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Contribution of OmpK36 to carbapenem susceptibility in KPC-producing *Klebsiella pneumoniae*

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Isolates of *Klebsiella pneumoniae* harbouring the carbapenemase KPC may have carbapenem MICs that remain in the susceptible range, and may therefore go unrecognized. To understand the mechanisms contributing to the variability in carbapenem MICs, 20 clinical isolates, all belonging to either of two clonal groups of KPC-possessing *K. pneumoniae* endemic to New York City, were examined. Expression of genes encoding KPC, the porins OmpK35 and OmpK36, and the efflux pump AcrAB was examined by real-time RT-PCR. Outer-membrane profiles of selected KPC-producing isolates were examined by SDS-PAGE, and proteins were identified by matrix-assisted laser desorption/ionization mass spectrometry. The identification of SHV and TEM β-lactamases and the genomic sequences of *ompK35* and *ompK36* were determined by PCR and DNA sequencing, respectively. For one clonal group, carbapenem MICs increased with decreasing expression of *ompK36*. A second clonal group also had carbapenem MICs that correlated with *ompK36* expression. However, all of the isolates in this latter group continued to produce OmpK36, suggesting that porin configuration may affect entry of carbapenems. For isolates that had the greatest expression of *ompK36*, carbapenem MICs tended to be lower when determined by the broth microdilution technique, and scattered colonies were seen around the Etest zones of inhibition. All of the KPC-producing isolates were highly resistant to ertapenem, regardless of *ompK36* expression. In conclusion, isolates of KPC-possessing *K. pneumoniae* that express *ompK36* tend to have lower MICs to carbapenems and therefore may be more difficult to detect by clinical laboratories. Regardless of *ompK36* expression, all of the KPC producers were consistently resistant to ertapenem.

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INTRODUCTION

Klebsiella pneumoniae possessing the carbapenemase KPC is now widespread in many medical centres in the north-eastern USA and is being increasingly reported worldwide (Bratu *et al.*, 2005a; Landman *et al.*, 2007; Queenan & Bush, 2007). The progressive spread of this β-lactamase, which is an efficient cephalosporinase and carbapenemase, can be partly explained by the difficulty clinical laboratories have in detecting isolates harbouring this enzyme. When carbapenem resistance is used as the gauge to identify KPC-possessing *K. pneumoniae*, a significant proportion will not be detected as they may have MICs that are below the breakpoint for resistance. In particular, automated systems may frequently misidentify these isolates as susceptible to carbapenems (Anderson *et al.*, 2007; Bratu *et al.*, 2005b; Tenover *et al.*, 2006). The reason for the variability in carbapenem MICs, particularly with imipenem and meropenem, is unknown.

It appears that there may be several factors besides carbapenemases that can contribute to carbapenem resistance in isolates of *K. pneumoniae*. Although unusual, *K. pneumoniae* lacking an efficient carbapenemase can still achieve resistance to carbapenems. These isolates typically possess an underlying extended-spectrum β-lactamase (ESBL) or AmpC-type β-lactamase (Bradford *et al.*, 1997; Cao *et al.*, 2000; Crowley *et al.*, 2002). In addition to a background of β-lactamase activity, alterations in porin production are an important contributor to carbapenem resistance. Many ESBL-carrying clinical isolates of *K. pneumoniae* lack OmpK35 (Hernández-Allés *et al.*, 1999a), which augments resistance to ceftazidime (Doménech-Sánchez *et al.*, 2003). The additional loss of OmpK36 leads to reduced susceptibility to cefotaxime, cephamycins and carbapenems (Ardanuy *et al.*, 1998; Crowley *et al.*, 2002; Doménech-Sánchez *et al.*, 2000, 2003;

Abbreviations: ESBL, extended-spectrum β-lactamase; MALDI, matrix-assisted laser desorption/ionization.

The GenBank/EMBL/DDBJ accession numbers for the sequences determined in this study are FJ577672 (*ompK35* from isolate CI507) and FJ555673 and FJ555675 (*ompK36* from isolates VM522 and CI839, respectively).

Martínez-Martínez *et al.*, 1996, 1999). Ertapenem may be particularly affected by the concomitant loss of OmpK35 and OmpK36 (Elliott *et al.*, 2006; Jacoby *et al.*, 2004; Mena *et al.*, 2006; Woodford *et al.*, 2007). Porin loss has been correlated with disruption of *ompK35* and *ompK36* by insertions or deletions in some isolates (Cai *et al.*, 2008; Hernández-Allés *et al.*, 1999b; Kaczmarek *et al.*, 2006; Mena *et al.*, 2006). In the small number of isolates of KPC-producing *K. pneumoniae* that have been studied, loss of OmpK35 (Woodford *et al.*, 2004; Yigit *et al.*, 2001) or OmpK36 (Cai *et al.*, 2008) has been observed. Finally, the AcrAB efflux system has been shown to affect a wide range of antimicrobial agents, including tetracyclines, chloramphenicol, trimethoprim and fluoroquinolones (Nikaido, 1996). Whether antibiotic efflux via this system is an important contributor to carbapenem resistance is unknown.

In this report, the mechanisms contributing to carbapenem resistance in clonal groups of KPC-possessing *K. pneumoniae* endemic to New York City were examined.

METHODS

Twenty clinical isolates of *K. pneumoniae* obtained from prior surveillance studies were chosen because they belong to important bla_{KPC}-possessing ribotypes endemic to our region (Bratu *et al.*, 2005a, b; Landman *et al.*, 2007). Susceptibility testing was performed using the Etest method (AB Biodisk); for isolates possessing bla_{KPC}, susceptibility testing for the carbapenems was also performed using the broth microdilution method (CLSI, 2006). For isolates possessing bla_{KPC}, carbapenem hydrolysis of crude cell extracts in 100 mM phosphate buffer was measured spectrophotometrically, as described previously (Woodford *et al.*, 2004). Student's *t*-test was used to compare hydrolysis rates between isolates; a two-tailed *P* value of $\leqslant 0.05$ was considered significant.

DNA amplification studies. To identify conserved regions, genetic sequences for a ribosomal housekeeping gene and for the target genes *ompK35*, *ompK36* and *acrB* were amplified and sequenced using the primers described in Table 1. DNA sequencing was performed using an automated fluorescent dye terminator sequencing system (Applied Biosystems) and analysed using BLAST. Plasmids were isolated by the alkaline lysis method (Sambrook & Russell, 2001). Plasmids were transformed into One Shot TOP10 Electrocomp *Escherichia coli* (Invitrogen) by electroporation. Plasmid DNA from the transformants was then screened for the presence of TEM, SHV and KPC β-lactamases, and identified using previously established primers and conditions (Bratu *et al.*, 2005a; Paterson *et al.*, 2003). Selected isolates were also examined for the presence of ACT-1 (Bradford *et al.*, 1997).

RT-PCR studies. Isolates were examined by real-time RT-PCR for expression of *ompK35*, *ompK36*, bla_{KPC} and *acrB*. DNase-treated RNA was obtained from late-exponential-phase cultures using an RNeasy kit (Qiagen), and 25 ng RNA was used for each sample. Primer and probe concentrations were adjusted to achieve efficiencies of 90–110 % and all experiments were performed in triplicate. The primers and probes used in these experiments are given in Table 1. Expression of each gene was normalized to that of a ribosomal housekeeping gene (*rrsA*). The relative expression of *ompK35* and *ompK36* was then calibrated against the corresponding expression by *K. pneumoniae* ATCC 13883 (an isolate with known expression of both porins).

Table 1. Primers and probes used for PCR amplification and real-time RT-PCR studies

Primer/probe	Sequence (5′→3′)
DNA amplification and sequencing	
ribofor	CAGCCACACTGGAACTGAGA
riboRev	TTATGAGGTCCGCTTGCTCT
ompK35for	AACTTATTGACGGCAGTGGC
ompK35rev	TTGGTAAACGATACCCACGG
ompK36for	GCAGTGGCATAATAAAAGGCA
ompK36rev	ACTGGTAAACCAGGCCCAG
acrBfor	TCAAACCAGGTGTGCAGGTA
acrBrev	TTAATACCCAGACCGGATGC
RT-PCR	
*ribo*for	GAAGAAGCACCGGCTAACTC
*ribo*rev	CACATCCGACTTGACAGACC
*ribo*probe	FAM-TGCCAGCAGCCGCGGTAATA-TAMRA
*ompK35*for	GTCTGGACCACCAATGGC
*ompK35*rev	GATCTGAGTTTCGCCTTTCA
*ompK35*probe	FAM-CCACCTATGCCCGTATCGGCC-TAMRA
*ompK36*for	GACCAGACCTACATGCGTGTA
*ompK36*rev	GTATTCCCACTGGCCGTAAC
*ompK36*probe	FAM-TGGGTTTCGCCTTTCACGCC-TAMRA
*kpc*for	CGTGACGGAAAGCTTACAAA
*kpc*rev	AGCCAATCAACAAACTGCTG
*kpc*probe	FAM-CTGGGCTCTGCACTGGCTGC-TAMRA
acrBfor	CAATACGGAAGAGTTTGGCA
acrBrev	CAGACGAACCTGGGAACC
acrBprobe	FAM-TCCTGGTTCACCTTCAGCAGGATG-TAMRA

Expression of *acrB* was calibrated against a susceptible control isolate, *K. pneumoniae* ATCC 11296. Expression of bla_{KPC} was initially calibrated against a previously described transformant possessing a plasmid with bla_{KPC-2} (Bratu *et al.*, 2007); the clinical isolate of *K. pneumoniae* with the lowest expression was then considered the control. For all controls, relative expression was equal to 1.0.

Isolation and characterization of outer-membrane proteins. Selected isolates had their outer-membrane proteins analysed by SDS-PAGE using standard techniques (Albertí *et al.*, 1995; Hernández-Allés *et al.*, 1999a). Briefly, isolates were grown in nutrient broth, sonicated and centrifuged. Cell membranes were obtained following centrifugation at 100 000 ***g***, and extracted with 1 % *N*-lauroylsarcosine. Protein concentrations were measured using the Bradford method, and each well contained ~4 μg total protein. Samples were boiled in Laemmli's sample buffer. SDS-PAGE was performed with a 10 % acrylamide gel, which was then stained with Coomassie blue, and selected proteins were identified using matrix-assisted laser desorption/ionization (MALDI) MS. Peptide sequences were compared with published outer-membrane proteins of *K. pneumoniae*.

RESULTS AND DISCUSSION

Isolates belonging to clonal group A

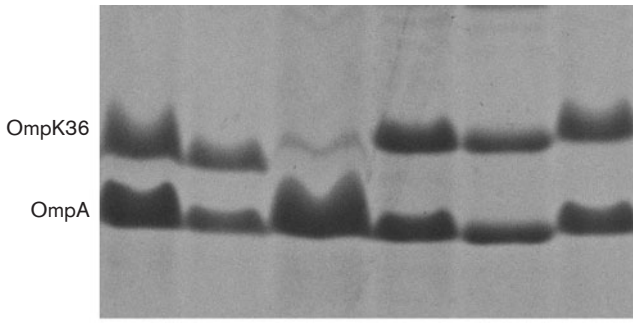
Eleven isolates belonged to clonal group A, a strain responsible for ~85 % of bla_{KPC}-possessing isolates in our region (Bratu *et al.*, 2005a). Five isolates did not harbour bla_{KPC} but did have ESBLs (either bla_{SHV-12}-like or bla_{SHV-11}-like) and had reduced susceptibility to ceftazidime (Table 2). The discrepancy in cephalosporin susceptibility for isolates CI504 and CI505 could not be explained by the information gathered from each isolate. All five had reduced expression of *ompK35*, and DNA sequencing revealed a frameshift mutation in this gene. All five expressed *ompK36* and, consistent with other reports, remained susceptible to cefoxitin and carbapenems (Ardanuy *et al.*, 1998; Crowley *et al.*, 2002; Doménech-Sánchez *et al.*, 2000, 2003; Martínez-Martínez *et al.*, 1996). Six isolates in this clonal group possessed bla_{KPC-2} (along with other β-lactamases). Despite variable expression of *ompK35*, all of these isolates possessed the same frameshift mutation, and OmpK35 was not evident by SDS-PAGE (Fig. 1) and MALDI MS. DNA sequencing revealed that the *ompK36* gene in this group was closely related to that found in the published genome of isolate MGH 78578. Expression of *ompK36* was variable in these six isolates and correlated with findings by SDS-PAGE (Fig. 1). Isolates with evidence of OmpK36 production had lower MICs to imipenem when determined by broth microdilution (Table 2) and had scattered colonies within the Etest zone

Table 2. Presence of other β-lactamases, expression by real-time RT-PCR of bla_{KPC}, *ompK35* and *ompK36* and MICs for β-lactams from clinical isolates belonging to clonal groups A and B

Isolate	β-Lactamase(s)	Presence of bla_{KPC-2}	Relative expression of:*			MIC (mg l^{-1})†					
			bla_{KPC}	*ompK35*	*ompK36*	IPM*	MEM	ERT	FOX	CAZ	FEP
Clonal group A											
CI504	SHV-11	No		0.54	4.58	0.19	0.032	0.064	0.5	>256	3
CI505	SHV-11	No		0.24	1.31	0.25	0.032	0.064	0.5	4	0.5
CI518	SHV-12	No		0.13	1.01	0.5	0.064	0.047	0.75	>256	2
VM522	SHV-12	No		0.18	0.88	0.5	0.032	0.047	0.75	>256	3
MA549	SHV-12	No		0.18	0.87	0.38	0.047	0.38	2	>256	8
CI516	SHV-11; TEM-1	Yes	1	2.74	4.19	12 (8)	16 (32)	24 (32)	48	128	16
DM834	SHV-12; TEM-1	Yes	1.25	0.54	2.94	>32 (8)	>32 (>32)	>32 (>32)	256	>256	24
VM9	SHV-11; TEM-1	Yes	1.1	0.49	1.7	12 (16)	24 (16)	24 (>32)	48	>256	192
MA31	SHV-12	Yes	1	0.06	0.25	>32 (32)	24 (32)	>32 (>32)	64	>256	24
CI507	SHV-11; TEM-1	Yes	3.75	0.17	0.06	>32 (512)	>32 (>32)	>32 (>32)	>256	>256	192
KC850	SHV-12; TEM-1	Yes	2.5	0.08	0.004	>32 (1024)	>32 (>32)	>32 (>32)	>256	>256	128
Clonal group B											
CI512	SHV-12; TEM-1	No		2.5	8.22	0.5	0.047	0.064	0.75	>256	24
CI511	SHV-12; TEM-1	No		1.25	3.11	0.5	0.125	0.5	4	>256	16
KB420	TEM-1	No		1.45	3.03	0.12	0.125	0.38	3	128	6
CI806	SHV-12; TEM-1	No		1.11	1.81	0.25	0.125	0.75	4	>256	16
CI513	SHV-12; TEM-1	Yes	18.75	0.94	7.71	24 (8)	6 (16)	24 (32)	128	>256	96
CI839	SHV-12; TEM-1	Yes	5	0.37	7.59	>32 (2)	>32 (>32)	>32 (>32)	64	>256	48
WO822	SHV-12; TEM-1	Yes	1.1	1.58	5.7	4 (8)	24 (8)	>32 (>32)	32	>256	256
WO555	SHV-12; TEM-1	Yes	5	1.8	4.47	>32 (32)	>32 (>32)	>32 (>32)	>256	>256	96
KB528	SHV-11; TEM-1	Yes	22.5	0.16	3.28	>32 (256)	>32 (>32)	>32 (>32)	>256	>256	256

*Relative expression compared with control (=1.0).

†IPM, imipenem; MEM, meropenem; ERT, ertapenem; FOX, cefoxitin; CAZ, ceftazidime; FEP, cefepime. Carbapenem MICs in parentheses were determined by the broth microdilution method.



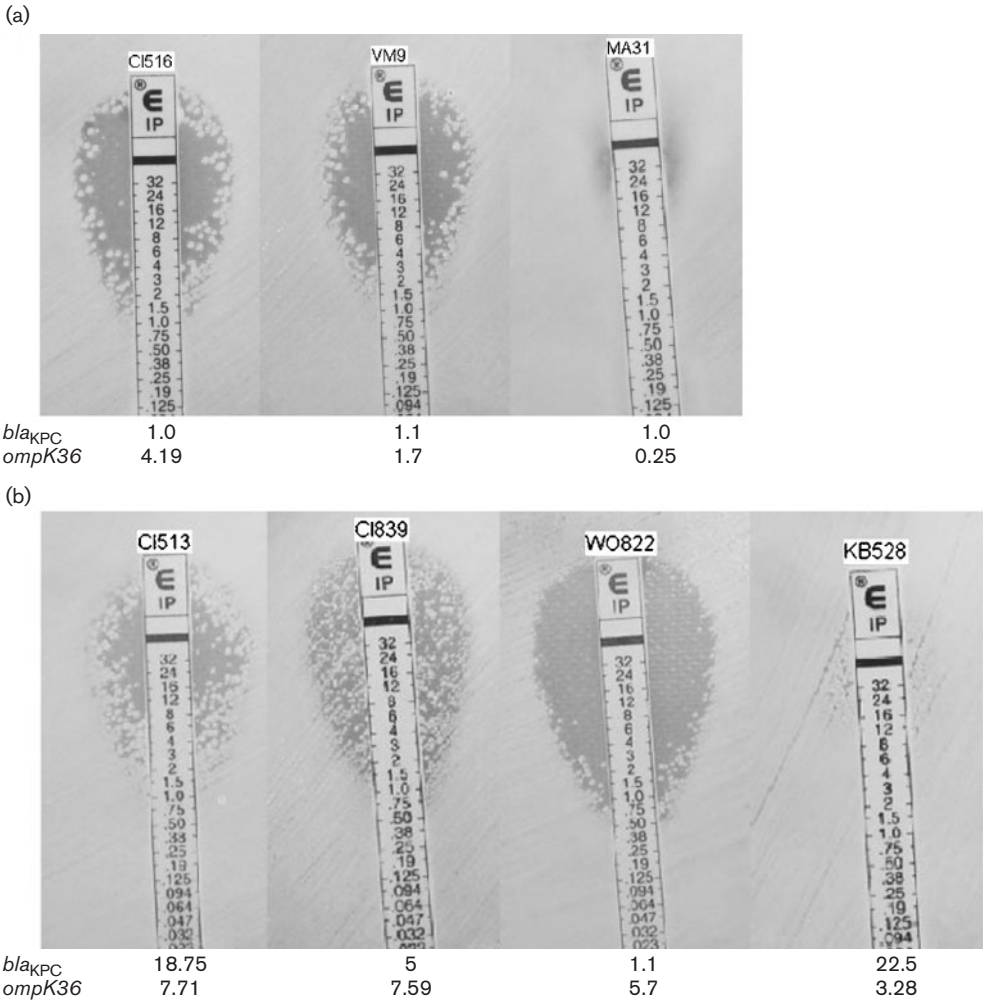
Clone	A	A	A	B	B	B
Isolate	CI516	DM834	CI507	WO822	CI839	KB528
ompK36	4.2	2.9	0.06	5.7	7.6	3.3

Fig. 1. Outer-membrane protein analysis of isolates of *K. pneumoniae* by SDS-PAGE. The clonal group, isolate name and relative expression of *ompK36* by real-time RT-PCR are provided for each lane. Labelled proteins were identified by MALDI MS.

of inhibition (Fig. 2a). All six were resistant to meropenem and ertapenem (Table 2). Expression of *acrB* did not correlate with the MICs of the carbapenems (data not shown).

Isolates belonging to clonal group B

Nine isolates belonged to clonal group B, a second important bla_{KPC}-possessing strain in our region (Bratu *et al.*, 2005a). Four ESBL-possessing isolates (lacking bla_{KPC}) expressed *ompK36* and remained susceptible to cefoxitin (and resistant to ceftazidime; Table 2). Five isolates possessed bla_{KPC-2}. Although most had evidence of transcription of *ompK35* by real-time RT-PCR, production of OmpK35 was not evident by SDS-PAGE (Fig. 1), suggesting post-transcriptional events affecting this porin. All of the isolates in this group had ample expression of *ompK36*, and this was also evident by SDS-PAGE (Fig. 1). Except for a 2 aa insertion (Gly-Asp) in the region of loop



bla_{KPC}	1.0	1.1	1.0	
ompK36	4.19	1.7	0.25	
bla_{KPC}	18.75	5	1.1	22.5
ompK36	7.71	7.59	5.7	3.28

Fig. 2. Imipenem Etest images of isolates with variable expression of bla_{KPC} and *ompK36*. (a) Isolates from clonal group A; (b) isolates from clonal group B. The relative expression of each gene for the isolate is given below its image.

3 of the porin, OmpK36 for isolates in this group was similar to that found in the susceptible control, *K. pneumoniae* ATCC 13883. Compared with OmpK36 found in clonal A group, the protein in this group had a 7 aa insert in the loop 6 region of the porin (in addition to several amino acid substitutions). Despite production of OmpK36 in these five isolates with bla_{KPC}, it is noteworthy that decreasing expression of *ompK36* still affected the broth microdilution MICs and Etest zones of inhibition of imipenem (Table 2, Fig. 2b). It appears that, despite OmpK36 production in this group, subtle quantitative or qualitative changes in the porin affected entry of β-lactams. It is also noteworthy that the meropenem, ertapenem and cefoxitin MICs in the group B isolates lacking bla_{KPC} were higher than those seen in the comparable group A isolates. Therefore, it appears that the OmpK36 phenotype in the group B isolates affected entry of β-lactams. Finally, the expression of *acrB* did not correlate with carbapenem resistance for isolates in this group (data not shown).

There appeared to be interplay between expression of bla_{KPC} and *ompK36* that affected the MICs to imipenem. Compared with isolate WO822, isolates CI513 and CI839, which had greater expression of bla_{KPC} and *ompK36*, had substantially higher imipenem MICs (Fig. 2b). Carbapenem hydrolysis studies supported the observations of the bla_{KPC} expression studies. Compared with isolates with an expression level of 1–1.25 times the control expression of bla_{KPC}, isolates with a $\geqslant$2.5-fold level of expression tended to have increased spectrophotometric hydrolysis rates of imipenem [0.22 ± 0.16 vs 0.13 ± 0.07 μg (mg protein)$^{-1}$ min^{-1}] and of meropenem [0.12 ± 0.09 vs 0.09 ± 0.04 μg (mg protein)$^{-1}$ min^{-1}], and significantly increased hydrolysis rates of ertapenem [0.09 ± 0.06 vs 0.05 ± 0.02 μg (mg protein)$^{-1}$ min^{-1}, $P=0.05$].

Expression of *ompK35* and *ompK36* was also assessed in four unrelated cephalosporin-susceptible clinical isolates. Compared with the control strain, *K. pneumoniae* ATCC 13883, expression of *ompK35* was similar (mean 1.9 times the control, range 0.43–4.4) in these susceptible isolates. However, expression of *ompK36* was increased compared with the control isolate (mean 4.4 times the control, range 2.0–5.4). Therefore, although *K. pneumoniae* ATCC 13883 is frequently used as a control in studies examining outer-membrane proteins, expression of the genes encoding the porins in this isolate is reduced compared with wild-type clinical isolates.

The rapid and, in some regions, unabated spread of *K. pneumoniae* with KPC β-lactamases has been especially troubling. Clinical laboratories have relied on carbapenem susceptibility to identify pathogens harbouring these enzymes; however, it has been well documented that many isolates with KPC β-lactamases will have MICs of carbapenems below the CLSI-defined breakpoint for susceptibility (Anderson *et al.*, 2007; Bratu *et al.*, 2005b; Tenover *et al.*, 2006). In this report, involving important nosocomial strains of *K. pneumoniae* endemic to New York City, the MICs of carbapenems were found to be dependent on an interaction between the presence of bla_{KPC} and expression of *ompK36*. It was also apparent that the OmpK36 phenotype may influence susceptibility. Isolates that abundantly expressed *ompK36* tended to have lower imipenem MICs by the broth microdilution method and had scattered colonies within the Etest zone of inhibition. Consistent with other reports, the MICs of ertapenem were consistently higher than those of imipenem and meropenem for the isolates possessing bla_{KPC} (Anderson *et al.*, 2007; Bratu *et al.*, 2005b). Isolates that had MICs of imipenem and meropenem in the susceptible or intermediate range continued to be frankly resistant to ertapenem. As all of our examined isolates had either diminished or defective production of OmpK35 (typical of many ESBL-possessing strains), the contribution of this porin to resistance involving ertapenem cannot be assessed in this report. However, prior studies have not found this porin to be important for the development of carbapenem resistance (Hernández-Allés *et al.*, 1999a). Other studies have found that restoring OmpK36 and OmpK37 improved susceptibility to ertapenem in KPC-producing isolates of *K. pneumoniae* (Jacoby *et al.*, 2004). However, OmpK37 is often not expressed (Doménech-Sánchez *et al.*, 1999), and its role in carbapenem resistance in clinical isolates is unknown.

Although cephamycins retain activity against many ESBL-producing isolates of *K. pneumoniae*, they are often not recommended as therapeutic agents due to the development of resistance (secondary to diminished OmpK36 production). A similar caution should be exercised for KPC-producing isolates that appear susceptible to imipenem or meropenem, as downregulation of *ompK36* expression will adversely affect the MICs to the carbapenems.

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REFERENCES

Albertí, S., Rodríquez-Quiñones, F., Schirmer, T., Rummel, G., Tomás, J. M., Rosenbusch, J. P. & Benedí, V. J. (1995). A porin from *Klebsiella pneumoniae*: sequence homology, three-dimensional model, and complement binding. *Infect Immun* **63**, 903–910.

Anderson, K. F., Lonsway, D. R., Rasheed, J. K., Biddle, J., Jensen, B., McDougal, L. K., Carey, R. B., Thompson, A., Stocker, S. & other authors (2007). Evaluation of methods to identify the *Klebsiella pneumoniae* carbapenemase in *Enterobacteriaceae*. *J Clin Microbiol* **45**, 2723–2725.

Ardanuy, C., Liñares, J., Domínguez, M. A., Hernández-Allés, S., Benedí, V. J. & Martínez-Martínez, L. (1998). Outer membrane profiles of clonally related *Klebsiella pneumoniae* isolates from clinical samples and activities of cephalosporins and carbapenems. *Antimicrob Agents Chemother* **42**, 1636–1640.

Bradford, P. A., Urban, C., Mariano, N., Projan, S. J., Rahal, J. J. & Bush, K. (1997). Imipenem resistance in *Klebsiella pneumoniae* is associated with the combination of ACT-1, a plasmid-mediated

AmpC β-lactamase, and the loss of an outer membrane protein. *Antimicrob Agents Chemother* **41**, 563–569.

Bratu, S., Landman, D., Haag, R., Recco, R., Eramo, A., Alam, M. & Quale, J. (2005a). Rapid spread of carbapenem-resistant *Klebsiella pneumoniae* in New York City. *Arch Intern Med* **165**, 1430–1435.

Bratu, S., Mooty, M., Nichani, S., Landman, D., Gullans, C., Pettinato, B., Karumudi, U., Tolaney, P. & Quale, J. (2005b). Emergence of KPC-possessing *Klebsiella pneumoniae* in Brooklyn, New York: epidemiology and recommendations for detection. *Antimicrob Agents Chemother* **49**, 3018–3030.

Bratu, S., Brooks, S., Burney, S., Kochar, S., Gupta, J., Landman, D. & Quale, J. (2007). Detection and spread of *Escherichia coli* possessing the plasmid-borne carbapenemase KPC-2 in Brooklyn, New York. *Clin Infect Dis* **44**, 972–975.

Cai, J. C., Zhou, H. W., Zhang, R. & Chen, G.-X. (2008). Emergence of *Serratia marcescens*, *Klebsiella pneumoniae*, and *Escherichia coli* isolates possessing the plasmid-mediated carbapenem-hydrolyzing β-lactamase KPC-2 in intensive care units of a Chinese hospital. *Antimicrob Agents Chemother* **52**, 2014–2018.

Cao, V. T. B., Arlet, G., Ericsson, B.-M., Tammelin, A., Courvalin, P. & Lanbert, T. (2000). Emergence of imipenem resistance in *Klebsiella pneumoniae* owing to combination of plasmid-mediated CMY-4 and permeability alteration. *J Antimicrob Chemother* **46**, 895–900.

CLSI (2006). *Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria that Grow Aerobically*, 7th edn. Approved Standard M7-A7. Wayne, PA: Clinical and Laboratory Standards Institute.

Crowley, B., Benedí, V. J. & Doménech-Sánchez, A. (2002). Expression of SHV-2 β-lactamase and reduced amounts of OmpK36 porin in *Klebsiella pneumoniae* results in increased resistance to cephalosporins and carbapenems. *Antimicrob Agents Chemother* **46**, 3679–3682.

Doménech-Sánchez, A., Hernández-Allés, S., Martínez-Martínez, L., Benedí, V. J. & Albertí, S. (1999). Identification and characterization of a new porin gene of *Klebsiella pneumoniae*: its role in β-lactam antibiotic resistance. *J Bacteriol* **181**, 2726–2732.

Doménech-Sánchez, A., Pascual, A., Suárez, A. I., Alvarez, D., Benedí, V. J. & Martínez-Martínez, L. (2000). Activity of nine antimicrobial agents against clinical isolates of *Klebsiella pneumoniae* producing extended-spectrum β-lactamases and deficient or not in porins. *J Antimicrob Chemother* **46**, 858–860.

Doménech-Sánchez, A., Martínez-Martínez, L., Hernández-Allés, S., del Carmen Conejo, M., Pascual, A., Tomás, T. M., Albertí, S. & Benedí, V. J. (2003). Role of *Klebsiella pneumoniae* OmpK35 porin in antimicrobial resistance. *Antimicrob Agents Chemother* **47**, 3332–3335.

Elliott, E., Brink, A. J., van Greune, J., Els, Z., Woodford, N., Turton, J., Warner, M. & Livermore, D. M. (2006). In vivo development of ertapenem resistance in a patient with pneumonia caused by *Klebsiella pneumoniae* with an extended-spectrum β-lactamase. *Clin Infect Dis* **42**, e95–e98.

Hernández-Allés, S., Albertí, S., Alvarez, D., Doménech-Sánchez, A., Martínez-Martínez, L., Gil, J., Tomás, J. M. & Benedí, V. J. (1999a). Porin expression in clinical isolates of *Klebsiella pneumoniae*. *Microbiology* **145**, 673–679.

Hernández-Allés, S., Benedí, V. J., Martínez-Martínez, L., Pascual, A., Aguilar, A., Tomás, J. M. & Albertí, S. (1999b). Development of resistance during antimicrobial therapy caused by insertion sequence interruption of porin genes. *Antimicrob Agents Chemother* **43**, 937–939.

Jacoby, G. A., Mills, D. M. & Chow, N. (2004). Role of β-lactamases and porins in resistance to ertapenem and other β-lactams in *Klebsiella pneumoniae*. *Antimicrob Agents Chemother* **48**, 3203–3206.

Kaczmarek, F. M., Dib-Hajj, F., Shang, W. & Gootz, T. D. (2006). High-level carbapenem resistance in a *Klebsiella pneumoniae* clinical isolate is due to the combination of bla_{ACT-1} β-lactamase production, porin OmpK35/36 insertional inactivation, and down-regulation of the phosphate transport porin PhoE. *Antimicrob Agents Chemother* **50**, 3396–3406.

Landman, D., Bratu, S., Kochar, S., Panwar, M., Trehan, M., Doymaz, M. & Quale, J. (2007). Evolution of antimicrobial resistance among *Pseudomonas aeruginosa*, *Acinetobacter baumannii* and *Klebsiella pneumoniae* in Brooklyn, NY. *J Antimicrob Chemother* **60**, 78–82.

Martínez-Martínez, L., Hernández-Allés, S., Albertí, S., Tomás, J. M., Venedi, V. J. & Jacoby, G. A. (1996). In vivo selection of porin-deficient mutants of *Klebsiella pneumoniae* with increased resistance to cefoxitin and expanded-spectrum cephalosporins. *Antimicrob Agents Chemother* **40**, 342–348.

Martínez-Martínez, L., Pascual, A., Hernández-Allés, S., Alvarez-Díaz, D., Suárez, A. I., Tran, J., Benedí, V. J. & Jacoby, G. (1999). Roles of β-lactamases and porins in activities of carbapenems and cephalosporins against *Klebsiella pneumoniae*. *Antimicrob Agents Chemother* **43**, 1669–1673.

Mena, A., Plasencia, V., García, L., Hidalgo, O., Ayestarán, J. I., Albertí, S., Borrell, N., Pérez, J. L. & Oliver, A. (2006). Characterization of a large outbreak by CTX-M-1-producing *Klebsiella pneumoniae* and mechanisms leading to in vivo carbapenem resistance development. *J Clin Microbiol* **44**, 2831–2837.

Nikaido, H. (1996). Multidrug efflux pumps of Gram-negative bacteria. *J Bacteriol* **178**, 5853–5859.

Paterson, D. L., Hujer, K. M., Hujer, A. M., Yeiser, B., Bonomo, M. D., Rice, L. B., Bonomo, R. A. & the International *Klebsiella* Group (2003). Extended-spectrum β-lactamases in *Klebsiella pneumoniae* bloodstream isolates from seven countries: dominance and widespread prevalence of SHV- and CTX-M-type β-lactamases. *Antimicrob Agents Chemother* **47**, 3554–3560.

Queenan, A. M. & Bush, K. (2007). Carbapenemases: the versatile β-lactamases. *Clin Microbiol Rev* **20**, 440–458.

Sambrook, J. & Russell, D. W. (2001). *Molecular Cloning: a Laboratory Manual*, 3rd edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.

Tenover, F. C., Kalsi, R. K., Williams, P. P., Carey, R. B., Stocker, S., Lonsway, D., Rasheed, J. K., Biddle, D. W., McGowan, J. E., Jr & Hanna, B. (2006). Carbapenem resistance in *Klebsiella pneumoniae* not detected by automated susceptibility testing. *Emerg Infect Dis* **12**, 1209–1213.

Woodford, N., Tierno, P. M., Jr, Young, K., Tysall, L., Palepou, M.-F. I., Ward, E., Painter, R. E., Suber, D. F., Shungu, D. & other authors (2004). Outbreak of *Klebsiella pneumoniae* producing a new carbapenem-hydrolyzing class A β-lactamase, KPC-3, in a New York Medical Center. *Antimicrob Agents Chemother* **48**, 4793–4799.

Woodford, N., Dallow, J. W. T., Hill, R. L. R., Palepou, M.-F. I., Pike, R., Ward, M. E., Warner, M. & Livermore, D. M. (2007). Ertapenem resistance among *Klebsiella* and *Enterobacter* submitted in the UK to a reference laboratory. *Int J Antimicrob Agents* **29**, 456–459.

Yigit, H., Queenan, A. M., Anderson, G. J., Doménech-Sánchez, A., Biddle, J. W., Steward, C. D., Albertí, S., Bush, K. & Tenover, F. C. (2001). Novel carbapenem-hydrolyzing β-lactamase, KPC-1, from a carbapenem-resistant strain of *Klebsiella pneumoniae*. *Antimicrob Agents Chemother* **45**, 1151–1161.