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Meropenem heteroresistance in clinical isolates of OXA-48–producing *Klebsiella pneumoniae*

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ABSTRACT

OXA-48–producing *Klebsiella pneumoniae* isolates often show growth of colonies within inhibition zones in carbapenem diffusion assays. The nature of these colonies was investigated in a series of clinical isolates of OXA-48–producing *K. pneumoniae* obtained in the context of a hospital outbreak, and they were found to be persistent colonies that reproduced again the same phenotype when they were collected and tested in diffusion assays again. The frequency of mutations conferring resistance to meropenem (8 μg/mL) was determined for the same isolates. The average mutation frequency was $5.47 \cdot 10^{-6}$ (range: $2.59 \cdot 10^{-8}$–$5.87 \cdot 10^{-5}$), and the analysis of several resistant mutants showed that all of them had mutations in the *ompK36* porin gene. Heteroresistance was investigated using population analysis profiling. The profiles were compatible with mutation frequency assays, and all the colonies analyzed were resistant mutants. In OXA-48–producing *K. pneumoniae*, the growth of persisters seems to be specific of diffusion assays.

1. Introduction

Carbapenem-resistant enterobacteria and, in particular, carbapenem-resistant *Klebsiella pneumoniae* have become an important problem in hospitals around the world during the last decade (Podschun and Ullmann, 1998; Schwaber and Carmeli, 2008). In enterobacteria, the major mechanisms that give rise to carbapenem resistance are the production of plasmid-encoded carbapenemases and decreased outer membrane permeability (Nordmann et al., 2012). OXA-48 is a class D β-lactamase highly active against penicillins and with lower activity against carbapenems (Queenan and Bush, 2007). OXA-48–producing *K. pneumoniae* isolates usually exhibit low-level resistance to carbapenems with meropenem and imipenem MICs frequently below clinical cut-off values (Oueslati et al., 2015). In *K. pneumoniae*, reduced susceptibility to carbapenems may arise also by a decrease in outer membrane permeability because of inactivation or altered expression of porins in strains producing β-lactamases with at least some hydrolytic activity against carbapenems. (Jacoby et al., 2004; Tsai et al., 2011; Wang et al., 2009).

High-dose meropenem-based therapy has been suggested to be useful to treat infections by OXA-48–producing *K. pneumoniae* if meropenem MICs are ≤4 μg/mL (Lowman and Schleicher, 2015). This is supported by previous studies that recommended meropenem to treat infections by KPC-producing *K. pneumoniae* if meropenem MICs were ≤8 μg/mL (Tumbarello et al., 2015). In both cases, the choice of carbapenem should be based on MIC data of the infecting strains, and this is particularly important because a significant degree of diversity in MIC values has been described in carbapenemase-producing *K. pneumoniae* isolates (Loli et al., 2006; Paño-Pardo et al., 2013; Tenover et al., 2006). Alterations in outer membrane permeability and carbapenemase genes may account for at least part of this variability (Loli et al., 2006). In addition, carbapenem heteroresistance has been described in VIM and KPC-producing *K. pneumoniae* isolates (Pournaras et al., 2010; Tato et al., 2010). Heteroresistance is a phenotypic phenomenon where a certain proportion of cells of a bacterial population exhibit a range of susceptibilities to a particular antibiotic. Heteroresistance may be the consequence of either the selection of bacterial mutants (with hereditary genetic changes) or the growth of persister cells (when the colonies with a susceptibility different from that of the original population are subcultured the same phenotype is reproduced again) (El-Halfawy and Valvano, 2015).

OXA-48–producing *K. pneumoniae* were first detected in our hospital in December 2010 and have spread through several hospital units since then (Paño-Pardo et al., 2013; Pérez-Blanco et al., 2018). Two major and several minor sporadic clones have been identified. The 2 major clones belong to sequence types ST405 and ST11. In addition to the OXA-48 carbapenemase gene, these 2 clones carry genes for CTX-M-15, OXA-1,

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SHV-76, and TEM-1 β-lactamases (Wesselink et al., 2012). Isolates from the 2 clones display a broad range of MIC values for carbapenems, including susceptible, intermediate, and resistant isolates, according to clinical breakpoints. Some isolates reproducibly display colony growth within inhibition zones in disk and gradient-diffusion assays, suggesting the existence of less susceptible cells within the population. The importance of accurate MIC determination to guide therapy led us to analyze in detail this phenomenon.

2. Material and methods

2.1. Bacterial strains and susceptibility testing

Twenty-four OXA-48–producing clinical isolates obtained in our hospital from April 2010 to April 2013 were included. They were arbitrarily selected to represent the whole range of meropenem MIC values. The isolates had been typed by multilocus sequence typing (MLST), Diversilab™ (bioMérieux, Marcy l'Étoile, France), or a clone-specific PCR assay (López-Camacho et al., 2014a). Antibiotic susceptibility was initially determined using microdilution MIC testing by panel 44 Microscan® (Siemens, USA) and interpreted according to the EUCAST guidelines (EUCAST, n.d.). Meropenem, ertapenem, and imipenem MICs were further analyzed by microdilution (ISO 20776-1 standard), gradient diffusion (Etests, bioMérieux), and disk diffusion assays (EUCAST). *Pseudomonas aeruginosa* ATCC® 27853™and *Escherichia coli* ATCC® 25922™ were used as controls.

2.2. Population analysis profile (PAP) curves

Cultures were grown overnight in Luria Bertani (LB) broth and serially diluted in saline. One-hundred-microliter aliquots of each dilution were spread on freshly prepared LB plates containing meropenem in a range of concentrations from 0.125 to 32 μg/mL. The plates were incubated overnight at 37 °C, and the colonies grown were counted. The proportion of resistant bacteria were calculated by dividing the number of colonies grown on antibiotic-containing plates by the colony counts from the same bacterial inoculum plated onto antibiotic-free plates. Results were represented on a semilogarithmic graph with relative resistant population frequencies on the vertical axis and drug concentration on the horizontal axis.

2.3. Mutation frequencies

Cells from overnight cultures (10^2–10^3) were inoculated in 5 mL of LB broth and grown overnight. Aliquots from serial dilutions were plated onto LB agar plates with and without antibiotic (rifampicin or meropenem at 300 μg/mL and 8 μg/mL, respectively). Colony counting was performed after 24 h. Experiments were done in triplicate from independent colonies.

2.4. Analysis of outer membrane proteins

Cells from 20-mL overnight cultures grown on LB broth were sonicated, and outer membrane proteins were extracted and analyzed by SDS-PAGE (López-Camacho et al., 2014b).

2.5. Molecular methods

Clones were identified by MLST (Diancourt and Passet, 2005) or by clone-specific real-time PCR for *K. pneumoniae* ST11 and ST405 (López-Camacho et al., 2014a). The *ompK36* mutants were analyzed by PCR using the primers K1ompK36-F (5′-GCACAATGAAATA GCCGACTG-3′) and K1ompK36-R (5′-ATCGAGGCTCCTCTTACCAG-3′) for the coding sequence and ompK36-Prom-F (5′-CCGCCCAGGAA TTATCTTAG-3′) and ompK36-Prom-R (5′-GTCGTTGATCTGGGTTTCG-3′) for the promoter sequence. The *ompK36* coding sequence and the promoter region were amplified separately with standard PCR conditions, except for a long extension step (5 min) to allow amplification of long DNA fragments in mutants carrying insertion elements. The final products were visualized by agarose gel electrophoresis. PCR amplification products were sequenced and compared with the *ompK36* sequence of the previously sequenced ST405 isolates KpO3210 (Wesselink et al., 2012) and *K. pneumoniae* 909957 (Genbank accession number NZ_AXUL00000000). Screening for OXA-48, SHV, CTX-M, TEM, and OXA-1 β-lactamase genes was done by PCR (Jones et al., 2009; Paño-Pardo et al., 2013).

3. Results

3.1. Bacterial populations

Twenty-four OXA-48–producing clinical isolates that exhibited decreased susceptibility to carbapenems and were resistant to most β-lactams (except the single ST45 isolate, which was susceptible to cephalosporins) and to amoxicillin/clavulanic acid and piperacillin/tazobactam combinations were studied. In addition to β-lactam resistance, most of the isolates presented combined resistance to fluoroquinolones, cotrimoxazole, aminoglycosides (gentamicin and tobramycin), and fosfomycin. Two isolates were porin-deficient mutants that had high carbapenem MICs (one ST405 and one ST846, both confirmed by SDS-PAGE). Twelve isolates belonged to sequence type ST405, 8 to ST11, and the remaining 4 isolates were characterized as ST45, ST147, ST323, and ST846 (Table 1). All of them showed growth of colonies within the meropenem inhibition zones in diffusion assays (either disks or gradient-strips). These colonies had the same MICs as the parental isolates and reproduced the colonies-within-zone phenotypes when retested, indicating they were persister cells.

3.2. Measurement of meropenem-resistance mutation frequencies

Table 1
OXA-48–producing isolates used in this work. The table indicates for each isolate the ST and the MICs of meropenem measured by E-test and microdilution, and the MICs of imipenem and ertapenem measured by microdilution.

Isolate	ST	Meropenem MIC (μg/mL) Microdilution	Meropenem MIC (μg/mL) E-test	Imipenem MIC (μg/mL) Microdilution	Ertapenem MIC (μg/mL) Microdilution
KpO1670	ST405	1	0.75	2	8
KpO3210	ST405	0.5	0.25	2	2
KpO8517	ST405	0.5	0.38	1	4
KpO0568	ST405	0.5	0.38	2	8
KpO1439	ST405	2	0.5	4	16
KpO1562	ST405	8	6	8	128
KpO2009	ST405	1	0.5	4	8
KpO2292[a]	ST405	32	>32	16	>128
KpO2307	ST405	32	>32	8	128
KpO3472	ST405	1	0.75	4	16
KpO6219	ST405	1	0.38	2	4
KpO5334	ST405	1	0.5	2	4
KpO7533	ST11	2	0.75	2	8
KpO0973	ST11	2	1	4	16
KpO5588	ST11	2	0.75	2	16
KpO0235	ST11	0.5	0.38	2	4
KpO2989	ST11	1	0.75	2	8
KpO1606	ST11	1	0.75	4	16
KpO7874	ST11	1	0.75	2	8
KpO4003	ST11	4	0.75	2	16
KpO6540	ST45	1	0.38	2	8
KpO6500	ST147	0.25	0.25	2	2
KpO1015	ST323	1	0.25	4	2
KpO6009[b]	ST846	16	>32	32	64

[a] Porin mutant: GACACC duplication into the *ompK36*-coding sequence at nucleotide position 409 (duplication in L3 loop).
[b] Porin mutant: *IS*4 insertion into the *ompK36*-coding sequence at nucleotide position 85.

Mutation to meropenem resistance was characterized by selecting mutants able to grow in plates with 8 μg/mL meropenem. Only the 2 porin mutants could grow (as expected from their MICs) in those plates, although a few resistant colonies were observed in some of the other isolates. The frequency of meropenem-resistant colonies was measured *in vitro* for 22 isolates. Under the experimental conditions used, no meropenem-resistant mutants were obtained from isolates KpO0235 (ST11), KpO6500 (ST147), and KpO1015 (ST323). Excluding these 3 isolates (and the 2 porin mutants), the average mutation frequency to 8 μg/mL meropenem resistance was $5.47 \cdot 10^{-6}$ (median: $2.82 \cdot 10^{-7}$), with a broad range of frequencies: $2.59 \cdot 10^{-8}$–$5.87 \cdot 10^{-5}$. For comparison, the frequencies of rifampicin resistance mutations were measured and were found to be similar for all the isolates, with an average mutation frequency of $2.38 \cdot 10^{-8}$ (median: $1.13 \cdot 10^{-8}$) and a comparatively narrow range of frequencies: $2.82 \cdot 10^{-9}$–$8.08 \cdot 10^{-8}$ (Fig. 1).

3.3. Analysis of the carbapenem resistant mutants

Ten meropenem-resistant colonies from each of 8 ST405 isolates and 3 ST11 isolates were randomly selected, and the *ompK36* gene of each one was analyzed. Out of 110 resistant mutants, in 52, the size of the *ompK36* gene amplification product was the same as the wild-type control (KpO3210) (Wesselink et al., 2012); in 56 colonies, the amplification product was 1 kb larger; and in 2 mutants, the *ompK36* gene could not be amplified. The *ompK36* amplicons from 15 randomly selected meropenem-resistant colonies of 6 ST405 isolates were sequenced (Table 2). The mutants that had amplicons of the same apparent size as the wild-type strain had nonsilent point mutations or small insertions in the *ompK36* coding sequence. The mutants with larger amplicons had an IS1 insertion element in the *ompK36* promoter region (Table 2). The outer membrane proteins of the IS1 insertion mutants and the KpO2307-1 mutant, which had a small DNA duplication into the *ompK36* coding sequence (Table 2), were analyzed by SDS-PAGE. The OmpK36 porin protein could not be detected in any insertion mutant (Fig. 2). The OmpK35 porin could not be detected in KpO3210 or any of the mutant isolates even though no mutations were found in the *ompK35* promoter region and coding sequences of KpO3210 (ST405).

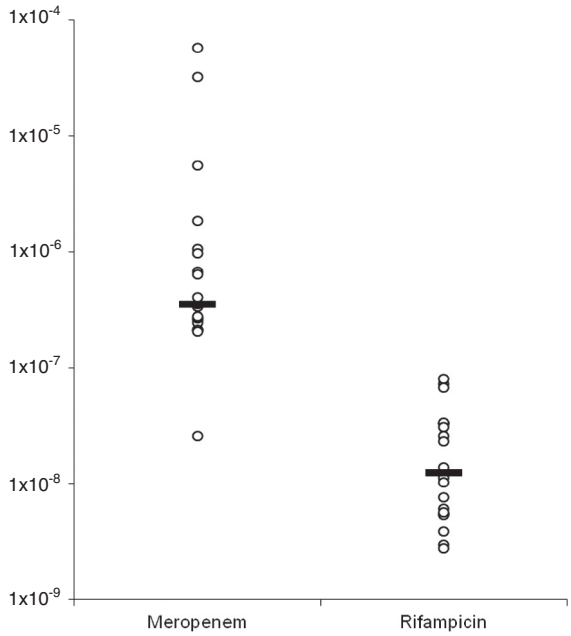

Fig. 1. Frequencies of mutation to meropenem and rifampicin resistance in the OXA-48–producing clinical isolates shown in Table 1. The line indicates the average value of the mutation frequencies.

Table 2
Mutations found in the *ompK36* porin gene in a set of spontaneous meropenem-resistant mutants from different ST405 isolates. Numbers in parentheses indicate the nucleotide positions of the mutations within the *ompK36* gene sequence. Negative numbers indicate positions upstream of the *ompK36* start codon. Premature stop codon numbers correspond to amino acid position of the unprocessed protein sequence.

Isolate	Genetic change (nucleotide position) and codon modification	Genetic lesion at protein level
KpO8517–1	(−95)::IS*1* (3′ → 5′)	NE
KpO8517–2	(−95)::IS*1* (3′ → 5′)	NE
KpO8517–5	(−23)::IS*1* (3′ → 5′)	NE
KpO0568–5	G → A (375); W125STOP	NE
KpO0568–7	ΔAG(271–272); FS	NE
KpO1439–6	ΔA(524); FS	NE
KpO1439–7	G → A (375); W125STOP	NE
KpO1439–8	(−4)::IS*1* (3′ → 5′)	NE
KpO2009–3	(−22)::IS*1* (3′ → 5′)	NE
KpO2009–10	(−36)::IS*1* (3′ → 5′)	NE
KpO2307–1	(409)::(GACACC); D137-T138 duplication	OmpK36V
KpO2307–2	(409)::(GACACC); D137-T138 duplication	OmpK36V
KpO2307–5	(−31)::IS*1* (5′ → 3′)	NE
KpO3472–3	(−72)::IS*1* (3′ → 5′)	NE
KpO3472–4	G → A (375); W125STOP	NE

FS = frameshift; NE = not expressed (no protein detected by SDS-PAGE); OmpK36V = OmpK36 variant.

3.4. Analysis of carbapenem heteroresistance in OXA-48–producing K. pneumoniae: PAP curves

To study heteroresistance, PAP of selected isolates from the 2 major clones was done. One isolate from a sporadic clone (ST45) that never had colonies within the inhibition zones was included. The porin-deficient OXA-48–producing mutant isolate KpO6009, which belongs to ST846, was used as a carbapenem-resistant control. This isolate did not present inhibition zone in diffusion assays. PAP curves show that colonies of both ST405 and ST11 could be obtained in the presence of meropenem at concentrations as high as 32 μg/mL (Fig. 3). The porin-deficient KpO6009 isolate expressed high carbapenem resistance homogeneously. The frequencies of resistant colonies obtained at different meropenem concentrations were determined. The curves vary slightly among the different isolates, but for meropenem concentrations ≥4 μg/mL, the colony frequencies were within the range of the mutation frequencies obtained at 8 μg/mL, suggesting that they might be meropenem-resistant mutants (Fig. 3). Six randomly selected colonies grown on plates with meropenem concentrations ≥4 μg/mL were further analyzed (4 colonies grown at 4 μg/mL of KpO5588 and 2 colonies of both KpO7533 and KpO1670 grown at 16 μg/mL). Meropenem

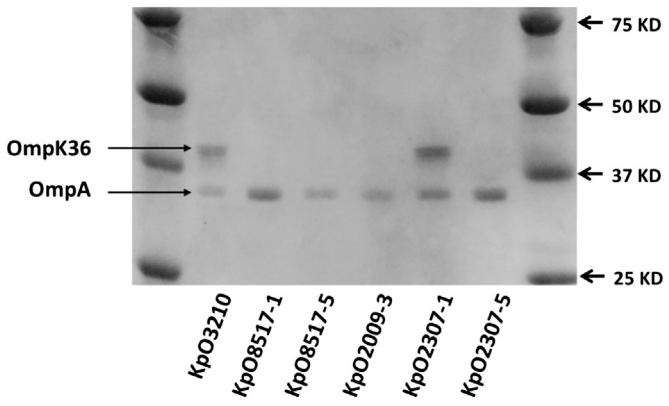

Fig. 2. SDS-PAGE analysis of outer membrane fractions obtained from selected carbapenem-resistant mutants obtained *in vitro* from several OXA-48–producing isolates. KpO3210 is the carbapenem-susceptible control. KpO8517-1, KpO8517-5, KpO2009-3, and KpO2307-5 are carbapenem-resistant IS1 insertion mutants. KpO2307-1 is a carbapenem-resistant mutant with a 6 nt DNA duplication in the *ompK36* gene that generates a 2–amino acid duplication in the internal L3 loop of the porin.

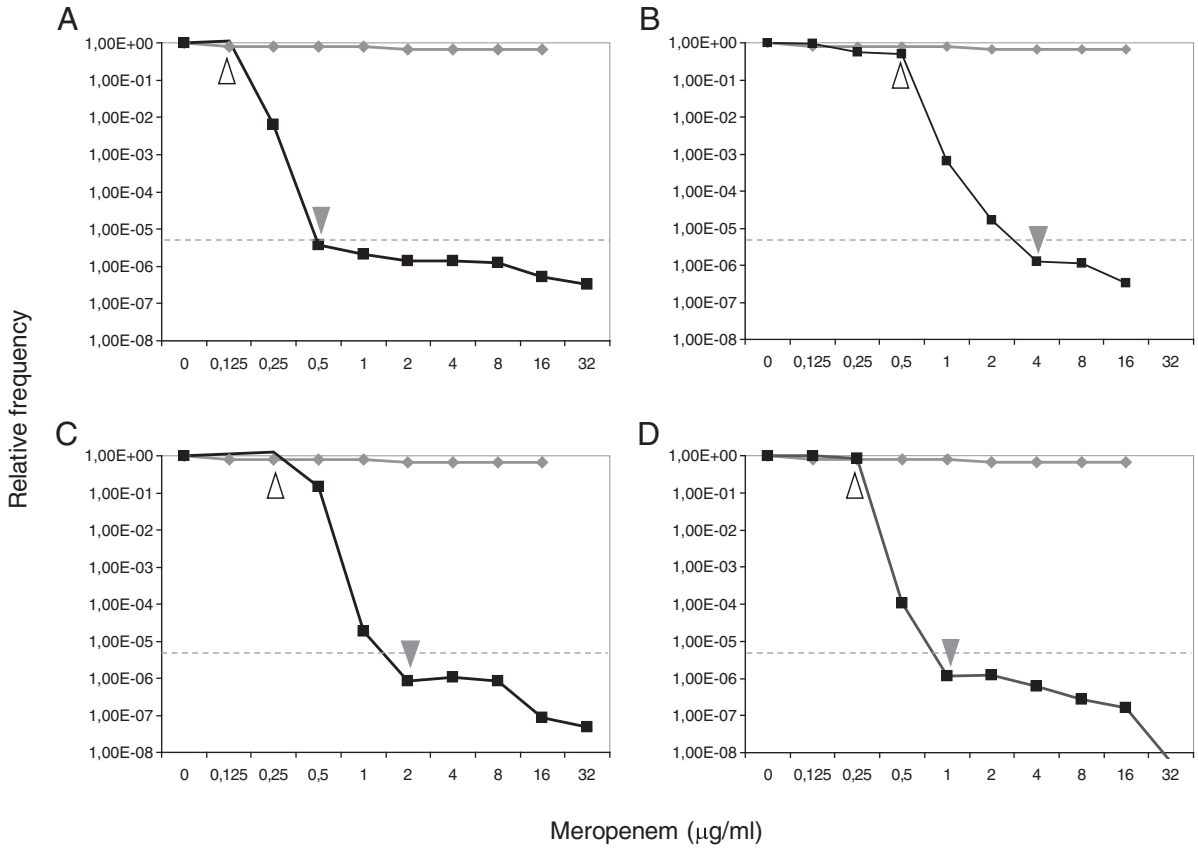

Fig. 3. Population analysis profiles of selected OXA-48–producing isolates plated in meropenem. The graphs represent the frequencies of colonies grown on the different meropenem concentrations relative to the plated viable counts. In all 4 panels, the profile of the meropenem-resistant isolate KpO6009 is included as a reference (gray line). The black lines show the different isolate profiles: A) KpO1670 (ST405), B) KpO7533 (ST11), C) KpO6540 (ST45, this isolate did not have colonies in the growth inhibition zones), D) KpO5588 (ST11). The dashed gray line shows the average meropenem-resistance mutation frequency obtained at a meropenem concentration of 8 μg/mL (5.47×10^{-6}). Open triangles point to the highest noninhibitory concentration. Gray triangles point to the lowest concentration exhibiting maximum inhibition. The difference between these 2 concentrations is in all cases ≤4 dilutions (El-Halfawy and Valvano, 2015).

Etest assays of these colonies showed that none of them presented inhibition zones (*i.e.*, MICs >32 μg/mL). All 6 colonies were retested again after 3 daily passages on liquid meropenem-free medium, and none of them presented inhibition zones, showing that they were stable meropenem-resistant mutants.

4. Discussion

In the course of the current expansion of OXA-48–producing *K. pneumoniae*, a broad range of MIC values for carbapenems has been observed among different isolates, even within those belonging to the same ST. Some isolates reproducibly display colonies within the inhibition zones in disk or gradient diffusion assays, independently of the MIC values measured by microdilution. We have analyzed the frequency and the nature of the meropenem-resistant colonies in OXA-48–producing *K. pneumoniae* clinical isolates obtained in the context of a hospital outbreak.

Analysis of the colonies obtained with meropenem concentrations ≥4 μg/mL in PAP experiments showed them to be stable resistant mutants, different to the colonies within inhibition zones. Their frequencies were similar to the meropenem resistance mutation frequencies of the same isolates determined at ≥8 μg/mL and much lower than the frequencies of persisters within inhibition zones. PAP of several OXA-48–producing isolates showed that the distance between the highest noninhibitory concentration and the lowest concentration exhibiting maximum inhibition was 2- to 3-fold, and if the criteria of El Halfalwy and Valvano were considered (El-Halfawy and Valvano, 2015), those isolates should not be defined as heteroresistant. Nevertheless, the growth of persisters inside inhibition zones in disk and gradient diffusion assays shows that there is indeed a subpopulation of cells which is different from that selected in high meropenem concentrations. This apparent conflict suggests that for OXA-48–producing *K. pneumoniae*, the methodological differences between PAP assays and diffusion assays are important. In mutation frequency determination and in PAP assays, the cells are exposed directly to certain antibiotic concentrations, while in diffusion assays, the antibiotic concentration builds up gradually as the antibiotic diffuses from the disks or the strips, and this gradual exposure might have a role in inducing persistence in a fraction of the cell population. Indeed, gradual increases of antibiotic concentration, as well as preexposure to low antibiotic concentrations, have been shown to increase persistence in *K. pneumoniae* (Li et al., 2018; Ren et al., 2015). Similarly, in diffusion assays, growth on a solid substrate in the presence of very low initial antibiotic concentrations could be related to the persistence phenomenon, perhaps because of a biofilm-like growth in solid media.

The analysis of several meropenem-resistant mutants showed that the most common mechanism is by far a deficiency of porin expression or activity. All the mutants analyzed had some mutation in the *ompK36* porin gene, and about half of them had IS1 insertions and no detectable OmpK36 protein. Interestingly, some of the mutations obtained had been described before in clinical isolates showing that the mutations selected *in vitro* are similar to those selected *in vivo*. One clinical isolate (KpO2292) and 1 mutant obtained *in vitro* (KpO2307-1) had the same 6-bp duplication (5′-GACACC-3′) in the *ompK36*-coding sequence that generated a duplication of 2 amino acid residues in the L3 loop of the protein, located in the inner part of the pore and probably having an

effect on the size or the functionality of the channel (Dutzler et al., 1999). The same mutation had been found in an ertapenem-resistant *K. pneumoniae* clinical isolate obtained in Italy (García-Fernández et al., 2010). Three of the mutants obtained independently from different isolates had the W125STOP mutation, which had been described previously in clinical isolates from the United Kingdom (Doumith et al., 2009).

In summary, when studying heteroresistance in OXA-48–producing *K. pneumoniae*, use of PAP assays and gradient diffusion assays may result in apparently contradictory, not completely congruent results, which can be related to the methodological differences between these 2 approaches. Analyses of mutants obtained in mutation frequency assays and in PAP assays show that porin deficiency is the major mechanism involved in MIC increases, and the high mutation frequencies (between 10^{-5} and 10^{-4}) measured in some isolates suggest that the risk of development of carbapenem resistance during therapy should not be disregarded.

Declarations

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Competing interests

No.

Ethical approval

Not required.

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