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Molecular mechanisms disrupting porin expression in ertapenem-resistant *Klebsiella* and *Enterobacter* spp. clinical isolates from the UK

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***Objectives*: The aim of this study was to investigate relatedness and molecular mechanism(s) of ertapenem resistance in clinical isolates of *Klebsiella* spp. ($n=28$) and *Enterobacter* spp. ($n=27$) referred from multiple hospitals to the UK national reference laboratory.**

***Methods*: Investigations included genotyping by PFGE, resistance gene analysis by PCR and antimicrobial susceptibility testing with and without inhibitors of efflux and β-lactamase activity. Outer membrane proteins (OMPs) were profiled by SDS–PAGE; porin genes were sequenced and their expression was examined by RT–PCR. The contribution of porin deficiency to resistance was investigated by restoring functional porin genes on plasmids.**

***Results*: PFGE showed significant clonal diversity among ertapenem-resistant isolates, with only small clusters identified. SHV- and CTX-M-type extended-spectrum β-lactamases were identified in the *Klebsiella* spp. isolates, whereas AmpC overexpression or KPC carbapenemase was detected in the *Enterobacter cloacae* isolates. SDS–PAGE showed that *Klebsiella pneumoniae* and *Enterobacter aerogenes* with high-level ertapenem resistance (MICs $\geq$16 mg/L) consistently lacked both of the two major non-specific porins, whereas variable patterns of OmpC and OmpF were seen in *E. cloacae* with lower-level ertapenem resistance. Various point mutations or insertion sequences were identified as disrupting the porin-coding sequences, as well as mutations in the promoter region. Functional restoration of OmpK35 or OmpK36 in *Klebsiella* and OmpC or OmpF in *Enterobacter* spp. isolates significantly decreased the MICs of all carbapenems, but particularly of ertapenem. We found no evidence of efflux contributing to resistance.**

***Conclusions*: Ertapenem resistance was exclusively due to combinations of β-lactamases with impermeability caused by loss of OMPs. Efflux was not implicated and there was no national spread of resistant clones.**

Keywords: impermeability, carbapenems, Enterobacteriaceae

Introduction

Carbapenems are frequently the only therapeutic options available for treatment of severe hospital or community-acquired infections caused by multiresistant AmpC- or extended-spectrum β-lactamase (ESBL)-producing Enterobacteriaceae. It is therefore disturbing that universal susceptibility to carbapenems in Enterobacteriaceae is no longer guaranteed; rather, carbapenem resistance can arise through the production of acquired metallo-β-lactamases (MBLs) such as VIM and IMP, or non-metallo-carbapenemases of the IMI/NMC, SME, OXA or KPC families.[1,2] Resistance can also arise when carbapenem accumulation is reduced in strains already producing ESBLs or AmpC β-lactamases. Thus, previous studies of sporadic ertapenem-resistant isolates have identified alterations or losses of major non-specific porins in *Klebsiella pneumoniae*,[3,4] *Escherichia coli*,[5] *Enterobacter cloacae*[6] and *Enterobacter aerogenes*, as well as suggestions of increased efflux activity.[6] A review of 171 clinical ertapenem-resistant *Klebsiella* and *Enterobacter* spp. isolates submitted to the UK reference laboratory between June 2004 and April 2006 found that very few had true carbapenemases. Rather, most had ESBLs or AmpC

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production combined with impermeability.[7] Although still rare in surveys, such isolates are increasingly referred.

The present study examined a wide collection of ertapenem-resistant *Klebsiella* and *Enterobacter* isolates, seeking to evaluate their genetic relationships and to investigate the relative contributions of impermeability, efflux and β-lactamase to their resistance, along with the molecular mechanism underlying impermeability.

Materials and methods

Strains and antimicrobial susceptibility testing

The clinical isolates of *Klebsiella* spp. ($n=28$) and *Enterobacter* spp. ($n=27$) were from 20 and 21 different UK microbiology laboratories, respectively, comprising 35 different sites in total; they were chosen from among clinical reference submissions received between April 2006 and March 2007 on the basis of ertapenem MICs ≥ 4 mg/L, except for two *Enterobacter* isolates with MICs of 1 and 2 mg/L. Isolates included *E. cloacae* ($n=19$), *E. aerogenes* ($n=8$) and *K. pneumoniae* ($n=28$). MICs were determined by BSAC agar dilution methodology or by Etest and, except for the higher ertapenem 'breakpoint', were interpreted in accordance with BSAC and the European Committee on Antimicrobial Susceptibility Testing (EUCAST) guidelines. *K. pneumoniae* strains ATCC 13883 and K20 (Table 3) and *E. cloacae* NCTC 10005 were used as carbapenem-susceptible controls for RT–PCR and outer membrane protein (OMP) extraction experiments. *E. cloacae* strains E684 and E684-con were used as inducible and derepressed controls of AmpC activity.[8] To investigate efflux, carbapenem MICs were determined by Etest on Mueller–Hinton agar containing 100 mg/L phenylalanine-arginine-β-naphthylamide (PAβN).

Screening tests for β-lactamases

MBLs belonging to the IMP, VIM, GIM, SPM and SIM families;[9] CTX-M enzymes of groups 1, 2, 8, 9 and 25;[10] plasmidic *ampC* genes of the ACC, CIT, DHA, ENT/EBC, FOX and MOX groups,[11] KPC carbapenemase,[12] TEM and SHV β-lactamases were sought by previously described PCR methods.[13,14] β-Lactamase genes from representative isolates were sequenced by the dideoxy chain-termination method using a Beckman CEQ 8000 Genetic Analysis System (Beckman Coulter, High Wycombe, UK). Sequences were edited with Bionumerics software (Applied Maths, Kortrijk, Belgium).

PFGE

Macrorestriction PFGE analysis of *Xba*I-digested genomic DNA was done by a previously described procedure.[15] Electrophoresis was for 30 h at 12°C with pulse times ranging from 5 to 35 s at 6 V/cm. Patterns were analysed using Bionumerics software to generate a dendrogram based on the unweighted pair group method with an arithmetic average from the Dice coefficient.

Examination of porin genes and porin expression

The coding sequences of (i) *ompK35* and *ompK36* from *K. pneumoniae*, (ii) *ompC* and *ompF* from *E. cloacae*, and (iii) *omp36* from *E. aerogenes*, all with their respective promoter regions, and also (iv) a 1021 bp fragment from the open reading frame (ORF) of *E. aerogenes omp35*, were amplified using the primers listed in Table 1 and sequenced as above. OMPs were isolated according to the rapid procedure of Carlone *et al.*,[16] then boiled in the presence of 6 M urea before SDS–PAGE in gels containing 12% acrylamide, 0.35% bisacrylamide and 0.1% SDS.

Analysis of gene expression

Analysis of mRNA was performed by RT–PCR. Total RNA was extracted using the RNeasy-kit (Qiagen, Crowley, UK) and treated with RNase-free DNase (Invitrogen, Paisley, UK). RT–PCR was then performed in a LightCycler (Roche, Burgess Hill, UK) using the one-step QuantiFast SYBR Green RT–PCR Kit (Qiagen) according to the manufacturer's protocols. The mRNA of the housekeeping *rpoB* gene was chosen as the endogenous reference RNA for relative quantification. Relative quantities of mRNA from each gene of interest were determined by the comparative threshold cycle method (C_T), where the C_T value was defined as the number of cycles needed for the fluorescence, which reflects the quantity of amplified RNA, to exceed a value of 0. The magnitude of expression (ΔC_T) of the gene of interest was then obtained by normalization against the housekeeping *rpoB* gene, to correct for variation in RNA content and amplification efficiency between samples. The equation $2^{-\Delta\Delta CT}$, where $\Delta\Delta C_T = \Delta C_{T\text{-target}} - \Delta C_{T\text{-reference}}$, then allowed the relative quantification of differences in gene expression levels between two strains, in our case ertapenem-resistant (target) and -susceptible (reference) strains.[17] Primers to examine gene expression of *ompK35*, *ompK36*, *ompK37*, *acrB*, *phoE*, *ompC*, *ompF* and *ampC* were designed in conserved DNA regions based on the sequences obtained from this study and those available in public databases (Table 1). All amplifications were carried out in triplicate from three different RNA preparations in a total volume of 20 μL, comprising 10–15 ng of RNA and 10 pmol of each primer. Expression level values are a mean of the three independent experiments. Negative controls without RT were performed to detect DNA contamination in the purified RNA.

Construction of plasmid bearing ompK35, ompK36 and ompC

To examine the contribution of porin loss to ertapenem resistance, plasmids encoding functional OmpK35, OmpK36, OmpC and OmpF were constructed. The *Sma*I–*Kpn*I fragment harbouring the xSm/Sp cassette in pHRP317[18] was first replaced by the *Nru*I–*Kpn*I fragment of pSHA4[19] containing the potassium tellurite resistance cassette (TeR) to generate p317tel. The *Hin*dIII fragment of p317tel carrying this TeR cassette and the flanking multicloning site was then isolated and ligated to the *Hin*dIII fragment of pSHA4 containing the origin of replication and a chloramphenicol resistance cassette yielding pTR. Internal-site primers in (i) the *ompK35*-flanking asparaginyl-tRNA synthetase and aspartate aminotransferase coding genes and (ii) *ompK36*-flanking thiamine biosynthesis lipoprotein and the putative two-component system sensor kinase coding genes were then used to amplify *ompK35* and *ompK36*, respectively, both still under regulation by their native promoter regions. Genomic DNA from *K. pneumoniae* strain ATCC 13883 was used as a template (Table 1). The resulting amplicons were cloned, blunt-ended, in the *Nru*I site of pTR, generating pTRompK35 and pTRompK36. The *E. cloacae* NCTC 10005 *ompC* and *ompF* genes, under the control of their native promoters, were amplified similarly, using the primers listed in Table 1, and cloned into pTR as *Sac*I–*Sph*I or *Nru*I blunt-ended fragments, yielding pTRompC and pTRompF.

For complementation, competent cells of clinical isolates were prepared according to standard methods, and plasmids were

Table 1. Primers used in this study

Gene(s)	Use	Sequence 5′→3′
rpoB-F	expression	AAGGCGAATCCAGCTTGTTCAGC
rpoB-R	expression	TGACGTTGCATGTTCGCACCCATCA
ompK35/*ompF*-F	expression	TCCCTGCCCTGCTGGTAG
ompK35-R	expression	CTGGTGTCGCCATTGGTGG
ompF-R	expression	TAAGTGTTGTCGCCATCGTTG
omp35-R	expression	CTGGTGTCACCATTGGTGG
ompK36/*ompC*-F	expression	GCGACCAGACCTACATGCGT
ompC-R	expression	TTCGTTCTCACCAGAGTTACCCT
ompK36-R	expression	AGTCGAAAGAGCCCGCGTC
ompF-F	cloning	AGACACCAAACTCTCATCAATAGTTC
ompF-R	cloning	CGCTATCAGGTTAACGGTA
ompK35-F	cloning	GCACGAAACAGATCGGCCAG
ompK35-R	cloning	TTACGTCACCGGCGTGCAGAA
ompK36/omp36-F	cloning	GACCCGCCAGAAGGTGCCCA
ompK36/omp36-R	cloning	TGATGTTGCCGGGGATCAGGGA
omp35-F	cloning	GCGCAATATTCTGGCAGTGG
omp35-R	cloning	TAAACGATACCAACCGCAGCCT
ompC-F	cloning	GAGCTCGTTAATGATGATAGCGAGAGTTAT
ompC-R	cloning	GCATGCACGTTCCCATGGTTTTGCCTTCCAGCA
ompK37-F	expression	GCCCTCGTTATTCCGGCTT
ompK37-R	expression	CCGTCTTTTTTCGAGTCGCTG
phoE-F	expression	TGATGGGCTTTGTGGCTTC
phoE-R	expression	CTTGCTGTCATAGTCGCTG
acrB-F	expression	CGATAACCTGATGTACATGTCC
acrB-R	expression	CCGACAACCATCAGGAAGCT
ampC-F	expression	GCATGGCGGTGGCCGTTAT
ampC-R	expression	CTGCTTGCCCGTCAGCTGT

Underlining indicates the *Sph*I and *Sac*I restriction sites introduced for cloning.

introduced by electroporation as described in the manufacturer's protocol using a GenePulser Electroporator (Bio-Rad, Hemel Hempstead, UK). Transformants were selected on Luria–Bertani agar containing 30 mg/L of potassium tellurite.

Results

Genetic diversity

The 28 ertapenem-resistant *Klebsiella* spp. and 27 *Enterobacter* spp. isolates, referred from 35 UK microbiology laboratories, were examined by PFGE of *Xba*I-digested DNA. Twelve of the 28 *Klebsiella* spp. isolates divided into five groups, each comprising two or three isolates from different laboratories, with >85% DNA similarity, along with 16 diverse isolates (Figure 1). Among the *Enterobacter* spp. isolates, only two pairs of related isolates were identified, each from a single hospital site (Figure 1). We concluded that most ertapenem-resistant isolates were diverse and that emerging resistance was not due to the dissemination of national clones.

Carbapenem susceptibility and β-lactamase expression

Carbapenem MIC distributions for the isolates studied are shown in Table 2. Ertapenem resistance tended to be greater in *Klebsiella* spp. than that in *Enterobacter* spp. MICs of imipenem and meropenem were raised compared with these for typical isolates, with this effect most pronounced for isolates with high-level ertapenem resistance (MICs > 16 mg/L). Imipenem and meropenem often remained moderately active against isolates with low-level ertapenem resistance, with MICs below EUCAST's 2 mg/L susceptible/intermediate breakpoints.

All 28 *Klebsiella* spp. isolates produced both TEM- and SHV-type enzymes and 27 had CTX-M ESBLs (25 had CTX-M group 1 types, 1 had CTX-M group 9 and 1 had both group 1 and 9 enzymes). None had a defined carbapenemase or plasmidic AmpC β-lactamase amplified by PCR. All the 27 *Enterobacter* spp. isolates produced TEM-type enzymes, but lacked metallo- or plasmidic AmpC-type β-lactamases, though most or all had chromosomal AmpC enzymes. One produced a KPC carbapenemase, seven had genes encoding SHV enzymes, one had a CTX-M group 9 enzyme and one harboured the genes encoding both SHV and CTX-M group 9 enzymes.

Nine *K. pneumoniae*, five *E. cloacae* and two *E. aerogenes* isolates, with ertapenem MICs from 4 to >16 mg/L, were used in further investigations (Table 3). Sequence analysis identified TEM-1, SHV-1/28, SHV-11, CTX-M-15 or CTX-M-33 variants among the *K. pneumoniae* isolates, and TEM-1, SHV-12 or KPC-4 in the *Enterobacter* spp. isolates (Table 3). The expression of *ampC* in the five *E. cloacae* isolates was analysed by RNA extraction and RT–PCR-specific amplification. The AmpC inducible strain *E. cloacae* E684 and its high-level AmpC constitutive

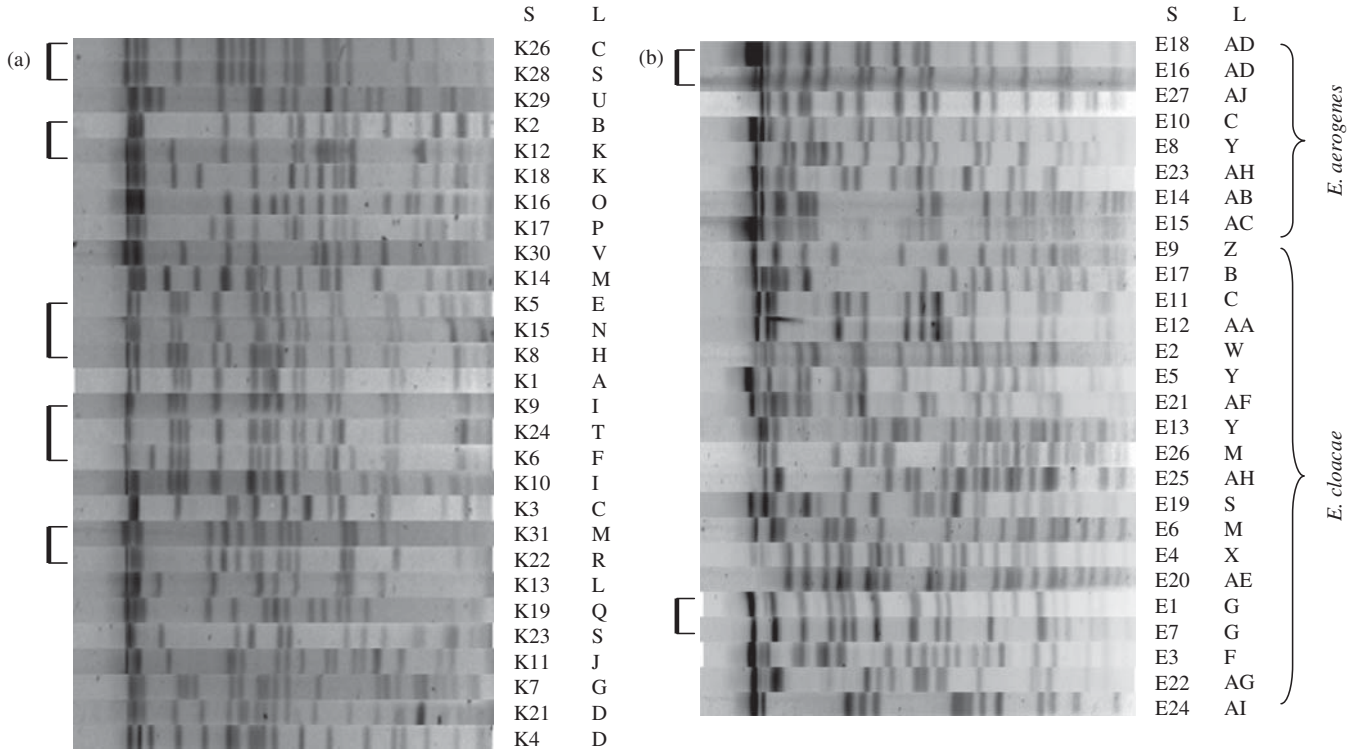

Figure 1. PFGE patterns for ertapenem-resistant clinical isolates of (a) *Klebsiella* and (b) *Enterobacter* spp. Dendrograms were generated with a band position tolerance of 1%. S and L correspond to strain and laboratory sources, respectively. Isolates with >85% similarity are joined with lines on the left-hand side of the figure.

mutant E684-con were used as controls, with RT–PCR analysis showing a 40-fold increased level of *ampC* mRNA in the latter. *Enterobacter* isolates E1 and E11 had 20- and 11-fold higher levels of *ampC* transcription than E684, whereas levels were similar to those of E684 in isolates E2, E24 and E25. In summary, all these *K. pneumoniae* and *E. cloacae* isolates, except E24, had either derepressed AmpC, KPC-4 or an ESBL. The elevated cephalosporin MICs for isolate K3 may not be attributed to the hydrolytic activity of TEM-1 and SHV-11 enzymes; the presence of an additional β-lactamase cannot be excluded.

Ertapenem resistance and efflux activity

The presence of 100 mg/L PAβN did not affect the growth of any of the 16 isolates studied and had no significant effect on carbapenem MICs, which were reduced by ≤2-fold. These data contraindicated efflux as a component in ertapenem resistance. Moreover, analysis of *acrB* expression by RT–PCR consistently revealed levels equal to those in carbapenem-susceptible *K. pneumoniae* ATCC 13883 and *E. cloacae* NCTC 10005 control strains.

Ertapenem resistance and impermeability

K. pneumoniae. SDS–PAGE analysis showed that the nine *K. pneumoniae* isolates examined lacked both OmpK35 and OmpK36 porins, but retained normal levels of OmpA, a structural protein (data not shown). Sequencing of the corresponding porin genes revealed various lesions. Point mutations predicted to result in early termination of translation or insertion sequences (ISs) interrupting the coding sequence of *ompK36* were identified in eight isolates, whilst the *ompK36* gene of the remaining isolate, K4, did not amplify, indicating either that mutations were present in the primer binding sites or that the DNA had been deleted (Table 4). Sequencing of *ompK36* amplified from the parent carbapenem-susceptible isolate K20 confirmed only the acquisition of the IS*1* insertion in the resistant isolate K21.

Sequencing of *ompK35* similarly revealed diverse types of disruption in seven of the nine isolates (the exceptions being isolates K3 and K21), including point mutations or the presence of ISs such as IS*1* and IS*10*. In isolate K2, IS*1* was inserted at the ATG start codon and, in K12, eight nucleotides upstream; in both cases, the result was to delete the ribosome binding site. The same insertion was located at the end of the ORF in isolates

Table 2. Carbapenem MIC distributions among ertapenem-resistant *Klebsiella* and *Enterobacter* spp. isolates

	MIC (mg/L)								
Genus/carbapenem	0.125	0.25	0.5	1	2	4	8	16	>16
Klebsiella spp., $n=28$									
imipenem			5	10	5	4	1	3	
meropenem				2	3	10	6	7	
ertapenem							6	4	18
Enterobacter spp., $n=27$									
imipenem			3	7	4	4	3	6	
meropenem	1	3	4	5	6	3	4	1	
ertapenem				1	1	6	8	3	8

Table 3. MICs and β-lactamases for the isolates studied

Strain	β-Lactamases	MIC (mg/L)												
		ETP	IPM	MEM	CTX	CTX/CLA	CAZ	CAZ/CLA	FOX	TZP	CIP	GEN	AMK	MIN
K. pneumoniae														
K2	TEM-1, SHV-11, CTX-M-15	>16	2	8	>256	>32	256	>32	64	>64	>8	>32	8	32
K3	TEM-1, SHV-11	8	4	4	32	8	256	32	64	64	>8	>32	16	16
K4	TEM-1, SHV-1/28, CTX-M-33	>16	8	16	>256	2	16	2	>64	>64	8	0.5	2	32
K6	TEM-1, SHV-11, CTX-M-15	>16	2	8	>256	>32	256	>32	>64	>64	>8	>32	1	4
K8	TEM-1, SHV-11, CTX-M-15	>16	2	8	>256	>32	256	32	64	>64	>8	>32	1	4
K9	TEM-1, SHV-11, CTX-M-15	16	1	4	>256	2	256	4	>64	>64	8	1	2	4
K10	TEM-1, SHV-11, CTX-M-15	16	0.5	2	>256	0.5	64	0.5	64	8	8	1	4	8
K12	TEM-1, SHV-11, CTX-M-15	>16	4	16	>256	>32	256	>32	64	>64	>8	0.5	1	4
K20	TEM-1, SHV-1/28, CTX-M-15	0.5	0.13	≤0.06	>256	0.25	256	1	32	>64	>8	>32	4	16
K21	TEM-1, SHV-1/28, CTX-M-15	>16	1	4	>256	8	256	4	>64	>64	>8	0.5	1	16
E. cloacae														
E1	TEM-1, AmpC(d)	8	1	0.5	>256	>32	256	>32	>64	>64	≤0.125	0.5	1	16
E2	TEM-1, SHV-12, AmpC	4	2	1	32	>32	256	>32	>64	32	4	0.5	1	32
E11	TEM-1, AmpC(d)	>16	8	4	>256	>32	256	>32	>64	>64	≤0.125	0.5	1	4
E24	TEM-1, AmpC	16	4	2	256	>32	256	>32	>64	>64	≤0.125	0.5	1	8
E25	TEM-1, AmpC, KPC-4	>16	8	8	256	>32	>256	>32	>64	>64	>8	0.5	1	>32
E. aerogenes														
E15	TEM-1, AmpC	16	16	4	>256	>32	8	>32	>64	>64	>8	2	1	4
E17	TEM-1, AmpC	16	16	4	>256	>32	64	>32	>64	64	>8	0.3	1	4

ETP, ertapenem; IPM, imipenem; MEM, meropenem; CTX, cefotaxime; CTX/CLA, cefotaxime + 4 mg/L clavulanic acid; CAZ, ceftazidime; CAZ/CLA; ceftazidime + 4 mg/L clavulanic acid; FOX, cefoxitin; TZP, piperacillin + 4 mg/L tazobactam; CIP, ciprofloxacin; GEN, gentamicin; AMK, amikacin; MIN, minocycline; AmpC(d), derepressed AmpC.
K20 and K21 were clonally identical ertapenem-resistant and -susceptible isolates from the same patient.

Table 4. Lesions causing disruption of porin genes

Porin lesion type	Strain														
	K. pneumoniae								*E. cloacae*					*E. aerogenes*	
	K2	K3	K4	K6	K8	K9	K10	K12	K21	E1	E2	E11	E24	E25	E15	E17
ompK36/ompC/omp36																
no PCR amplification			+													
premature stop codon	172	141		125		248							154		224	
disruption by IS					IS*2*$_{(+562)}$		IS*26*$_{(+368)}$	IS*903*$_{(+104)}$	IS*1*$_{(+57)}$			IS*1*$_{(+18)}$				A$_{(+997)}$
frameshift mutation																
ompK35/ompF/omp35																
translational stop (amino acid)			42							234		27			810	
disruption by IS	IS*1*$_{(+2)}$			IS*1*$_{(+1048)}$	IS*1*$_{(+1048)}$	IS*1*$_{(+1048)}$	IS*10*$_{(+952)}$	IS*1*$_{(-8)}$								
mutation in promoter										*Pr*$_{(-51)}$			*Pr*$_{(-51)}$	*Pr*$_{(-51)}$		

Numbers between parentheses correspond to the nucleotide positions of insertions; + and − indicate the position, upstream and downstream, of the A of the start codon.
Premature stop codon numbers correspond to amino acid position.
Pr and IS*n* indicate mutation in the promoter region and the presence of insertion sequences, respectively.

K6, K8 and K9, resulting in a truncated porin lacking the last 10 amino acids, which includes a phenylalanine residue necessary for the insertion of porin into the outer membrane.[20] Sequencing did not identify any lesion in the coding sequence or the promoter region for OmpK35 in isolates K3 and K21; moreover, mRNA levels for *ompK35* in these two isolates were similar to those in the carbapenem-susceptible control isolates. Nevertheless, the porin itself was absent from the outer membrane, possibly indicating a failure of translation or a membrane change that precluded insertion.

A postulated association between loss of major porins and overexpression of the minor porins OmpK37 and PhoE was examined by RT–PCR. The results showed similar levels of mRNA for these minor porins in both ertapenem-resistant and -susceptible control isolates, discounting the hypothesis.

E. cloacae. SDS–PAGE analysis for *E. cloacae* revealed diverse OMP profiles among the five isolates studied (Figure 2): E11 and E24 lacked both OmpC and OmpF, E1 lacked OmpF and had normal expression of OmpC, E2 expressed low levels of both OmpC and OmpF and E25 had low levels of expression of OmpC but lacked OmpF. The mRNA levels for *ompC* and *ompF* were found to be 15- to 20-fold less in isolates E2 and E25 compared with the carbapenem-susceptible *E. cloacae* NCTC 10005 reference strain. All the isolates retained the normal level of expression of OmpA. These patterns correlated to the levels of carbapenem resistance: thus, E11 and E24, which lacked both porins, had high-level carbapenem resistance, as did E25, which had a KPC enzyme; in contrast, isolates E1 and E2, with less pronounced resistance, expressed moderate amounts of at least one of these non-specific major porins.

Sequence analysis identified point mutations and IS, disrupting the coding sequence of *ompC* and *ompF* genes in isolates E1, E11 and E24, whereas point mutations were identified in the promoter region of *ompF* in E24 and E25 isolates (Table 4).

E. aerogenes. Isolates E15 and E17 lacked both Omp35 and Omp36. Sequencing revealed that *omp36* was inactivated by a frameshift produced by a 1 bp deletion in E17 and by a point mutation in E15, creating premature stop codons (Table 4). A 1021 bp fragment of the *omp35*-coding sequence was amplified by PCR. Sequencing of this identified a premature stop codon in the product from E15 but a wild-type sequence in that from isolate E17. The absence of Omp35 in the outer membrane of isolate E17 therefore may reflect mutational alterations in the unamplified sequences of the ORF or down-regulation at the transcriptional or translational levels.

Figure 2. OMP profiles of *E. cloacae* isolates on a 12.5% SDS polyacrylamide gel. Lane M: markers of 27, 34.6 and 42.7 kDa.

Complementation studies

To elucidate the respective contributions of β-lactamases and porin loss to resistance phenotypes, competent cells of eight *K. pneumoniae* isolates were prepared and transformed with plasmid pTRompK36 (we failed to obtain competent cells from isolate K6 despite several attempts). The restoration of functional OmpK36 resulted in significant decreases in the MICs of all three carbapenems for all eight isolates where transformants were obtained (Table 5). Ertapenem MICs were reduced from 8 to >16 mg/L to 0.25–2 mg/L and those of imipenem and meropenem were reduced to <1 mg/L. SDS–PAGE analysis confirmed that the missing OmpK36 was restored in all transformants, but in variable expression levels; isolates for which ertapenem MICs were reduced to 1–2 mg/L, subsequent to transformation with pTRompK36, expressed less OmpK36 than transformants with greater reductions in ertapenem MICs (data not shown).

The addition of clavulanic acid (4 mg/L) resulted in further reduction of carbapenem MICs, especially for ertapenem (1- to 5-doubling-dilutions reduction in MIC), underscoring the contribution of ESBL activity, even in the presence of functional porins. Six of these isolates were also transformed with pTRompK35, causing significant MIC reductions for carbapenems as did pTRompK36 (Table 5). Thus, restoration to full function of either of the two non-specific porins reinstated susceptibility to carbapenems. The complementation experiments with pTRompK35 failed to increase susceptibility to carbapenems in isolates K3 and K21, which had no identified mutation in their *ompK35* gene. OMPs extraction from both transformants showed no detectable expression levels of OmpK35, suggesting possible inhibition at the translation level in these isolates.

Restoration of either functional OmpC or OmpF by introducing pTRompC or pTRompF into *E. cloacae* E11 and E24 considerably decreased all carbapenem MICs, indicating that the expression of either porin alone was sufficient to allow rapid entry of these antibiotics (Table 5). The derepressed *ampC* expression in E1 and E11, and the KPC-4 carbapenemase in E25, contributed to increasing the MICs of carbapenems, notably that of ertapenem. When comparing isolates E11 and E24 (both of which lacked OmpF and OmpC), the overexpression of AmpC in E11 (but not E24) resulted in slightly higher MICs of all carbapenems, and the contribution of this enzyme was confirmed for the E11/pTRompC transformant by the addition of boronic acid, 100 mg/L, which resulted in an 8-fold decrease in the ertapenem MIC.

Complementation of *E. aerogenes* E15 isolate with pTRompC resulted in significant reduction of carbapenem MICs, just as for the *E. cloacae* isolates, indicating cross-species function.

Table 5. Effects of introduction of plasmids encoding functional porins into porin-deficient clinical isolates

Strain	Transformant	MIC (mg/L) ertapenem	meropenem	imipenem
K. pneumoniae				
K2	/pTR	32	8	2
	/pTRompK36	1	0.12	0.25
	/pTRompK35	0.12	0.12	0.12
	/pTRompK36/CLA	0.06	0.12	0.03
K3	/pTR	8	4	4
	/pTRompK36	1	1	0.5
	/pTRompK35	8	4	4
	/pTRompK36/CLA	1	0.5	0.5
K4	/pTR	>32	16	8
	/pTRompK36	0.25	0.5	0.12
	/pTRompK35	0.5	0.5	0.5
	/pTRompK36/CLA	0.12	0.25	0.12
K8	/pTR	>32	8	2
	/pTRompK36	0.25	0.25	0.03
	/pTRompK36/CLA	0.12	0.25	0.03
K9	/pTR	16	4	1
	/pTRompK36	2	0.12	0.12
	/pTRompK35	0.25	0.12	0.06
	/pTRompK36/CLA	0.12	0.12	0.03
K10	/pTR	16	2	0.5
	/pTRompK36	2	0.12	0.12
	/pTRompK36/CLA	0.25	0.12	0.03
K12	/pTR	32	16	4
	/pTRompK36	2	0.25	0.5
	/pTRompK35	0.03	0.25	0.06
	/pTRompK36/CLA	0.06	0.12	0.03
K21	/pTR	>32	4	1
	/pTRompK36	2	0.12	0.25
	/pTRompK35	>32	4	1
	/pTRompK36/CLA	0.25	0.12	0.12
E. cloacae				
E11	/pTR	>32	4	8
	/pTRompC	0.25	0.03	0.25
	/pTRompF	0.01	0.12	0.19
	/pTRompC/BA	0.03	0.03	0.25
E24	/pTR	16	2	4
	/pTRompC	0.25	0.12	0.12
	/pTRompF	0.03	0.03	0.25
	/pTRompC/BA	0.03	0.12	0.12
E. aerogenes				
E15	/pTR	16	4	16
	/pTRompC	0.25	0.03	0.125
	/pTRompC/BA	0.25	0.03	0.125

CLA, +4 mg/L clavulanic acid; BA, +100 mg/L boronic acid.

Discussion

Resistance among Enterobacteriaceae to expanded-spectrum cephalosporins is a serious and growing clinical problem, leading to an increased reliance on carbapenems. Acquired carbapenem resistance is still rare among Enterobacteriaceae but can arise due to the presence of carbapenemases such as KPC types, or via combinations of reduced permeability with an ESBL or a strongly expressed AmpC enzyme.

In this study, we examined: (i) the genetic relationships among ertapenem-resistant *Klebsiella* and *Enterobacter* spp. isolates referred to the UK reference laboratory; and (ii) their mechanisms of resistance. PFGE data indicated that the ertapenem-resistant clinical isolates did not belong to

disseminated national clones and synergy studies with PAβN contraindicated efflux as a major component in resistance. Furthermore, no up-regulation of the AcrAB RND efflux pump was detected in any of the ertapenem-resistant isolates tested. Thus, although increased efflux has been reported to be involved in ertapenem resistance in clinical isolates,[6] our results would suggest that it is not a frequent or major contributor in *Klebsiella* and *Enterobacte*r spp. Rather, the combination of impermeability with ESBLs, AmpC or, in just one case, a KPC enzyme seemed critical.

The absence or reduced expression of the two major porins (OmpK35 and OmpK36 in *K. pneumoniae*,[21,22] OmpC and OmpF in *E. cloacae* and *E. coli* or Omp35 and Omp36 in *E. aerogenes*[5,23,24]) in combination with various β-lactamases has been implicated in carbapenem resistance by previous authors, and the present data support their conclusions. We then went to investigate the basis of porin loss, which was found to be the result of: (i) point mutations or, more frequently, insertional interruptions in the coding sequences, leading to premature translational termination; (ii) point mutations in the promoter region, possibly affecting transcription; or (iii) unidentified mechanisms working at the translational level or to prevent porin insertion into the outer membrane. In this last case there was an intact porin gene and an mRNA transcript, but there was no porin in the outer membrane (e.g. isolates K3 and K21).

The diversity of lesions observed suggests the absence of mutational hot spots as origins of porin inactivation. Rather, different ISs including IS*1*, IS*5*, IS*26*, IS*102* and IS*903*—as found here—have been reported as a cause for porin alterations.[25] Multiple copies of these ISs may be present in the chromosome, and their transposition to sites in porin genes may be selected under antibiotic pressure. The present study indicated that a loss of two major porins was required for high level (MICs $\geq$ 16 mg/L) resistance to ertapenem in both genera and that, for a strain lacking both porins, complementation with either porin alone decreased the MICs of the three carbapenems significantly. Ertapenem MICs were variably reduced in transformants (range 0.25–2 mg/L), correlating with different expression levels of the plasmid porin-encoding genes used for complementation, possibly due to variation in the regulation at the transcriptional or translational levels between the studied isolates. The difference between the types of lesions disrupting the major porin genes in the same isolates suggests that these disruptions are the results of separate events. It should be noted here that many ESBL-producing *K. pneumoniae* already lack OmpK35, reported to be the major porin through which ceftazidime penetrates the Gram-negative outer membrane.[22,26]

In conclusion, ertapenem resistance in clinical isolates of *Klebsiella* spp. and *Enterobacter* spp. was not the result of dissemination of national clones in the UK but rather reflected repeated independent emergence in different strains. Mechanistically, this resistance was associated with porin deficiency in combination with β-lactamase expression, with a variety of deletion, point mutation, insertional inactivation and unknown mechanisms leading to the loss of porin expression. The fact that ertapenem is more affected than other carbapenems by this mechanism may reflect slower penetration through the minor porins that must take over when major porins are lost, and may perhaps be contingent on its larger size and more negative charge.[26] Although the prevalence of isolates with these mechanisms is still extremely low, their occurrence deserves careful monitoring; not least because such phenotypes have recently also been reported among *E. coli*,[5] the main target species for ertapenem. Careful antibiotic usage is required so as to minimize the new selection of this mode of resistance and, in this context, it is notable that although ertapenem is the most-affected carbapenem, there are reports of selection by all three commercially available carbapenems.[3,27]

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References

1. Livermore DM. The impact of carbapenemases on antimicrobial development and therapy. *Curr Opin Investig Drugs* 2002; **3**: 218–24.

2. Walsh TR, Toleman MA, Poirel L *et al.* Metallo-β-lactamases: the quiet before the storm? *Clin Microbiol Rev* 2005; **18**: 306–25.

3. Elliott E, Brink AJ, van Greune J *et al. In vivo* development of ertapenem resistance in a patient with pneumonia caused by *Klebsiella pneumoniae* with an extended-spectrum β-lactamase. *Clin Infect Dis* 2006; **42**: e95–8.

4. Jacoby GA, Mills DM, Chow N. Role of β-lactamases and porins in resistance to ertapenem and other β-lactams in *Klebsiella pneumoniae*. *Antimicrob Agents Chemother* 2004; **48**: 3203–6.

5. Lartigue MF, Poirel L, Poyart C *et al.* Ertapenem resistance of *Escherichia coli*. *Emerg Infect Dis* 2007; **13**: 315–7.

6. Szabo D, Silveira F, Hujer AM *et al.* Outer membrane protein changes and efflux pump expression together may confer resistance to ertapenem in *Enterobacter cloacae*. *Antimicrob Agents Chemother* 2006; **50**: 2833–5.

7. Woodford N, Dallow JW, Hill RL *et al.* Ertapenem resistance among *Klebsiella* and *Enterobacter* submitted in the UK to a reference laboratory. *Int J Antimicrob Agents* 2007; **29**: 456–9.

8. Livermore DM, Oakton KJ, Carter MW *et al.* Activity of ertapenem (MK-0826) versus Enterobacteriaceae with potent β-lactamases. *Antimicrob Agents Chemother* 2001; **45**: 2831–7.

9. Ellington MJ, Kistler J, Livermore DM *et al.* Multiplex PCR for rapid detection of genes encoding acquired metallo-β-lactamases. *J Antimicrob Chemother* 2007; **59**: 321–2.

10. Woodford N, Fagan EJ, Ellington MJ. Multiplex PCR for rapid detection of genes encoding CTX-M extended-spectrum β-lactamases. *J Antimicrob Chemother* 2006; **57**: 154–5.

11. Perez-Perez FJ, Hanson ND. Detection of plasmid-mediated AmpC β-lactamase genes in clinical isolates by using multiplex PCR. *J Clin Microbiol* 2002; **40**: 2153–62.

12. Yigit H, Queenan AM, Anderson GJ *et al*. Novel carbapenem-hydrolyzing β-lactamase, KPC-1, from a carbapenem-resistant strain of *Klebsiella pneumoniae*. *Antimicrob Agents Chemother* 2001; **45**: 1151–61.

13. Leflon-Guibout V, Heym B, Nicolas-Chanoine M. Updated sequence information and proposed nomenclature for bla_{TEM} genes and their promoters. *Antimicrob Agents Chemother* 2000; **44**: 3232–4.

14. Yuan M, Aucken H, Hall LM *et al*. Epidemiological typing of klebsiellae with extended-spectrum β-lactamases from European intensive care units. *J Antimicrob Chemother* 1998; **41**: 527–39.

15. Turton JF, Kaufmann ME, Warner M *et al*. A prevalent, multiresistant clone of *Acinetobacter baumannii* in Southeast England. *J Hosp Infect* 2004; **58**: 170–9.

16. Carlone GM, Thomas ML, Rumschlag HS *et al*. Rapid microprocedure for isolating detergent-insoluble outer membrane proteins from *Haemophilus* species. *J Clin Microbiol* 1986; **24**: 330–2.

17. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. *Methods* 2001; **25**: 402–8.

18. Parales RE, Harwood CS. Construction and use of a new broad-host-range *lacZ* transcriptional fusion vector, pHRP309, for Gram$^-$ bacteria. *Gene* 1993; **133**: 23–30.

19. Martinez-Martinez L, Pascual A, Hernandez-Alles S *et al*. Roles of β-lactamases and porins in activities of carbapenems and cephalosporins against *Klebsiella pneumoniae*. *Antimicrob Agents Chemother* 1999; **43**: 1669–73.

20. Struyve M, Moons M, Tommassen J. Carboxy-terminal phenylalanine is essential for the correct assembly of a bacterial outer membrane protein. *J Mol Biol* 1991; **218**: 141–8.

21. Crowley B, Benedi VJ, Domenech-Sanchez A. Expression of SHV-2 β-lactamase and of reduced amounts of OmpK36 porin in *Klebsiella pneumoniae* results in increased resistance to cephalosporins and carbapenems. *Antimicrob Agents Chemother* 2002; **46**: 3679–82.

22. Martinez-Martinez L, Hernandez-Alles S, Alberti S *et al*. *In vivo* selection of porin-deficient mutants of *Klebsiella pneumoniae* with increased resistance to cefoxitin and expanded-spectrum-cephalosporins. *Antimicrob Agents Chemother* 1996; **40**: 342–8.

23. Bornet C, Davin-Regli A, Bosi C *et al*. Imipenem resistance of *Enterobacter aerogenes* mediated by outer membrane permeability. *J Clin Microbiol* 2000; **38**: 1048–52.

24. Hopkins JM, Towner KJ. Enhanced resistance to cefotaxime and imipenem associated with outer membrane protein alterations in *Enterobacter aerogenes*. *J Antimicrob Chemother* 1990; **25**: 49–55.

25. Hernandez-Alles S, Benedi VJ, Martinez-Martinez L *et al*. Development of resistance during antimicrobial therapy caused by insertion sequence interruption of porin genes. *Antimicrob Agents Chemother* 1999; **43**: 937–9.

26. Nikaido H, Rosenberg EY, Foulds J. Porin channels in *Escherichia coli*: studies with β-lactams in intact cells. *J Bacteriol* 1983; **153**: 232–40.

27. Ehrhardt AF, Sanders CC, Thomson KS *et al*. Emergence of resistance to imipenem in *Enterobacter* isolates masquerading as *Klebsiella pneumoniae* during therapy with imipenem/cilastatin. *Clin Infect Dis* 1993; **17**: 120–2.