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Characterization of imipenem resistance mechanisms in *Pseudomonas aeruginosa* isolates from Turkey

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Abstract

The emergence of carbapenem resistance in *Pseudomonas aeruginosa* threatens the efficacy of this important anti-pseudomonal antibiotic class. Between 2003 and 2006, an increase in the number of carbapenem-resistant *P. aeruginosa* isolates at the Zonguldak Karaelmas University Hospital was observed (Zonguldak, Turkey). To assess the imipenem resistance mechanisms emerging in these *P. aeruginosa* isolates, they were characterized by amplified fragment length polymorphism typing, which revealed diversity among imipenem-resistant isolates as well as two clonally related outbreak groups. The molecular mechanism of carbapenem resistance was characterized in a representative isolate from each clonal group. Mutational disruption of *oprD* was the most frequently encountered resistance mechanism (23/27 isolates).

Keywords: Imipenem, *oprD*, *Pseudomonas aeruginosa*, Turkey

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Pseudomonas aeruginosa is a major cause of nosocomial infections and is associated with hospital-acquired pneumonia as well as urinary tract, surgical site and bloodstream infections [1,2]. These infections are difficult to control with antibiotics and disinfectants because of the high intrinsic resistance of this organism. The use of broad-spectrum antimicrobial agents such as the carbapenem antibiotics is recommended for suspected *P. aeruginosa* infections as they have excellent activity against this species [3,4]. However, resistance to carbapenem antibiotics such as imipenem has risen among *P. aeruginosa* and can also emerge during therapy causing treatment failure [5]. This study characterizes the mechanisms contributing to imipenem resistance in *P. aeruginosa* clinical isolates from Turkey, a region where carbapenem resistance rates are high [6,7].

Seventy-four *P. aeruginosa* isolates exhibiting imipenem resistance were identified at Zonguldak Karaelmas University Hospital between 2003 and 2006 with increasing prevalence in their isolation over the study interval (5, 6, 19 and 43 isolates per year). Antibiotic susceptibilities testing showed that amikacin was the most effective antimicrobial against all the isolates (81%) *in vitro*, followed by cefepime (64%) and piperacillin–tazobactam (63%).

To assess whether the increased isolation rate of carbapenem-resistant *P. aeruginosa* isolates was caused by a clonal outbreak, strain typing by amplified fragment length polymorphism (AFLP) was performed [8]. The AFLP fingerprinting showed that the 74 imipenem-resistant strains belonged to 27 divergent clonal lineages indicating strain diversity. However, two larger clonal groups were identified (n20 and n10), which contributed to the increase in numbers in 2005 and 2006. The first cluster of isolates (n20) appeared in April 2005 and the second group (n11) first appeared in January 2006, followed by an increase in the numbers isolated. Isolates from both clusters were no longer detected after September 2006. Despite the two clonal transmission episodes, emergence of imipenem resistance occurred in independent strains. A single representative strain from each of the 27 AFLP clonal groups was chosen for further investigation into the mechanisms of imipenem resistance in the diverse *P. aeruginosa* clinical strains isolated.

As imipenem resistance could be explained by the dissemination of a transferrable resistance mobile element among different strains, a representative isolate from each AFLP clonal group was screened for the presence of diverse carbapenem resistance genes, including bla_{OXA-23}, bla_{OXA-24}, bla_{OXA-51}, bla_{OXA-58}, bla_{GIM-1}, bla_{SIM-1}, bla_{SPM-1}, bla_{IMP}, *and* bla_{VIM} as previously described using isolated bacterial DNA as a template [9]. No previously characterized carbapenemase resistance genes were identified, suggesting that the imipenem

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resistance observed is not the result of these transferrable resistance genes. In addition, all isolates were negative for metallo-β-lactamase production according to the Etest® MBL strip assay. The transfer of carbapenem resistance from resistant isolates to a susceptible recipient strain by conjugation could not be demonstrated (data not shown), suggesting that imipenem resistance in these strains is unlikely to be transferrable and consequently may be the result of chromosomal resistance mechanisms.

As almost all (73 out of 74) imipenem-resistant strains also exhibited chloramphenicol resistance, we hypothesized that the LysR-type transcriptional regulator, MexT, might be involved in this phenotype. MexT directly regulates the MexEF-oprN efflux system that confers resistance to a number of antibiotics including chloramphenicol [10,11]. In addition, MexT has also been shown to play a role in the down-regulation of the outer membrane porin, OprD, which facilitates uptake of imipenem into the cell [12]. As determined by quantitative RT-PCR, the ratio of detected *mexE* transcript relative to that of *proC* (internal control) in PA14*nfxC* (*mexEF-oprN* over-expressing control) was 262 ± 73-fold higher than the ratio detected in PA14 wild-type cells (Tables 1 and 2). In contrast, none of the isolates exhibited a *mexE* : *proC* expression ratio that was significantly higher than that of PA14 wild-type, confirming that *mexE* is not induced in these strains. This suggests that MexT is not constitutively active in these strains and that imipenem and chloramphenicol resistance occurred independent of the MexT regulatory pathway.

Mutational inactivation of *oprD* is known to occur in *P. aeruginosa* clinical isolates leading to carbapenem resistance [1]. To establish whether this could have occurred in the 27 representative imipenem-resistant isolates from this study, the *oprD* gene was sequenced in each isolate. This revealed that the *oprD* sequence was modified in about 85% of the imipenem-resistant isolates. The mutations observed in *oprD* included small and large deletions, insertions and duplications causing frame shifts in the *oprD* coding sequence as well as substitutions resulting in early translational termination (Table 2).

TABLE 1. Probes and primers used in quantitative reverse transcription–polymerase chain reaction analysis

Target gene	UPL probe[a]	Primers[b]
proC	#20 (5′-CCAGCCAG-3′)	5′-CTTCGAAGCACTGGTGGAG-3′ 5′-TTATTGGCCAAGCTGTTCG-3′
mexE	#61 (5′-CTGGGCAA-3′)	5′-CACCCTGATCAAGGACGAAG-3′ 5′-CCAGGACCAGCACGAACT-3′
oprD	#132 (5′-TGCTGCTC-3′)	5′-GGGTTCATCGAAGACAGCAG-3′ 5′-GCCGTCACGGTTGAAATAGT-3′
ampC	#23 (5′-GGGCTGGG-3′)	5′-CCATCGCGGTTACTACAAGG-3′ 5′-GAGATCGGCCAGTCGTAGG-3′

[a]Probes from Roche Universal ProbeLibrary™ (Roche Applied Biosystems, Burgess Hill, UK) labelled at the 5′-terminal with fluorescein (*FAM*) and 3′-proximal with a dark quencher dye.
[b]Specific primers for probe hydrolysis reaction.

Characterization of *oprD* allelic diversity was performed by comparing the observed mutations with those deposited in the Pseudomonas Genome Database [13,14], which showed that the isolates were broadly distributed among three major OprD cluster groups (Group A-6 isolates, Group B-11 isolates, Group C-10 isolates). In addition to the identified mutations, 13 of the *oprD* sequences contained silent nucleotide polymorphisms that had not previously been described, and that are reported here as novel alleles (Table 2). Half of those novel alleles belong to the OprD cluster Group A. These findings suggest that the imipenem resistance phenotypes observed can be attributed to independent mutational events that led to the inactivation of *oprD*.

Four imipenem-resistant isolates (TY5010, TY5017, TY5018 and TY5041) had functional *oprD* genes based on sequence analysis. This suggests that a resistance mechanism other than MexT-mediated OprD down-regulation or defective OprD mutations were responsible for the imipenem resistance observed in these strains. Additional analysis also ruled out the presence of the bla_{KPC}, bla_{GES} and bla_{OXA-40} carbapenemase genes. Moreover, additional phenotypic analysis (Hodge Test) excluded other undetected carbapenemase genes in these strains [15]. To assess whether the down-regulation of *oprD* or up-regulation of *ampC* could account for imipenem resistance in these strains, quantitative RT-PCR was employed to assess the level of *oprD* and *ampC* expression in each strain. Only TY5010 exhibited significant down-regulation of *oprD*, which was approximately nine-fold lower than expression levels in PA14 (Table 2). AmpC was not induced in TY5041, but was highly induced in TY5017 and TY5018 (*ampC* : *proC* ration of 2690 and 4340, respectively), which could account for their increased imipenem resistance levels. To assess whether AmpC contained a mutation in its DNA sequence that subsequently led to increased expression levels, sequence analysis of AmpC was performed. Interestingly both isolates (TY5017 and TY5018) contained the recently described T105A mutation, which has been shown to lead to an increased affinity for imipenem and as such is classified as an extended-spectrum AmpC [16]. However, some authors suggest that T105A is only a polymorphism, therefore additional analysis will be required to clarify the exact role of this polymorphism in those strains. [17]. In addition, TY5017 also had a previously described R79Q mutation in addition to T105A, which have previously been

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TABLE 2. Molecular investigation of chromosomal resistance mechanisms in imipenem-resistant *Pseudomonas aeruginosa* isolates

Strain	Imipenem MIC (μg/mL)	Resistance gene expression[a]				*oprD* gene sequence
		mexE	*ampC*	*oprD*	DOM[b]	Non-synonymous/frame shift mutations
TY5003	16	1.33	NA	NA	yes	large 427-bp deletion from NT 47
TY5005	16	0.39	NA	NA	yes	1-bp insertion after NT 1205 → Frame shift → stop codon at NT 1423-5
TY5007	32	2.98	NA	NA	yes	large 427-bp deletion from NT 47
TY5009	16	0.45	NA	NA	yes	C-T base substitution at NT 1279 → stop codon at NT 1279-81
TY5011	32	0.02	NA	NA	yes	11-bp deletion of NT96-106 → Frame shift → stop codon at NT 147-50
TY5014	16	0.95	NA	NA	yes	1-bp deletion of NT38 → Frame shift → stop codon at NT280-2
TY5021	16	14.17	NA	NA	yes	G-T base substitution at NT 901 → early stop codon
TY5027	32	1.09	NA	NA	yes	duplication of CT at NT 670-1 → Frame shift → stop codon at NT 688-90
TY5028	8	0.90	NA	NA	yes	1-bp deletion of NT 903 → Frame shift → stop codon at NT 1033-5
TY5030	16	0.54	NA	NA	yes	28-bp deletion from of NT 662-690 → Frame shift → stop codon at NT 760-2
TY5031	32	0.52	NA	NA	yes	A-G base substitution at NT 1251 → early stop codon
TY5033	32	1.28	NA	NA	yes	duplication of G at NT 455 → Frame shift → stop codon at NT 219-21
TY5034	16	0.30	NA	NA	yes	4-bp deletion of NT 869-872 → Frame shift → stop codon at NT 1030-2
TY5036	8	0.30	NA	NA	yes	1-bp deletion of NT 737 → Frame shift → stop codon at NT 1033-5
TY5040	32	1.24	NA	NA	yes	C-A base substitution at NT 1197 → early stop codon
TY5043	>32	1.88	NA	NA	yes	duplication of CCTC at NT 69-72 → Frame shift → stop codon at NT 691-693
TY5046	16	0.01	NA	NA	yes	C-T base substitution at NT 703 → stop codon at NT 703-5
TY5053	32	0.02	NA	NA	yes	duplication of G at NT 455 → Frame shift → stop codon at NT 219-21
TY5056	16	0.01	NA	NA	yes	13-bp deletion of NT 63-75 → Frame shift → stop codon at NT 1021-3
TY5062	16	0.11	NA	NA	yes	large 427-bp deletion from NT 47
TY5066	16	0.78	NA	NA	yes	duplication of G at NT 455 → Frame shift → stop codon at NT 219-21
TY5067	32	0.03	NA	NA	yes	G-A base substitution NT 195 → early stop codon
TY5076	32	0.04	NA	NA	yes	C-T base substitution NT 1295 → early stop codon
TY5010	32	0.55	0.34	0.11	no	–
TY5017	>32	0.90	2690.16	0.53	no	–
TY5018	16	0.38	4340.36	0.54	no	C-G base substitution at NT 452 (Ala-Gly)
TY5041	>32	0.21	0.19	0.53	no	–

[a]Target gene : *proC* ratio in clinical isolates relative to PA14.
[b]NA, not applicable.
[c]DOM, defective OprD mutation. These *oprD* sequences are predicted to encode non-functional Opr porins.

reported to coincide [16]. Overall, the combined down-regulation of *oprD* and the induction of *ampC* could account for the imipenem phenotype observed.

A single imipenem-resistant isolate (TY5040) exhibited no defective mutations in *oprD*, had levels of *oprD* expression comparable to PA14 and did not over-express *ampC*. A possible explanation could be the post-transcriptional down-regulation of *oprD*, which has been previously suggested [18]. Alternatively, an as yet uncharacterized resistance mechanism could contribute to imipenem resistance in this strain. However, the majority of imipenem-resistant clinical strains show mutational inactivation of OprD as the predominant pathway leading to imipenem resistance in the isolates obtained in the Zonguldak Karaelmas University Hospital in Turkey.

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Transparency Declaration

The authors declare no conflicts of interest.

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