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Contribution of OqxAB efflux pumps to quinolone resistance in extended-spectrum-β-lactamase-producing *Klebsiella pneumoniae*

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Objectives: The aims of this study were to analyse the presence of *oqxA* and *oqxB* genes in a collection of extended-spectrum β-lactamase (ESBL)-producing *Klebsiella pneumoniae* strains, to determine their chromosomal and/or plasmidic locations and to analyse expression levels in relation to susceptibility or resistance to quinolones.

Methods: A collection of 114 non-repetitive isolates of ESBL-producing *K. pneumoniae* was used. *K. pneumoniae* ATCC 27799 and *K. pneumoniae* ATCC 700603 were also included. Detection of *oqxA* and *oqxB* genes was performed by PCR. Testing for chromosomal and/or plasmidic location was carried out using plasmid DNA and subsequent hybridization. *oqxA* gene expression was analysed using real-time RT–PCR. Transfer of the plasmid-encoded OqxAB was evaluated.

Results: The prevalence of both *oqxA* and *oqxB* detected in *K. pneumoniae* was high: 76% and 75%, respectively. Hybridization assays showed that *oqxA* (16%) and *oqxB* (13%) were simultaneously present in locations on the chromosome and on large plasmids. The plasmids were transferable by transformation into *K. pneumoniae*. RT–PCR assays showed higher expression (4-fold) in strains with reduced susceptibility to quinolones than in susceptible strains. Interestingly, *K. pneumoniae* ATCC 700603 showed an 18-fold higher expression than *K. pneumoniae* ATCC 27799. These differences were in accordance with quinolone susceptibility.

Conclusions: The prevalence of the OqxAB efflux pump (both chromosomal and plasmid encoded) in ESBL-producing *K. pneumoniae* is high in Spain and represents a potential reservoir for the spread of these genes. High expression of this pump contributes to reduced susceptibility to quinolones in clinical isolates of ESBL-producing *K. pneumoniae*.

Keywords: ESBLs, *K. pneumoniae*, PMQR

Introduction

Plasmid-borne genes conferring quinolone resistance are increasingly being recognized.[1–3] Some years ago, the plasmid-encoded efflux pump, OqxAB, conferring resistance to quinoxaline-di-*N*-oxide olaquindox and used as a promoter of growth in pigs, was discovered in *Escherichia coli* isolates of porcine origin in Denmark and Sweden.[4–6] OqxAB is encoded by the *oqxA* and *oqxB* genes, located on a 52 kb conjugative plasmid, designated pOLA52, and confers resistance to multiple agents, including fluoroquinolones such as nalidixic acid, ciprofloxacin and norfloxacin, as well as biocides such as triclosan and chlorhexidine.[4,7] Recently, for the first time in a human clinical isolate, the genes for the multidrug efflux pump OqxAB were found on a plasmid in *E. coli*—in which IS*26*-like sequences flanked the plasmid-mediated *oqxAB* genes, suggesting that they had been mobilized as part of a composite transposon—as well as on the chromosome of *Klebsiella pneumoniae*.[8]

The aims of this study were: (i) to analyse the presence of *oqxA* and *oqxB* genes in a collection of extended-spectrum β-lactamase (ESBL)-producing *K. pneumoniae* strains; (ii) to determine their chromosomal and/or plasmidic location; and (iii) to analyse their expression levels relative to susceptibility or resistance to quinolones.

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Materials and methods

Bacterial strains

A multicentre collection of 114 ESBL-producing *K. pneumoniae* isolates recovered from urine, wounds, blood cultures, respiratory tract and other samples was analysed. Clinical and epidemiological data were reported previously.[9–11] *K. pneumoniae* ATCC 27799 and *K. pneumoniae* ATCC 700603 were included as reference strains.

Antimicrobial susceptibility testing

Antimicrobial susceptibility was determined by microdilution and disc diffusion, following CLSI recommendations.[12] The antimicrobials tested were ciprofloxacin (Bayer, Leverkusen, Germany), moxifloxacin (Bayer), levofloxacin (Roussel Uclaf, Paris, France), norfloxacin (Sigma, Madrid, Spain), chloramphenicol (Oxoid, Madrid, Spain) and trimethoprim (Sigma). The following biocides were also tested, following CLSI guidelines: 40 g/L sodium hypochlorite (domestic bleach), 20% chlorhexidine digluconate (Sigma–Aldrich, Spain) and 50% benzalkonium chloride (Sigma–Aldrich, Denmark).

Screening for oqxA and oqxB genes

The *oqxA* and *oqxB* genes were screened using a PCR-based technique. The presence of *oqxA* and *oqxB* was detected using primers oqxAF (5′-CTCGGCGCGATGATGCT-3′) and oqxAR (5′-CCACTCTTCACGGGAGACGA-3′), with products of 392 bp, and oqxBs (5′-TTCTCCCCCGGCGGGAAGTAC-3′) and oqxBa2 (5′-CTCGGCCATTTTGGCGCGTA-3′), with products of 512 bp, as described previously.[8] The amplicons obtained were confirmed by sequencing both chains.

Additionally, the association of IS*26* with *oqxA*, as reported previously,[7,8] was investigated by PCR. The presence of IS*26* was performed using primers IS26a (5′-TTACATTTCAAAAACTCTGCTTACC-3′) and IS26b (5′-ATGAACCCATTCAAAGGCCG-3′), producing a fragment of 704 bp.[13] The association of IS*26* with *oqxA* was determined using primers IS26-F (5′-GCTGTTACGACGGGAGGAG-3′)[14] and oqxAR.

Plasmid analysis and Southern hybridization

Plasmid size was determined by Kieser extraction[15] and electrophoresis using a 0.7% agarose gel. *E. coli* 50192 harbouring 154, 66, 48 and 7 kb plasmids was used as a reference strain. Incompatibility groups were characterized as described previously by Caratolli *et al.*[16]

Purified plasmids were run on a 0.7% agarose gel and transferred to a positively charged nylon membrane (Amersham Hybond-N+). Membrane hybridization was performed using PCR-amplified *oqxA* and *oqxB* genes as probes.

Analysis of mutations in the quinolone resistance-determining region (QRDR)

Mutations of *gyrA* and *parC* genes in the QRDR were analysed by PCR and sequenced for all isolates included in the RT–PCR assays (see below), as described previously.[17]

Conjugation and transformation assays

Conjugation experiments were carried out in Luria–Bertani (LB) broth with sodium azide-resistant *E. coli* J53 as the recipient. Transconjugants were selected by plating onto LB agar plates containing sodium azide at 150 mg/L and ciprofloxacin at 0.05 mg/L for selection of plasmid-mediated fluoroquinolone resistance.

In all cases, plasmids were not transferable by conjugation and a transformation assay was performed. Plasmids extracted by the Kieser method were electroporated into *E. coli* DH10B and *K. pneumoniae* ATCC 27799 and plated onto LB agar containing ciprofloxacin at 0.05 and 0.2 mg/L, respectively.

Semi-quantitative real-time RT–PCR

To detect *oqxA* gene expression, RT–PCR was carried out using a set of 24 *K. pneumoniae* clinical strains, whose *oqxA* and *oqxB* genes were only encoded chromosomally. *K. pneumoniae* ATCC 27799 and *K. pneumoniae* 700603 were included as references. The LightCycler Fast Start DNA Master SYBR-Green I Kit (Roche) was used for amplification. Normalized expression levels of the target gene transcripts were calculated relative to *rpoB*, using the $2^{-\Delta\Delta CT}$ method.[18] The primers used for gene amplification were OqxA-Fw-RT (5′-GTAACCTGGTCACCGCGGGC-3′) and OqxA-Rw-RT (5′-TGCCCTGGTGGGGGTAACCC-3′), which amplified an internal product of 207 bp.

Time–kill experiments

Time–kill kinetic assays were conducted on Mueller–Hinton broth at drug concentrations of 1× MIC, 4× MIC and 1 mg/L ciprofloxacin. Antimicrobial agent-free broth was evaluated in parallel as a control. Cultures were incubated at 37°C with shaking. Viable counts were determined by serial dilution at 0, 2, 4, 6, 8 and 24 h after adding the drug and plating 100 μL of control, test culture or dilutions at the indicated times onto Mueller–Hinton agar plates. Plates were incubated for 24 h and, after the colonies were counted, the $\log_{10}$ of viable cells (cfu/mL) was determined. The time needed to achieve a 3 $\log_{10}$ drop in bacterial viability was also determined as an index of bactericidal activity.

Analysis of outer membrane proteins (OMPs)

OMPs were studied by SDS–PAGE using 10% polyacrylamide gels.[19]

Statistical methods

The Student's *t*-test for paired samples was used for analysis. A *P* value of <0.05 was considered statistically significant.

Results and discussion

PCR was used to screen a total of 114 ESBL-producing *K. pneumoniae* clinical isolates for *oqxA* and *oqxB* genes. Strains positive for both *oqxA* and *oqxB* genes were confirmed by sequencing of the PCR products. Eighty-seven (76.3%) and 85 (74.6%) of 114 *K. pneumoniae* isolates were provisionally classified as positive for *oqxA* and *oqxB*, respectively. All the strains used in this study were clonally unrelated as determined by both repetitive element palindromic ('REP')-PCR and PFGE (data not shown).[9] Ten amplicons of each gene were sequenced and a BLAST search of nucleotide sequence similarity of the *oqxA* and *oqxB* PCR products gave more than 97% identity with pOLA52 (GenBank accession number EU370913) and 100% amino acid identity. There was also 100% amino acid identity of OqxA and OqxB proteins with *K. pneumoniae* MGH 78578 (GenBank accession number NC009648), showing a high degree of conservation for these genes. The data support the idea that *oqxAB* genes seem to be conserved chromosomal genes in *K. pneumoniae*.[7,8,20]

In spite of this, we tested the chromosomal or plasmidic location of the genes in our collection. Hybridization assays

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Table 1. Summary of the characteristics of selected strains of *K. pneumoniae*

Strain	*oqxA*/*oqxB* PCR result	PMQR genes	CIP MIC (mg/L)	QRDR mutations		Southern blotting result for *oqxA*/*oqxB*
				GyrA	ParC	
5/01	+/+	*qnrA1*	2	WT	WT	C/C
7/16	+/+	—	0.06	WT	WT	C/C
8/14	+/+	—	0.015	WT	WT	C/C
20/18	+/+	—	0.25	WT	WT	C/C
24/41	+/+	—	0.008	WT	WT	C/C
25/21	+/+	*aac(6′)Ib-cr*	0.06	WT	WT	C/C
25/55	+/+	*aac(6′)Ib-cr*	0.5	WT	WT	C/C
39/18	+/+	*aac(6′)Ib-cr*	0.06	WT	WT	C/C
39/75	+/+	—	0.015	WT	WT	C/C
39/77	+/+	*aac(6′)Ib-cr*	0.06	WT	WT	C-P/C-P
45/28	+/+	—	0.125	S83Y	WT	C/C
43/12	+/+	*qnrB1*	0.5	D87G	WT	C/C
45/42	+/+	*aac(6′)Ib-cr*	0.125	S83Y+D87Y	WT	C/C
24/04	+/+	—	0.125	S83Y	WT	C/C
6/15	+/+	—	64	S83I	S80I	C/C
6/16	+/+	—	16	S83I	S80I+E84K	C/C
7/04	+/+	—	16	S83I	S80I	C/C
11/06	+/+	—	16	S83I	S80I	C/C
16/04	+/+	—	32	S83F+D87N	E84K	C/C
49/01	+/+	—	32	S83Y	S80I	C/C
27/20	+/+	—	16	S83I	S80I	C/C
4/07	+/+	—	0.125	S83F	WT	C-P/C-P
4/14	+/+	—	0.25	WT	WT	C-P/C-P
39/39	+/+	*aac(6′)Ib-cr*	0.125	WT	WT	C-P/C-P
39/63	+/+	*aac(6′)Ib-cr*	0.125	WT	WT	C-P/C-P
41/05	−/−	—	0.015	WT	WT	C (low signal)/C (low signal)
43/28	−/−	—	0.25	S83Y	WT	−/−
43/29	−/−	*aac(6′)Ib-cr*	64	S83F+D87A	E84K	−/−
ATCC 27799	+/+	—	0.03	WT	WT	C/C
ATCC 700603	+/+	—	0.25	WT	WT	C/C

CIP, ciprofloxacin; WT, wild-type; C, chromosomal location; P, plasmidic location.

confirmed the PCR results, showing strong signals and bands co-migrating with chromosomal DNA for both *oqxA* and *oqxB* genes in all positive isolates tested by PCR. We also found simultaneous *oqxA* and *oqxB* signals in both chromosomal and large plasmid (>160 kb) locations in 16% (n=18) and 13% (n=15) of *oqxA* and *oqxB* genes, respectively (Table 1 and Figure 1). These assays confirmed that about 25% of repeatedly PCR-negative *K. pneumoniae* strains were also negative by Southern hybridization using *oqxA* and *oqxB* probes.

We selected four different *K. pneumoniae* clinical strains (4/07, 4/14, 39/63, 41/29) that showed strong signals of both *oqxA* and *oqxB* in plasmid DNA and carried out plasmid transfer assays. We did not find direct transfer of *oqxAB*, by either conjugation or electroporation, using *E. coli* as receptor (both J53 and DH10B genetic backgrounds). We did, however, obtain *K. pneumoniae* transformants harbouring a plasmid encoding OqxAB in all cases of *K. pneumoniae* ATCC 27799 being used as a receptor (confirmed by hybridization, data not shown) (Table 2). This finding suggests that the *oqxA*–*oqxB*-hybridizing plasmids were non-conjugative, at least under our laboratory conditions. The MICs of fluoroquinolones increased 16- to 128-fold and the MIC of trimethoprim 16- to 32-fold, according to the substrate specificity previously described for this pump (Table 2).[4] All these plasmids belonged to the IncF group, as has been previously described.[14]

In this collection, 62.2% of strains were of intermediate susceptibility or resistant to ciprofloxacin and 9.6% and 24.5% harboured *qnr* (*qnrA*, *qnrB* or *qnrS*) genes or *aac(6′)Ib-cr*, respectively, as previously reported.[9–11] A summary of the characteristics of selected *K. pneumoniae* strains used in this study is provided in Table 1. There was a correlation between the MICs of ciprofloxacin and the presence of plasmid-mediated quinolone resistance (PMQR) genes and QRDR substitutions. To determine whether the expression of *oqxAB* genes was associated with resistance to quinolones, we used RT–PCR to analyse the level of expression of chromosome-encoded *oqxA* in 24 selected *K. pneumoniae* clinical strains (these strains did not contain plasmid-located *oqxAB* genes; Figure 2a). We divided this set of strains into ciprofloxacin-susceptible clinical strains (zone of inhibition around a 5 μg ciprofloxacin disc of more than 33 mm, MIC of

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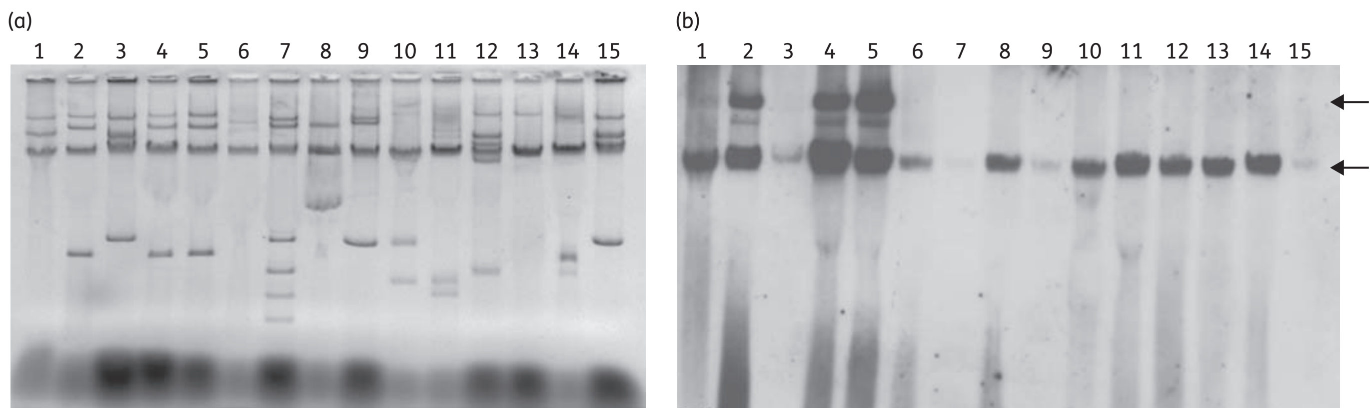

Figure 1. Gel electrophoresis (a) and Southern hybridization with an *oqxA* probe (b) of plasmid DNA preparations. Lane 1, *K. pneumoniae* ATCC 27799; lane 2, positive plasmid control (clinical isolate 4/07); lanes 3 and 15, *E. coli* 50192 (ladder); lanes 4–14, *K. pneumoniae* clinical isolates 4/14, 4/07, 5/01, 5/09, 5/13, 5/23, 6/11, 6/13, 6/15, 6/16 and 6/19, respectively. The top arrow indicates plasmid DNA and the bottom arrow indicates the location of chromosomal DNA and/or sheared plasmid DNA.

Table 2. MICs of fluoroquinolones and trimethoprim for four different clinical isolates of *K. pneumoniae* containing plasmid-mediated *oqxA* and *oqxB* genes and their transformant derivatives in *K. pneumoniae* ATCC 27799

	MIC (mg/L)				
Strain	CIP	LVX	MFX	NOR	TMP[a]
4/07	0.125	0.125	0.125	0.25	0.5
4/07 TF	0.5	1	2	4	64
4/14	0.25	0.25	0.25	0.5	1
4/14 TF	2	4	4	16	128
39/63	0.125	0.125	0.125	0.25	0.25
39/63 TF	2	4	4	16	128
41/29	0.125	0.06	0.125	0.25	0.5
41/29 TF	1	2	2	8	128
K. pneumoniae ATCC 27799	0.03	0.03	0.06	0.25	4

CIP, ciprofloxacin; LVX, levofloxacin; MFX, moxifloxacin; NOR, norfloxacin; TMP, trimethoprim.
[a]Co-resistance to chloramphenicol was not used as a marker since *K. pneumoniae* ATCC 27799 was resistant.

ciprofloxacin ≤0.06 mg/L, n=8) and strains with reduced susceptibility to ciprofloxacin at least (zone of inhibition around a 5 μg ciprofloxacin disc was less than 30 mm, MIC of ciprofloxacin ≥0.125 mg/L, n=16). This division was made on the basis that OqxAB efflux pumps themselves confer a low level of quinolone resistance. RT–PCR assays showed significantly higher *oqxA* expression (about 4-fold) in strains with reduced susceptibility to quinolones than in fully susceptible strains (Figure 2a, P=0.003). These data suggest that the level of expression of chromosomally encoded OqxAB could contribute to reduced susceptibility to quinolones in *K. pneumoniae*.

No differences were found in the promoter regions of *oqxA* (TTGCAC −35 box, TACAAT −10 box and surrounding sequences) among *K. pneumoniae* ATCC 27799 and ATCC 700603 strains and various clinical isolates that differed in *oqxA* transcript levels (data not shown). In every case, these sequences were identical to those of *K. pneumoniae* MGH 78578 (GenBank accession number NC009648). Thus, the increased expression of *oqxA* in these strains does not appear to be due to mutations in the putative promoter and it may be that, as previously suggested, other undefined regulatory elements are implicated.[8] Recently it has been shown that global regulators such as *ramA* or *rarA*, belonging to the AraC-type transcriptional regulators family, and the local regulator *oqxR*, encoding a GntR-type regulator adjacent to the *oqxAB* operon, are implicated in up-regulation or down-regulation, respectively, of the expression of the *oqxAB* efflux pump.[21,22] Additionally, although all strains containing plasmids coding for OqxAB were IS*26* positive, no collinearity was found between this insertion sequence and *oqxA* in contrast to what is observed for pOLA52.[7]

Interestingly, we observed that *K. pneumoniae* ATCC 700603 showed an 18-fold higher expression than *K. pneumoniae* ATCC 27799 (Figure 2b). These two strains showed identical QRDR amino acid sequences for both GyrA and ParC as *K. pneumoniae* MGH 78578 (GenBank accession number NC009648) and the two OMPs OmpK35 and OmpK36 were also present in the two strains, as verified by SDS–PAGE (data not shown). We also found no PMQR genes in these reference strains. The data suggest that the observed differences in MIC of ciprofloxacin (0.25 and 0.03 mg/L for *K. pneumoniae* ATCC 700603 and *K. pneumoniae* ATCC 27799, respectively; Table 1) were in line with the differences in expression observed, supporting the role of these efflux pumps in terms of reduced susceptibility to quinolones in *K. pneumoniae*. We also observed that *K. pneumoniae* ATCC 700603 that overexpressed *oqxA* showed higher MICs of various disinfectants used in healthcare, such as sodium hypochlorite (2-fold), chlorhexidine digluconate (4-fold) or benzalkonium chloride (4-fold), in accordance with previous results.[4]

Killing-curve assays showed a selective advantage for survival at 1 mg/L ciprofloxacin for *K. pneumoniae* ATCC 700603 that overexpressed the *oqxA* gene relative to *K. pneumoniae* ATCC 27799 (basal level of *oqxA* expression) (Figure 3). This ciprofloxacin concentration defines the borderline for establishing

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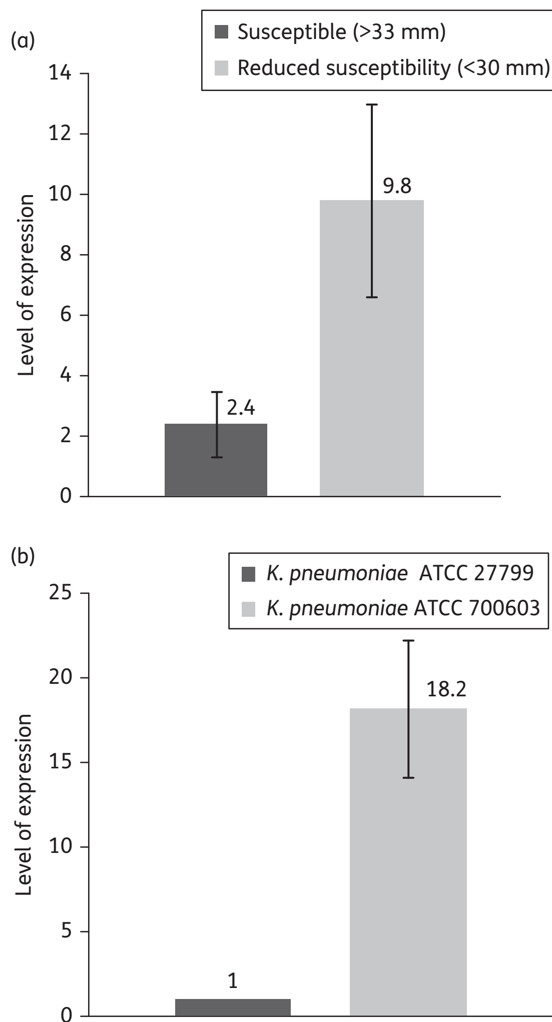

Figure 2. Mean relative expression of the *oqxA* gene in *K. pneumoniae* strains. (a) Ciprofloxacin-susceptible clinical strains (inhibition zone of 5 μg ciprofloxacin disc was more than 33 mm) compared with strains with at least reduced susceptibility (inhibition zone of 5 μg ciprofloxacin disc was less than 30 mm). (b) *K. pneumoniae* ATCC 27799 (basal expression level of *oqxA*) compared with *K. pneumoniae* ATCC 700603 (*oqxA* overexpression). The expression level of *K. pneumoniae* ATCC 27799 was used as a reference with a value of 1.

susceptibility, intermediate susceptibility or resistance (according to CLSI and EUCAST guidelines) in Enterobacteriaceae.[12,23] Ciprofloxacin at 1 mg/L caused a marked reduction in the number of viable *K. pneumoniae* ATCC 27799 bacteria after 2 h of incubation. After 4 h, bacterial regrowth was noted. The marked reduction in viable bacteria was not observed for *K. pneumoniae* ATCC 700603, which overexpressed *oqxA* at 1 mg/L, supporting the potential impact of the OqxAB efflux pump on bacterial viability. No clear differences in bacterial viability were observed between the two strains at 1× MIC or 4× MIC (Figure 3). Taken together, the findings support the idea that low-level quinolone resistance mechanisms such as the OqxAB system and PMQR[24,25] could have an important impact in terms of fluoroquinolone activity.

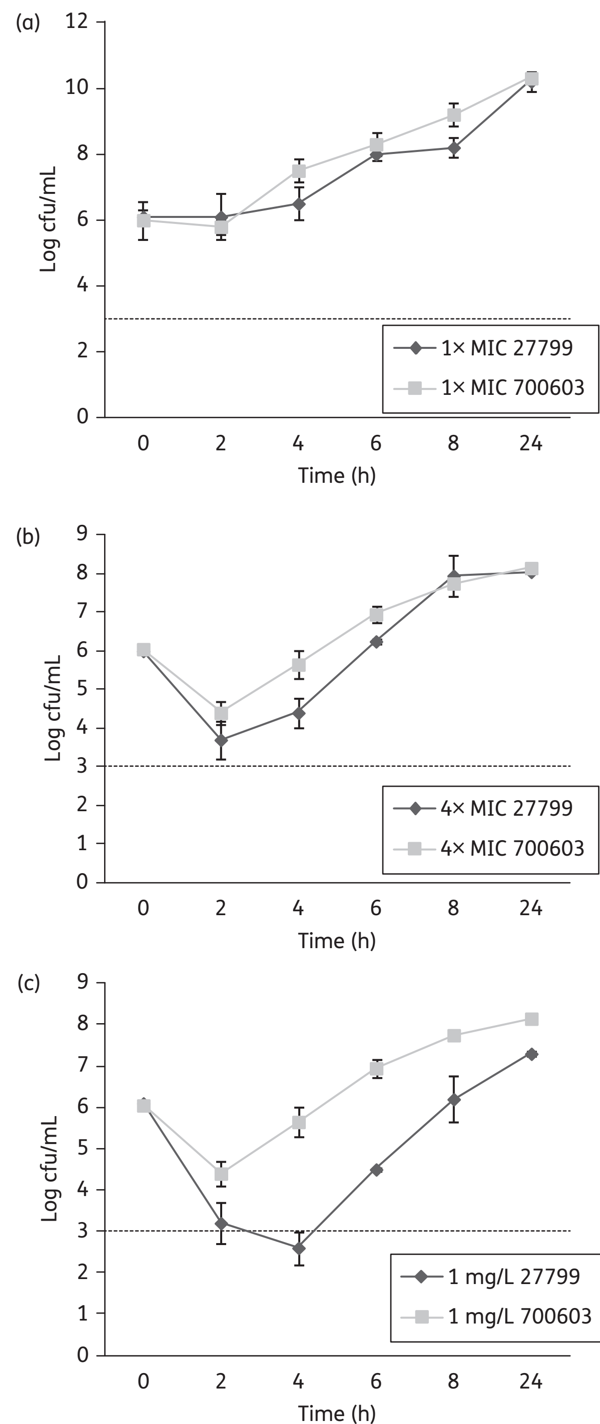

Figure 3. *In vitro* time–kill studies (in broth) of ciprofloxacin against *K. pneumoniae* ATCC 27799 (basal expression of *oqxA*) and *K. pneumoniae* ATCC 700603 (*oqxA* overexpression): (a) 1× MIC; (b) 4× MIC; and (c) 1 mg/L. The horizontal broken line indicates the level for a 3 $\log_{10}$ reduction of viable bacteria.

In conclusion, the prevalence of the OqxAB efflux pump (both chromosomal and plasmidic) is high in ESBL-producing *K. pneumoniae* in Spain and represents a potential reservoir for the spread of *oqxAB*. High expression of this pump seems to

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contribute to the reduced susceptibility to quinolones in clinical isolates of *K. pneumoniae*.

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Transparency declarations

None to declare.

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