Characterization of clinical isolates of *Pseudomonas aeruginosa* heterogeneously resistant to carbapenems

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Fourteen apparently carbapenem-susceptible *Pseudomonas aeruginosa* clinical isolates that exhibited colonies within the inhibition zone around carbapenem discs were analysed. MICs of carbapenems were determined and the isolates were genotyped by PFGE. Population analysis, one-step selection of carbapenem-resistant mutants and growth curves of progenitors and carbapenem-resistant subpopulations were performed. Agar dilution MICs of imipenem and meropenem ranged from 0.5 to 4 mg l⁻¹ and from 0.25 to 2 mg l⁻¹, respectively. Population analysis confirmed subpopulations that grew in concentrations of up to 18 mg l⁻¹ and 12 mg l⁻¹ of imipenem and meropenem, respectively, at frequencies ranging from 6.9×10^{-5} to 1.1×10^{-7} , suggesting that they might not be detected by standard agar dilution MIC testing. The minority subpopulations exhibited MICs for imipenem ranging from 10 to 20 mg l⁻¹ and for meropenem from 4 to 14 mg l⁻¹. The one-step 8 mg l⁻¹ selection of imipenem-resistant mutants test showed growth in all isolates at frequencies ranging from 3.8×10^{-4} to 5.1×10^{-7} . Growth curves revealed a prolonged lag phase and a short exponential phase for the heterogeneous subpopulations compared with their respective native subpopulations. These findings may be indicative that the use of carbapenems can lead to selection of *P. aeruginosa* resistant subpopulations that subsequently cause infections and result in treatment failure.

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INTRODUCTION

Carbapenems, mainly imipenem and meropenem, have been extensively used for the treatment of infections caused by multi-resistant *Pseudomonas aeruginosa* strains. However, pseudomonads may develop resistance to carbapenems through combined mechanisms such as target inaccessibility, stable derepression of AmpC β -lactamase, overexpression of efflux systems and production of metallo- β -lactamases (MBLs) (Kohler *et al.*, 1999; Livermore, 2002). Recent data suggest that almost 15% of *P. aeruginosa* isolates are resistant to either imipenem or meropenem (Gales *et al.*, 2006).

Heteroresistance to carbapenems has been described in population studies of methicillin-resistant staphylococci (Kayser *et al.*, 1989). Among Gram-negative species, population heterogeneity has been detected in *Acinetobacter baumannii* as subcolonies within the apparent zone of inhibition in either disc diffusion or Etest assays (Pournaras *et al.*, 2005a). However, in *P. aeruginosa* heteroresistance to carbapenems has not been investigated,

though a recent study has shown that subpopulations with reduced susceptibility to meropenem may selectively be amplified during exposure to the drug (Tam *et al.*, 2005). In our hospitals MBL-producing or efflux-pump overexpressing carbapenem-resistant *P. aeruginosa* isolates have emerged in recent years (Pournaras *et al.*, 2003, 2005b). These issues prompted the cautious evaluation of susceptibility testing in pseudomonads that were systematically collected in our clinical laboratories. Disc diffusion or Etest results gave indications that heterogeneous populations with reduced susceptibility to carbapenems may exist in a number of *P. aeruginosa* strains that appear to be carbapenem susceptible by conventional automated susceptibility assays. In the present study we attempted to characterize this nonuniformity in the phenotypic expression of carbapenem resistance among unrelated *P. aeruginosa* clinical isolates.

METHODS

Bacterial isolates and susceptibility testing. The study included all *P. aeruginosa* isolates that were recovered consecutively from clinical sites of separate patients at the University Hospital of Larissa from July to October 2005 and exhibited one or a few

Abbreviation: MBL, metallo- β -lactamase.

colonies within the apparent zone of inhibition in disc diffusion assays. The isolates were identified using the API 20NE system (bioMérieux).

MICs of imipenem, meropenem and the remaining antipseudomonal antibiotics (amikacin, aztreonam, ciprofloxacin, cefepime, ceftazidime, gentamicin, netilmicin, piperacillin, piperacillin/tazobactam, ticarcillin, tobramycin) were performed by the agar dilution method (Clinical and Laboratory Standards Institute, 2003). The agar plates for carbapenems were prepared with 2 mg l⁻¹ increments of concentrations from 2 to 32 mg l⁻¹. MICs of carbapenems were also performed by Etest (AB Biodisk). *P. aeruginosa* ATCC 27853 was used as a control and susceptibility status was defined according to the interpretative criteria of the Clinical and Laboratory Standards Institute (2005). The isolates were screened by Etest MBL (AB Biodisk) and imipenem-EDTA double-disc synergy test, in order to identify possible MBL producers.

PFGE assay. PFGE of *Xba*I-digested genomic DNA of *P. aeruginosa* isolates was performed with a CHEF-DRIII system (Bio-Rad) and banding patterns were compared to published criteria (Tenover *et al.*, 1995).

Population analysis. Analysis of the cell subpopulations with reduced susceptibility to carbapenems was performed as described by Tomasz *et al.* (1991) and Hiramatsu *et al.* (1997) with some modifications. Briefly, 0.1 ml starting bacterial suspension with an optical density corresponding to McFarland 0.5 and serial dilutions of this were spread onto Muller-Hinton agar (MHA) containing imipenem or meropenem in serial twofold dilutions for concentrations ranging from 0.5 to 128 mg l⁻¹. Concentrations of imipenem or meropenem between 2 and 32 mg l⁻¹ were made with 2 mg l⁻¹ increments to detect more precisely the changes in the susceptibilities of the heteroresistant populations. Tenfold serial dilutions of the starting bacterial suspension were also plated onto drug-free MHA to determine the exact size of the inoculum. The plates were incubated at 37 °C for 48 h before the c.f.u. were counted. The analysis was performed twice for all isolates and the mean values were estimated. The number of resistant cells in the 0.1 ml starting cell

suspension was calculated and plotted on a semi-logarithmic graph. The stability of the heterogeneous subpopulations carbapenem MIC was tested by agar dilution with 2 mg l⁻¹ increments, after subculturing the colonies for one week in a drug-free medium. Population analysis of the tested isolates was compared with a carbapenem-susceptible *P. aeruginosa* isolate with a large inhibition zone and no subcolonies in the disc diffusion assay (PA3240, MICs of 1 mg imipenem l⁻¹ and 0.5 mg meropenem l⁻¹), a carbapenem-resistant *P. aeruginosa* isolate (PA105, MICs of 64 mg imipenem l⁻¹ and 32 mg meropenem l⁻¹) and *P. aeruginosa* ATCC 27853.

One-step selection of imipenem-resistant mutants. To test whether the parental *P. aeruginosa* isolates have the capacity to generate carbapenem-resistant mutants, a one-step imipenem selection assay was performed. A 0.1 ml portion of approximately 10⁸ c.f.u. ml⁻¹ cell suspension was spread on MHA containing 8 mg imipenem l⁻¹ and tenfold serial dilutions were plated on drug-free medium to determine the size of the inoculum. The plates were incubated at 37 °C for 48 h and screened. The frequency of emergence of imipenem resistance was calculated by dividing the number of c.f.u. on the imipenem containing agar by the inoculum size.

Growth curves of bacterial isolates. Growth curves were determined by diluting 0.1 ml overnight culture of the progenitor strains and their respective resistant subpopulations in 15 ml Muller Hinton broth, followed by incubation at 37 °C for 10 h under constant shaking. A volume of 0.1 ml was diluted tenfold serially to 10⁻⁶ every 30 min and 0.1 ml each dilution was plated on MHA and incubated for 18 h in 37 °C. The number of c.f.u. were counted and plotted on a semi-logarithmic graph.

RESULTS

During the study period, 14 non-repetitive *P. aeruginosa* isolates were collected, in which one or a few subcolonies appeared within the zone of inhibition of both carbapenems. These isolates represented 27.5 % of the apparently

Table 1. Clinical source of isolation, PFGE profile and agar dilution MICs for imipenem and meropenem for the isolates included in this study

Strain	Ward	Specimen	PFGE type	MIC (mg l ⁻¹)		Resistance to other anti-pseudomonal antimicrobials*
				Imipenem	Meropenem	
1	Gastrointestinal	Pus	I	1	0.5	GEN, TOB, TIC
2	Pneumology	Sputum	II	4	0.25	–
3	Medical	Sputum	III	2	0.25	GEN, TOB, TIC
4	ICU	Bronchial	IV	2	1	–
5	Dermatology	Skin infection	V	2	1	–
6	Surgical	Wound infection	VI	4	1	TIC
7	Medical	Blood	VII	2	1	–
8	ICU	Bronchial	VIII	2	1	–
9	ICU	Urinary catheter	V	4	2	PIP, TIC
10	Dialysis	Urine	IX	1	0.5	CIP, TIC
11	Medical	Urine	X	2	2	–
12	Paediatrics	Throat swab	II	0.5	0.25	–
13	Surgical	Pus	VI	2	0.5	PIP, TIC
14	Paediatrics	Urine	VIII	2	0.5	GEN, PIP, TIC

ICU, intensive care unit; CIP, ciprofloxacin; GEN, gentamicin; PIP, piperacillin; TIC, ticarcillin; TOB, tobramycin.

*All isolates were susceptible or intermediate to amikacin, aztreonam, cefepime, ceftazidime, netilmicin and piperacillin/tazobactam.

Fig. 1. Population analysis of isolates 1, 4, 5 and 13 (Table 2), in comparison with PA3240, PA105 and ATCC 27853, substantiating resistant subpopulations that exceeded the meropenem MIC of the native population. The curves are representative of experiments performed twice for each isolate.

carbapenem-susceptible isolates of the study period. The agar dilution MICs of imipenem and meropenem ranged from 0.5 to 4 mg l⁻¹ and 0.25 to 2 mg l⁻¹, respectively, while their Etest MICs ranged from 0.5 to 4 mg l⁻¹ and 0.19 to 2 mg l⁻¹, respectively. However, colonies growing within the zone of inhibition in Etest were observed at imipenem or meropenem concentrations of 8 to 24 mg l⁻¹. The isolates were susceptible to most anti-pseudomonal antimicrobials

and PFGE revealed ten distinct genotypes (Table 1). All were negative for MBL production by phenotypic methods.

Population analysis showed distinct subpopulations that grew in concentrations of up to 18 mg imipenem l⁻¹ and 12 mg meropenem l⁻¹ (Fig. 1). PFGE patterns of the resistant and native populations were identical, eliminating the possibility that mixed cultures were responsible for the findings observed. The ratio of heterogeneity ranged from 6.9×10^{-5} to 1.2×10^{-7} for imipenem and 2.1×10^{-5} to 1.1×10^{-7} for meropenem as calculated from the colonies grown in the highest concentration of each carbapenem (Table 2). Susceptibility testing on these colonies revealed MICs that ranged from 10 to 20 mg l⁻¹ for imipenem and 4 to 14 mg l⁻¹ for meropenem (Table 2). In the resistant subclones MICs of ceftazidime were within the susceptible range (1 to 4 mg l⁻¹) being equal to or different by one dilution from their native isolates, implying that AmpC overproduction does not contribute to heteroresistance. However, resistant subclones from all but three ciprofloxacin-susceptible native isolates, formed on either imipenem or meropenem agar plates, showed a fourfold increase in the MIC of ciprofloxacin, suggesting the potential contribution of efflux pump systems.

At the one-step 8 mg l⁻¹ selection of imipenem-resistant mutants, growth was observed in all 14 *P. aeruginosa* isolates at frequencies ranging from 3.8×10^{-4} to 5.1×10^{-7} . No growth was detected for the carbapenem-susceptible PA3240 and ATCC 27853 strains. Growth curves showed in all cases a prolonged lag phase and a short exponential phase for the heterogeneous subpopulations in comparison with their respective native populations (Fig. 2), indicating

Table 2. The highest concentration where subclones were observed by population analysis, frequency of appearance of subclones at the highest carbapenem concentration and MICs of *P. aeruginosa* subclones after subculturing for 1 week in a drug-free medium

Isolate	Highest concentration where growth occurred in population analysis		Frequency of appearance of subclones		MIC after 1 week passage in drug-free medium	
	Imipenem	Meropenem	Imipenem	Meropenem	Imipenem	Meropenem
1	16	4	6.9×10^{-5}	1.4×10^{-7}	20	4
2	12	6	2.3×10^{-7}	3.8×10^{-7}	16	10
3	8	4	5.1×10^{-6}	6.6×10^{-6}	12	4
4	14	12	1.2×10^{-7}	4×10^{-6}	16	14
5	18	2	5.7×10^{-7}	5.2×10^{-7}	20	6
6	10	6	4.3×10^{-6}	4.3×10^{-7}	14	10
7	8	6	1.7×10^{-6}	9.6×10^{-6}	10	6
8	14	4	2.2×10^{-7}	1.6×10^{-5}	16	8
9	18	6	3×10^{-7}	2.2×10^{-7}	18	10
10	8	8	4.3×10^{-7}	1.1×10^{-7}	12	10
11	8	8	2.8×10^{-6}	2.6×10^{-7}	12	12
12	8	6	4.4×10^{-5}	2.1×10^{-5}	12	8
13	10	4	2.6×10^{-6}	3.7×10^{-7}	14	8
14	12	2	5.7×10^{-7}	6.4×10^{-7}	16	6

Fig. 2. Growth curve of the native and the heterogeneous population of a representative study isolate (isolate 1).

that an increase of the incubation time in phenotypic tests is needed in order to detect resistant subpopulations.

DISCUSSION

Heteroresistance in its broadest sense may represent mixed populations of drug-resistant and drug-susceptible cells of a single bacterial strain (Rinder, 2001). However, among different pathogens and antimicrobials, detection of heteroresistance may be affected by variables such as the screening method, test conditions, local epidemiology, antibiotic selective pressure and the unstable nature of the resistance phenotype (Tomasz *et al.*, 1991; Yamazumi *et al.*, 2003; Plipat *et al.*, 2005). This is to the best of our knowledge the first report that characterizes heterogeneous subpopulations with reduced susceptibility to carbapenems in *P. aeruginosa*. These subpopulations were initially indicated as mature subcolonies of lower susceptibility or resistance. Subsequently, the heterogeneous subclones were demonstrated by using a detailed population analysis. The assay showed the presence of multiple subpopulations with a virtually continuous spectrum of intermediate MICs between the MIC of the majority of the cells and the MIC for the most highly resistant subclones. The latter subclones exhibited substantially higher levels of resistance than the parental strains, in contrast to a previous observation in *A. baumannii*, where parental and daughter isolates had similar susceptibility profiles (Pournaras *et al.*, 2005a). Furthermore, the heterogeneous subpopulations retained their resistance levels implying a rather stable expression of the phenotype similar to heteroresistance to vancomycin in enterococci (Alam *et al.*, 2001).

The heterogeneously carbapenem-resistant pseudomonads pose a challenge in terms of treatment. The optimization of meropenem administration regimens to suppress resistant *P. aeruginosa* mutants has been recently proposed (Tam *et al.*, 2005). Carbapenem-resistant subpopulations might thus proliferate preferentially and lead to treatment failure or recurrence of infection. It is plausible that an association exists between the frequency of heterogeneous compositions and the emergence of resistant mutants during exposures to drug levels not far exceeding the MIC of the organisms. In

our region the extensive use of carbapenems for multidrug-resistant *P. aeruginosa* infections may have contributed to the frequent carbapenem-heteroresistant phenotype. Although a carbapenem-resistant *P. aeruginosa* strain did not emerge from any of our patients, the detection of heterogeneous growth in susceptibility assays would be of major importance. Conventional agar dilution MICs using the standard 10^4 per spot inoculum may miss carbapenem-resistant mutants with lower rates of occurrence. In such cases, higher inocula or prolonged incubation time might be used to define more precisely the highest drug concentration where resistant colonies occur and allow the administration of appropriately higher carbapenem dosages to suppress amplification of the resistant subclones.

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