Investigation of carbapenem heteroresistance among different sequence types of *Pseudomonas aeruginosa* clinical isolates reveals further diversity

Pseudomonas aeruginosa is a leading infectious agent causing difficult-to-treat nosocomial infections due to the multiresistant phenotype that it often exhibits. Expression of the intrinsic AmpC β -lactamase, overexpression of Mex-Opr-type efflux pumps as well as repression of porin OprD have been associated with β -lactam resistance in this species (Lister *et al.*, 2009). Also, different genotypes of carbapenem-susceptible *P. aeruginosa* have been documented to produce carbapenem-heteroresistant subpopulations (Pournaras *et al.*, 2007).

Briefly, besides their elevated carbapenem MICs, the carbapenem-heteroresistant *P. aeruginosa* subpopulations were found to have a prolonged lag phase and a short exponential phase in the growth curve relative to their native populations (Pournaras *et al.*, 2007). This might explain their delayed phenotypic detection, up to 48 h of incubation at 37 °C for some isolates, as distinct colonies within the inhibition zone of carbapenem Etests or discs. Also, the heterogeneous subpopulations were found to have the intrinsic efflux pump systems MexAB-OprM and MexXY-OprM upregulated while porin OprD was downregulated, relative to the native populations (Ikonomidis *et al.*, 2008); the combination of these antimicrobial resistance mechanisms was suggested to contribute to carbapenem heteroresistance in *P. aeruginosa*.

This report investigates the distribution of carbapenem heteroresistance among several *P. aeruginosa* sequence types (STs) and documents the further diversity of this phenotype.

All single *P. aeruginosa* clinical isolates that were initially identified as susceptible to at least one carbapenem (imipenem or meropenem) by the VITEK 2 automated system (bioMérieux) in the Laboratory of

Microbiology, University Hospital of Larissa, Greece, were included in the study. The study period lasted from late November 2007 to early December 2008. MICs of imipenem, meropenem, piperacillin/tazobactam, ceftazidime, ciprofloxacin, amikacin and gentamicin were tested by agar dilution according to Clinical and Laboratory Standards Institute criteria (CLSI, 2008). Reference strain *P. aeruginosa* ATCC 27853 as well as previously characterized carbapenem-heteroresistant *P. aeruginosa* clinical isolate 6315 (Pournaras *et al.*, 2007) were used as controls. The genetic relationship of the tested isolates was investigated by PFGE of *Xba*I-digested genomic DNA; banding patterns were compared using criteria proposed by Tenover *et al.* (1995). Also, multilocus sequence typing (MLST) was performed according to a previously described scheme (Curran *et al.*, 2004) and STs were assigned through the *P. aeruginosa* MLST database (<http://pubmlst.org/paeruginosa/>).

Population analysis profile assays (PAPs) with imipenem and meropenem were performed by spreading approximately 10^8 c.f.u. (100 μ l of a starting inoculum containing approx. 10^9 c.f.u. ml⁻¹) on Mueller–Hinton agar plates with imipenem or meropenem in serial dilutions for concentrations ranging from 1 to 32 mg l⁻¹ and incubating the plates for 48 h. The analysis was performed three times for all isolates and mean values of viable c.f.u. were calculated. The frequency of appearance of heteroresistant subpopulations at the highest drug concentration was calculated by dividing the number of colonies grown on the antibiotic-containing plate by the colony counts from the same bacterial inoculum grown on antibiotic-free plates. For each strain, three distinct colonies grown at the highest drug concentration were subcultured daily in antibiotic-free

medium for 1 week and carbapenem MICs were tested by agar dilution on Mueller–Hinton agar plates to check the stability of heteroresistant phenotypes.

During the study period, 283 *P. aeruginosa* clinical isolates were recorded from which 195 (68.9%) were susceptible to imipenem and/or meropenem. Twenty-one of the latter isolates were selected at random and further investigated with respect to their putative heteroresistant phenotype. MICs of imipenem and meropenem ranged from <1 to 16 mg l⁻¹ and from <1 to 32 mg l⁻¹, respectively. The susceptibility to the remaining antipseudomonal antimicrobials is shown in Table 1. The isolates belonged to at least 15 distinct genotypes as shown by PFGE analysis, and 12 ST types as shown by MLST analysis. Of note, seven isolates belonged to ST696, which is a single locus variant of ST235, a member of international clonal complex CC11 (Libisch *et al.*, 2008), which has been associated with European epidemic clones (Nemec *et al.*, 2010).

In PAPs using imipenem, heteroresistant subpopulations showed two- to fourfold-higher MICs versus the native populations when retested after subculture in the absence of antibiotics. Five isolates (23.81%) did not exhibit imipenem-heteroresistant subpopulations (PA124, PA140, PA226, PA243 and PA273) but two of them were meropenem heteroresistant (PA140 and PA273). In PAPs using meropenem, heteroresistant subpopulations showed two- to >fourfold-higher MICs when retested after free-of-drug subcultures versus the native populations. Six isolates (28.57%) did not exhibit meropenem-heteroresistant subpopulations (PA10, PA33, PA63, PA124, PA226 and PA243) but three of them were imipenem heteroresistant (PA10, PA33 and PA63). Overall, three out of the 21 tested isolates (14.29%) did not produce stable heteroresistant

Table 1. Characteristics of the isolates of the study

Differences of onefold between agar dilution and PAP were not evaluated. Numbers in bold and underlined refer to the cases in which the heterogeneous subpopulations returned to the parental phenotype. IMP, Imipenem; MEM, meropenem.

Isolate	PFGE	ST	MIC of native population		Highest drug concn in PAPs		Retest of heterogeneous subpopulation grown in the highest IMP concn in PAP after free-of-drug subcultures		Retest of heterogeneous subpopulation grown in the highest MEM concn in PAP after free-of-drug subcultures	
			IMP	MEM	IMP	MEM	IMP	MEM	IMP	MEM
PA1	I	688	2	<1	8	8	16	8	16	8
PA2	II	267	2	<1	16	4	16	8	16	8
PA6	III	110	2	<1	8	4	16	8	8	4
PA10	IV	546	2	<1	8	<u>8</u>	16	8	8	<u>2</u>
PA26	V	696	2	4	4	32	8	16	8	16
PA33	V	390	4	32	16	64	32	32	4	32
PA63	VI	696	2	4	4	4	8	8	8	4
PA84	VII	677	2	<1	8	4	8	4	8	4
PA86	VII	631	2	<1	4	4	16	8	4	8
PA124	VIII	696	8	32	16	64	16	32	16	32
PA132	IX	696	<1	4	8	16	8	4	8	16
PA140	IV	102	4	2	8	8	8	4	8	16
PA151	IX	677	8	<1	16	8	32	8	32	8
PA159	X	272	1	<1	16	8	32	8	32	8
PA165	XI	102	1	<1	8	8	16	8	16	8
PA200	XII	644	4	4	16	16	32	16	32	16
PA215	XIII	696	<1	2	4	8	8	16	16	16
PA226	XIII	696	8	32	8	64	16	32	8	32
PA243	XIII	696	16	16	16	32	16	16	16	16
PA261	XIV	102	2	<1	8	8	16	16	32	16
PA273	XV	654	2	<1	<u>8</u>	8	<u>2</u>	<1	16	16
ATCC 27853			2	<1	8	2	16	4	16	4

subpopulations (PA124, PA226 and PA243). In addition, in two isolates (PA10 and PA273) the heterogeneous subpopulations of meropenem and imipenem, respectively, returned to the parental phenotype. Interestingly, carbapenem-heteroresistant subpopulations showing more than twofold-higher carbapenem MICs were also recovered from reference strain ATCC 27853. This could be attributed to the higher inoculum used in this study relative to the original publication (Pournaras *et al.*, 2007). The frequency of imipenem-heteroresistant subpopulations ranged from 5×10^{-7} to 10^{-2} and that of meropenem-heteroresistant subpopulations ranged from 4×10^{-7} to 2×10^{-2} .

Although there is not yet a formal definition of heteroresistance, it could be defined as resistance manifested by a fraction of a microbial population that is

generally considered to be susceptible to the respective antimicrobial (Falagas *et al.*, 2008). According to Balaban *et al.* (2004), a bacterial population might survive antibiotic treatment through resistant mutants which show stable higher MICs to the antimicrobial or through persistent cells that return to the parental phenotype when the antibiotic pressure is withdrawn. This report shows that *P. aeruginosa* might produce both types of heteroresistant subpopulations when a carbapenem is used. Even within the same genotype, for example genotype XIII, isolates might exhibit different phenotypic profiles when carbapenem is used, varying from homogeneous susceptibility (PA243) to production of heteroresistant subpopulations with stable higher MICs (PA215). Moreover, a subpopulation might survive antimicrobial pressure through both a fraction of persisters and a fraction of stable resistant mutants as

shown by some isolates in our collection (data not shown).

Overall, although carbapenem heteroresistance seems a dispersed trait among *P. aeruginosa*, it appears diverse even within the same bacterial population. In addition to the detected phenotypic diversity it should be noted that the original observations on Mex-Opr-type efflux pumps and porin OprD (Ikonomidis *et al.*, 2008) were not replicated in all of the isolates of this study. Indicatively for strain ATCC 27853, imipenem and meropenem subpopulations showed a $+0.66 (\pm 0.14)$ - and $+0.36 (\pm 0.16)$ -fold change in gene *mexB* expression respectively, relative to the native population, whereas expression of gene *mexY* showed no difference. Of interest, gene *oprD* showed a $+0.83 (\pm 0.20)$ - and $+1.07 (\pm 0.15)$ -fold change and a $-0.35 (\pm 0.07)$ - and -0.31

(± 0.11)-fold change in gene *mexE* expression in imipenem and meropenem subpopulations, respectively. Similar observations were also made for other isolates (data not shown), which further complicates the issue.

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Olga Oikonomou,¹ Maria Panopoulou² and Alexandros Ikononmidis¹

¹Laboratory of Clinical Microbiology, School of Medicine, University of Thessaly, Larissa, Greece

²Laboratory of Clinical Microbiology, School of Medicine, Democritus University of Thrace, Alexandroupolis, Greece

Correspondence: Alexandros Ikononmidis (ado@med.uth.gr)

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