studies examining the mechanisms of carbapenem resistance have involved controlled laboratory-derived strains; however, we analyzed the mechanisms of carbapenem resistance in clinical isolates of MDR *P. aeruginosa*. Multiple carbapenem resistance mechanisms were found in a quarter of the isolates in the current study. Although OprD downregulation and MexAB-OprM overexpression were the most common mechanisms, carbapenem resistance was usually associated with multiple mechanisms. This study showed that MHT and ECD are easy tests for the early recognition of carbapenemase production in *P. aeruginosa,* but the results should be confirmed by molecular tests. Rapid tests with high sensitivity and specificity should be developed for the detection of carbapenemase production in *P. aeruginosa*. The clustering rate obtained by PFGE typing revealed that carbapenem-resistant isolates mainly originated from epidemiologically unrelated sources. Additionally, there was no strict relationship between PFGE profiles and carbapenem-resistance mechanisms. The high consumption of carbapenems in hospitals worldwide exerts a strong selective pressure on clinical populations of *P. aeruginosa*, promoting the emergence, and spread, of resistant clones. Carbapenem-resistant *Pseudomonas* spp. is one of the most important bacteria causing treatment failures. Determination of antibiotic resistance rates and mechanisms responsible for antibiotic resistance will contribute to the management of *P. aeruginosa* infections with multiple drug resistance. Molecular epidemiological methods are important tools in the control and prevention of healthcare-associated infections. Early detection of MBL-producing *P. aeruginosa* will help to choose proper antimicrobial therapies and avoidance of dissemination of these MDR isolates. Additionally, regional surveillance, early detection of carbapenemase producers, and knowledge of resistance mechanisms are imperative for the improvement of resistance prevention strategies. A thorough understanding of resistance mechanisms will

allow the development of new antimicrobials that target these mechanisms in the future.

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Corresponding author

Tuba Muderris, MD.
Department of Medical Microbiology, Atatürk Training and Research Hospital, Bilkent Street, 06800, Ankara, Turkey.
Phone: +90 0505 502 51 43
Fax: +90 0312 291 25 25
e-mail: tubamuderris@yahoo.com

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