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Pseudomonas aeruginosa carbapenem resistance mechanisms in Spain: impact on the activity of imipenem, meropenem and doripenem

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Objectives: To investigate the mechanisms of carbapenem resistance in the 175 *Pseudomonas aeruginosa* isolates (39%; 175/448) showing non-susceptibility (European Committee on Antimicrobial Susceptibility Testing breakpoints) to imipenem (35%), meropenem (33%) and/or doripenem (33%) recovered in 2008–09 from 16 Spanish hospitals during the Comparative Activity of Carbapenem Testing (COMPACT) surveillance study.

Methods: MICs (Etest), clonal relatedness (PFGE) and metallo-β-lactamase (MBL) production (Etest-MBL, PCR and sequencing) were determined. Mutation-driven resistance was studied in 60 non-MBL producers according to the doripenem MICs (15 isolates from each of four MIC groups: ≤1, 2–4, 8–16 and ≥32 mg/L). The expression of *ampC*, *mexB*, *mexY*, *mexD* and *mexF* was determined by real-time reverse transcription–PCR and the presence of mutations in *oprD* by PCR and sequencing. Isogenic mutants expressing combinations of mutation-driven carbapenem resistance were constructed.

Results: Twelve (6.9%) isolates were MBL (VIM-20, VIM-2 or VIM-13) producers and all showed high-level resistance (MIC 32 mg/L) to all three carbapenems. Regarding mutation-driven resistance, all but 1 of the 60 isolates were non-susceptible (MIC >32 mg/L) to imipenem, linked to oprD inactivation. In addition, 50% of the isolates overexpressed *ampC*, 33% *mexY*, 32% *mexB* and 15% *mexF*, while none overexpressed *mexD*. Increasing prevalence of *ampC* overexpression correlated with increasing doripenem MICs (≤1, 13%; 2–4, 53%; 8–16, 60%; and ≥32, 73%) while overexpression of efflux pumps correlated only with moderate resistance. Doripenem showed slightly higher activity than meropenem against isolates overexpressing ampC, especially *mexB* or *mexY*. The analysis of a collection of isogenic laboratory mutants supported this finding.

Conclusions: Although the prevalence of MBL producers is increasing, mutation-driven resistance is still more frequent in Spain. Imipenem resistance was driven by OprD inactivation, while additional AmpC and particularly efflux pump hyperproduction had a lower impact on the activity of doripenem compared with meropenem.

Keywords: antibiotic resistance mechanisms, *P. aeruginosa*, carbapenemases, AmpC, efflux pumps, OprD

Introduction

The growing threat of antimicrobial resistance in *Pseudomonas aeruginosa* relies on the one hand on the extraordinary capacity of this organism for developing resistance to almost any available antibiotic through mutations in chromosomal genes, and on the other hand to the increasing prevalence of transferable resistance determinants, particularly those encoding class B carbapenemases [or metallo-β-lactamases (MBLs)] or extended-spectrum β-lactamases (ESBLs), frequently co-transferred with genes encoding aminoglycoside-modifying enzymes.[1,2]

Although there are a few molecules under clinical development which may help to mitigate to some extent these resistance concerns in the future,[3–5] the currently available carbapenems in Europe (imipenem, meropenem and doripenem) are still the first-line agents for combating infections due to antibiotic-resistant *P. aeruginosa*. While all three carbapenems share some common properties, among them susceptibility to hydrolysis by MBLs, there appear to be important differences regarding their *in vitro* antipseudomonal potency,[6] probably due to differences in intrinsic activity [efficiency of binding to the essential penicillin-binding proteins (PBPs)][7] and/or susceptibility to intrinsic and

mutational resistance mechanisms.[8] Among the mutation-mediated resistance mechanisms, particularly noteworthy are those leading to the repression or inactivation of the porin OprD, the hyperproduction of the chromosomal cephalosporinase AmpC and the up-regulation of one of the several efflux pumps encoded in the *P. aeruginosa* genome.[9–12]

A deep understanding of the ever-changing epidemiology and impact of *P. aeruginosa* carbapenem resistance mechanisms is crucial, along with pharmacokinetic/pharmacodynamic (PK/PD) modelling, to optimize antimicrobial therapy in order to prevent and combat infections by multidrug-resistant (MDR) *P. aeruginosa*. Thus, this work aimed to investigate the mechanisms of carbapenem resistance and their impact on the activity of imipenem, meropenem and doripenem among a large collection of *P. aeruginosa* isolates recovered in 2008–09 from 16 Spanish hospitals during the Comparative Activity of Carbapenem Testing (COMPACT) surveillance study.[13] Additionally, the information obtained from the clinical strains was complemented by an analysis of a collection of isogenic laboratory mutants expressing several combinations of the most relevant mutation-driven carbapenem resistance mechanisms.

Materials and methods

Clinical strains, susceptibility testing and molecular epidemiology

The 175 *P. aeruginosa* isolates (39% of the 448 *P. aeruginosa* isolates tested) showing non-susceptibility, according to EUCAST breakpoints, to imipenem (MIC >4 mg/L, 35%), meropenem (MIC >2 mg/L, 33%) or doripenem (MIC >1 mg/L, 33%) recovered in 2008–09 from 16 Spanish hospitals during the COMPACT surveillance study were evaluated.[13] The overall prevalence of carbapenem-non-susceptible isolates from Spain was close to the average for all countries participating in the COMPACT study.[14] All isolates were recovered from patients with intra-abdominal infections, bacteraemia or pneumonia. Identification and susceptibility testing were performed with the semiautomatic WIDER system (Francisco Soria Melguizo, S.A., Madrid, Spain). Additionally, MICs of imipenem, meropenem and doripenem were also determined by Etest (bioMérieux, Durham, NC, USA). Clonal relatedness was studied in all isolates by PFGE.

Characterization of mutational antibiotic resistance mechanisms

The levels of expression of *ampC* and efflux pump encoding genes (*mexB*, *mexD*, *mexY* and *mexF*) were determined by real-time reverse transcription (RT)–PCR following previously described protocols.[15,16] Briefly, strains were grown in 10 mL of LB broth at 37°C and 180 rpm to late log phase ($OD_{600}=1$) and collected by centrifugation. Total RNA was isolated using the RNeasy Mini Kit (Qiagen), dissolved in water and treated with 2 U of TURBO DNase (Ambion) for 30 min at 37°C to remove contaminating DNA. A 50 ng sample of purified RNA was then used for one-step reverse transcription and real-time PCR amplification using the QuantiTect SYBR Green RT–PCR Kit (Qiagen) in a SmartCycler II (Cepheid). Previously described primers[15,16] were used for amplification of *ampC*, *mexB*, *mexD*, *mexY*, *mexF* and *rpsL* (used as reference to normalize the relative amount of mRNA). Appropriate controls, including reactions without RT, were used to rule out the presence of contaminating DNA. According to previous studies,[17] strains were considered positive for *ampC*, *mexD*, *mexF* or *mexY* overexpression when the corresponding mRNA level was at least 10-fold higher than that of *P. aeruginosa* strain PAO1, negative if lower than 5-fold, and borderline if between 5- and 10-fold. Strains were considered positive for *mexB* overexpression when the corresponding mRNA level was at least 3-fold higher than that of PAO1, negative if lower than 2-fold, and borderline if between 2- and 3-fold.[17] Mean values (±SD) of mRNA levels obtained in the three independent duplicate experiments were considered. Previously obtained PAO1 mutants overexpressing these mechanisms were used as controls.[17] The presence of mutations in *oprD* was explored by PCR and sequencing using previously described primers and conditions.[10]

Detection of MBLs

The presence of MBLs was evaluated in all isolates non-susceptible to carbapenems and ceftazidime with Etest-MBL strips and by PCR followed by sequencing. Previously described primers and conditions were used to amplify the genes encoding VIM-1, VIM-2, IMP-1 and IMP-2 enzymes.[10] After PCR amplification, sequencing reactions were performed with the BigDye Terminator kit (PE Applied Biosystems, Foster City, CA, USA), and sequences were analysed on an ABI Prism 3100 DNA sequencer (PE Applied Biosystems). The resulting sequences were then compared with those available at GenBank (www.ncbi.nih.gov/BLAST).

Construction and characterization of isogenic laboratory mutants showing several combinations of resistance mechanisms

The complete list of laboratory strains and plasmids used or constructed in this study is shown in Table 1. PAO1 mutants showing several combinations of resistance mechanisms, including OprD inactivation, MexAB-OprM overexpression (*mexR* inactivation) and AmpC overexpression (*ampD* inactivation) were constructed following well-established procedures[16] based on the *cre-lox* system for gene deletion and antibiotic resistance marker recycling in *P. aeruginosa*.[18] The phenotypes of the mutants were confirmed by SDS–PAGE analysis of outer membrane protein (OMP) profiles (*oprD* and *mexR* mutants) and *ampC* expression (*ampD* mutants) following previously described procedures.[16,19] MICs of imipenem, meropenem and doripenem for all mutants and isogenic parent strains were determined by Etest in duplicate experiments.

Results and discussion

Impact of transferable MBLs in carbapenem resistance

The 175 *P. aeruginosa* isolates (39% of the total of 448 *P. aeruginosa* isolates tested) showing non-susceptibility, according to EUCAST breakpoints, to imipenem, meropenem or doripenem were evaluated. Twelve (6.9%) of the 175 *P. aeruginosa* isolates showing reduced susceptibility to at least one of the carbapenems were found to be MBL producers. Thus, the overall prevalence of MBL production in *P. aeruginosa* isolates from the 2008–09 COMPACT surveillance study was 2.7%, slightly higher than the 1% documented for bloodstream isolates in a contemporary, recently published multicentre study in Spain.[17] This slightly higher figure in our study could be associated with the particular selection of isolates in the COMPACT study, most of them from complicated infections in intensive care unit patients,[13] and with the outbreak situation in one of the hospitals (see below). In any case, while this prevalence of MBLs is still lower than that reported in certain countries from South America,[20] the Far East[21] and Europe,[22] it denotes an ∼30-fold increase compared with a multicentre study performed in Spain 5 years earlier,[10] in which the prevalence of MBLs was 0.08%. All MBL-producing isolates showed high-level resistance

Table 1. Strains and plasmids used or constructed in this study

Strain or plasmid	Genotype/relevant characteristics	Reference or source
P. aeruginosa		
PAO1	reference strain completely sequenced	laboratory collection
PAΔD	PAO1 Δ*ampD*::*lox; ampD* encodes a negative regulator of the chromosomal β-lactamase AmpC	Juan *et al*.[16]
PAOD1	spontaneous *oprD* null mutant (W65X) of PAO1	Moya *et al*.[3]
PAOMxR	PAO1 Δ*mexR*::*lox; mexR* encodes the negative regulator of MexAB-OprM efflux pump	X. Mulet & A. Oliver (unpublished results)
PAOD1ΔD	PAOD1 Δ*ampD*::*lox*	this work
PAOD1MxR	PAOD1 Δ*mexR*::*lox*	this work
PAΔDMxR	PAΔD Δ*mexR*::*lox*	this work
E. coli		
XL-1 blue	F'::Tn10 *proA*$^{+}$*B*$^{+}$ *lac1*^{q} Δ*(lacZ)M15/recA1 endA1 gyrA96* (Nal^{R}) *thi hsdR17* ($r_k^-m_k^-$) *mcrB1*	laboratory collection
S17.1	RecA pro (RP4-2Tet::Mu Kan::Tn*7*)	laboratory collection
Plasmids		
pEX100Tlink	Ap^{R}, *sacB*, pUC19-based gene replacement vector with a multicloning site	Quénée *et al*.[18]
pUCGm*lox*	Ap^{R}, Gm^{R}, pUC18-based vector containing the *lox*-flanked *aacC1* gene	Quénée *et al*.[18]
pCM157	Tc^{R}, *cre* expression vector	Quénée *et al*.[18]
pEXADGm	pEX100Tlink containing 5′ and 3′ flanking sequence of *ampD*::Gm*lox*	Juan *et al*.[16]
pEXMxRGm	pEX100Tlink containing 5 and 3′ flanking sequence of *mexR*::Gm*lox*	X. Mulet & A. Oliver (unpublished results)

(MICs >32 mg/L) to the three carbapenems. Moreover, MBLs were detected in up to 41.4% of the isolates showing doripenem MICs >32 mg/L. Eleven of the MBLs belonged to the VIM-2 cluster (two VIM-2 and nine VIM-20) and one to the VIM-1 cluster (VIM-13). All VIM-20 isolates were recovered from a single hospital and belonged to a single clone highly disseminated in the institution.[23] All of the MBL-producing isolates showed a pattern of MDR including ceftazidime, cefepime, piperacillin/tazobactam, gentamicin, tobramycin, amikacin (all but one isolate), ciprofloxacin and levofloxacin. Seven of the 12 isolates were additionally resistant to aztreonam and all were susceptible to colistin.

Role of mutational mechanisms in carbapenem resistance

The presence of mutation-driven carbapenem resistance mechanisms was investigated in 60 of the 175 isolates showing reduced susceptibility to at least one of the carbapenems. Selection was based on the distribution of doripenem MICs. Fifteen isolates from each of the following MIC groups were randomly selected: ≤1 mg/L (susceptible); 2–4 mg/L (reduced susceptibility, EUCAST intermediate category); 8–16 mg/L (resistant, but target might be attainable at high dose and prolonged infusion according to PK/PD models[24–26]); and ≥32 mg/L (high-level resistant). MBL producers were excluded from the analysis of mutation-driven resistance mechanisms. A total of 28 different PFGE profiles were detected among the 60 studied strains. The natural distribution of the MICs from the 175 isolates into the four established categories was as follows: 18.9%, ≤1 mg/L; 34.9%, 2–4 mg/L; 29.1%, 8–16 mg/L; and 17.1%, ≥32 mg/L.

All but 1 of the 60 studied isolates were non-susceptible (MIC ≥8 mg/L) to imipenem. Mutational inactivation of *oprD* is known to be the main mechanism of imipenem resistance in the absence of acquired carbapenemases. Thus, to confirm the involvement of *oprD* inactivation in the carbapenem-resistant phenotypes, *oprD* was sequenced in the single imipenem-susceptible isolate and in 18 randomly selected imipenem-non-susceptible isolates. As expected, the imipenem-susceptible isolate showed the wild-type *oprD* sequence. This isolate showed borderline meropenem and doripenem susceptibility (MIC 2 mg/L) and overexpressed both *ampC* and *mexB*. On the other hand, all 18 imipenem-non-susceptible isolates had a non-functional OprD caused by diverse mutations leading to frameshifts (12 isolates) or premature stop codons (6 isolates). These data therefore confirm that OprD inactivation is a nearly universal signature of imipenem resistance.

While *oprD* inactivation also increases the MICs of meropenem and doripenem, clinical resistance to these carbapenems is thought to require additional mechanisms, such as AmpC or efflux pump (particularly MexAB-OprM) overexpression.[10,27] We therefore investigated the impact of the overexpression of AmpC and the four major efflux pumps (MexAB-OprM, MexXY-OprM, MexCD-OprJ and MexEF-OprN) on the activity of meropenem and doripenem. The prevalence of these resistance mechanisms according to the doripenem MIC category is shown in Figure 1. In the complete collection of studied isolates, up to 50% overexpressed *ampC*, while the prevalence of overexpression of efflux pump encoding genes was 33.3% for *mexY*, 31.7% for *mexB*, 15% for *mexF* and 0% for *mexD*.

As shown in Figure 1, increased prevalence of *ampC* overexpression correlated with increasing doripenem resistance, the prevalence ranging from 13.3% in the ≤1 mg/L group to 73.3%

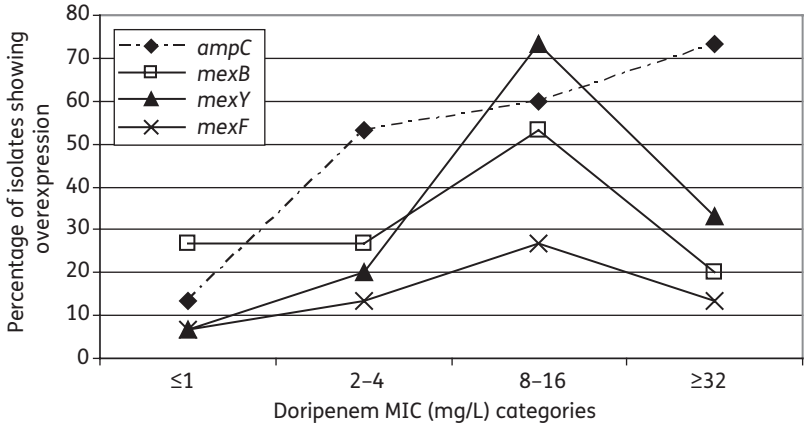

Figure 1. Prevalence of *ampC*, *mexB*, *mexF* and *mexY* overexpression according to doripenem MIC categories.

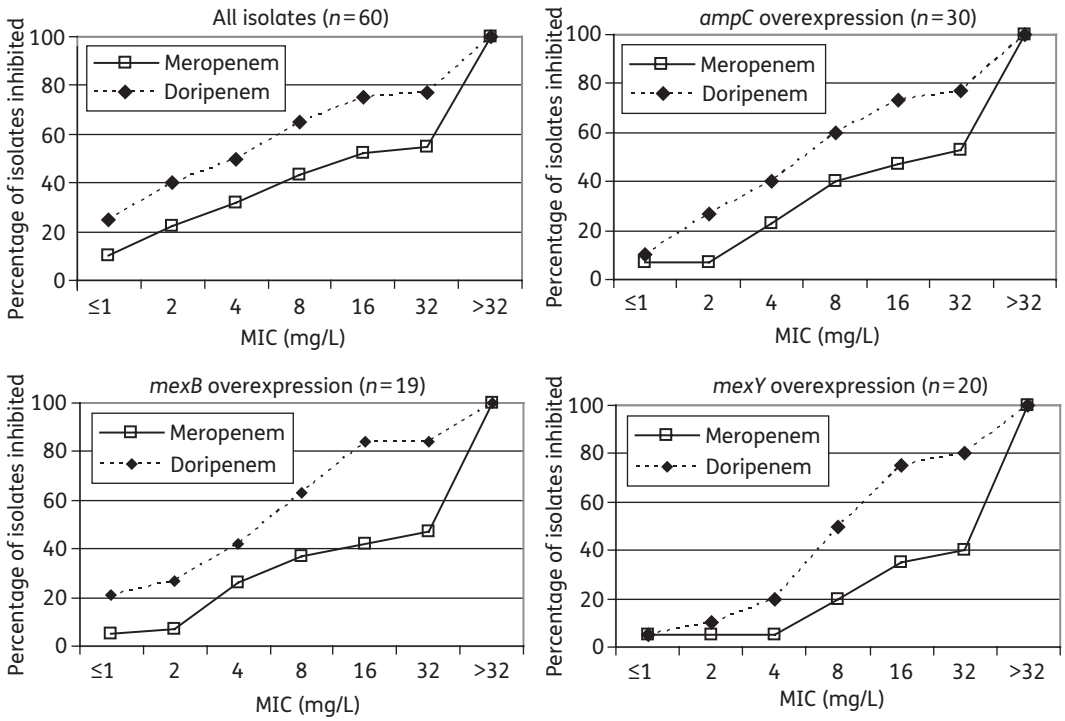

Figure 2. Comparative activity of meropenem and doripenem against isolates showing different mechanisms of resistance.

in the ≥32 mg/L group. On the other hand, efflux pump overexpression correlated with moderate doripenem resistance, but not with high-level resistance. For all efflux pumps, the highest prevalence peaks were obtained in the 8–16 mg/L group: 73.3% for *mexY*, 53.3% for *mexB* and 26.7% for *mexF*.

The comparative activity of meropenem and doripenem against isolates expressing the different mechanisms of resistance studied is shown in Figure 2. As shown, for the complete collection the percentage of isolates inhibited at each antibiotic concentration was ~20% higher for doripenem than for meropenem. The comparative activity for the subsets of isolates overexpressing *ampC* was very similar to that obtained for the complete collection. On the other hand, the differences were increased further among isolates overexpressing *mexB* or *mexY*. For example, 80% of isolates overexpressing *mexY* or *mexB* were inhibited at a concentration ≤16 mg/L of doripenem, in contrast to only 40% for meropenem.

One further factor, not evaluated in this work, that could also modulate carbapenem resistance level is the presence of certain polymorphisms, such as T105A in the AmpC sequence, leading to the denominated extended-spectrum AmpCs.[12,28] Nevertheless, a recent study detected these polymorphisms also in wild-type strains, and failed to demonstrate any link with carbapenem resistance.[29]

Table 2. Activity of imipenem, meropenem and doripenem against laboratory mutants showing different resistance mechanisms

Strain	MIC (mg/L)		
	imipenem	meropenem	doripenem
PAO1 (wild-type)	2	0.38	0.19
PAOD1 (*oprD*$^-$)	>32	4	1.5
PAΔD (*ampD*$^-$, AmpC hyperproduction)	2	1.5	1
PAOMxR (*mexR*$^-$, MexAB-OprM hyperproduction)	1.5	2	0.38
PAOD1ΔD (*oprD*$^-$, *ampD*$^-$)	>32	12	8
PAOD1ΔmR (*oprD*$^-$, *mexR*$^-$)	>32	>32	4
PAΔDMxR (*ampD*$^-$, *mexR*$^-$)	2	6	1.5

Comparative activity of imipenem, meropenem and doripenem against isogenic laboratory mutants expressing several combinations of resistance mechanisms

Additionally, the activities of imipenem, meropenem and doripenem were investigated against a collection of isogenic laboratory mutants expressing different combinations of resistance mechanisms, including OprD inactivation, AmpC hyperproduction (*ampD* inactivation) and MexAB-OprM hyperproduction (*mexR* inactivation). The phenotypes of the *oprD* and *mexR* mutants were confirmed by analysis of OMP profiles and those of the *ampD* mutants by the demonstration of increased *ampC* expression as described previously.[16]

In agreement with the data on clinical strains and previous studies, doripenem was the most potent carbapenem against the wild-type PAO1 strain, yielding MICs 2-fold and 12-fold lower than those of meropenem and imipenem, respectively (Table 2). While the inactivation of the porin OprD determined high-level resistance to imipenem (MIC >32 mg/L), it produced only moderate resistance (intermediate susceptibility according to EUCAST breakpoints) to meropenem (4 mg/L) and doripenem (1.5 mg/L). Nevertheless, the sharpest differences in the activity of doripenem compared with meropenem were observed, in agreement with the above data on clinical strains, for the mutants showing MexAB-OprM overexpression; the MIC of doripenem (0.38 mg/L) for the *mexR* mutant was 5-fold lower than that of meropenem (2 mg/L). Moreover, while simultaneous OprD inactivation and MexAB-OprM overexpression produced very high-level meropenem resistance (MIC >32 mg/L), the MIC of doripenem (4 mg/L) remained within the EUCAST intermediate category. On the other hand, the overexpression of AmpC, alone or combined with OprD inactivation, had a very similar impact on the activity of doripenem and meropenem, significantly raising MICs by 2- to 4-fold. Our results confirm and extend the data from a previous study showing that doripenem appeared to be less affected by *P. aeruginosa* mutational resistance mechanisms than other carbapenems, tending to be less prone to develop resistance during *in vitro* exposure to the antibiotic.[8] Nevertheless, our data from the collection of clinical isolates and laboratory mutants indicates that OprD inactivation plus AmpC overexpression is probably the most effective mutational resistance mechanism for doripenem. On the other hand, although we show, in agreement with previous data,[8] that doripenem MICs are raised upon MexAB-OprM overexpression, the impact of this resistance mechanism on the activity of meropenem is significantly higher.

Conclusions

Although the prevalence of MBL-producing *P. aeruginosa* has increased significantly in recent years, mutation-driven resistance is still far more frequent. Imipenem resistance was mainly driven by OprD inactivation, while additional AmpC and particularly efflux pump hyperproduction had a lower impact on the activity of doripenem when compared with meropenem. These results support further the potential advantage of doripenem, compared with the other available carbapenems, in the fight against antibiotic resistance in *P. aeruginosa*, particularly when optimizing the dose to fulfil PK/PD target attainment.[24]

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