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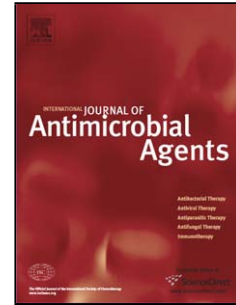
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Presence of plasmid-mediated quinolone resistance in *Klebsiella pneumoniae* and *Escherichia coli* isolates possessing *bla*_{VIM-1} in Greece

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ABSTRACT

Among nalidixic acid-resistant, ciprofloxacin-susceptible *Escherichia coli* and *Klebsiella pneumoniae* clinical isolates recovered over a 5-month period from inpatients and outpatients of Attikon University General Hospital (Athens, Greece), only one *E. coli* was positive for *qnrB2* and one *K. pneumoniae* was positive for *qnrA1*. Both isolates were extended-spectrum β -lactamase-negative, metallo- β -lactamase-positive and carried the *bla*_{VIM-1} gene. Neither of the isolates had mutations in *gyrA* and *parC* or carried *aac(6')-Ib-cr* or *qepA*. The *K. pneumoniae* isolate also harboured *bla*_{CMY-13} on the same transferable plasmid with *qnrA1*. This is the first report of a *qnrA1*-positive *K. pneumoniae* and *qnrB2*-positive *E. coli* harbouring a concurrent *bla*_{VIM-1} gene.

1. Introduction

Quinolone resistance in Enterobacteriaceae is mostly mediated by point mutations in the quinolone resistance-determining regions (QRDRs) of the gyrase and topoisomerase IV genes, leading to target modification [1]. More recently, three plasmid-mediated quinolone resistance mechanisms have been described: Qnr peptides, capable of protecting DNA gyrase and topoisomerase IV from quinolones; Aac(6')-Ib-cr aminoglycoside acetyltransferase, modifying quinolones with a piperazinyl substituent (e.g. ciprofloxacin); and the quinolone efflux pump QepA [1]. *qnrA1* from a clinical strain of *Klebsiella pneumoniae* isolated in Alabama was first described by Martinez-Martinez et al. in 1998 [2]. To date, *qnrA*, *qnrB* and *qnrS* comprise three families differing from each other by $\geq 40\%$ in nucleotide sequence. Within each family, minor ($\leq 10\%$) variation in sequence has defined a growing number of alleles. A total of 6 *qnrA*, 23 *qnrB*, 4 *qnrS* and 1 each of *qnrC* and *qnrD* variants have been described in the literature and are listed in the database maintained at the website <http://www.lahey.org/qnrStudies> (updated 16 February 2010).

qnr genes are widely distributed in clinical Enterobacteriaceae isolates around the world and are usually associated with mobile genetic elements (complex *sul1*-type integrons) [1]. Their products lead to increased minimum inhibitory concentrations (MICs) of quinolones, which in many cases are still below the approved susceptibility breakpoints and require the coexistence of an additional mechanism to lead to frank resistance [1]. *qnr* genes were found to be carried on the same plasmid with various extended-spectrum or AmpC-type β -lactamases genes [1]. Recently, Mendes et al. [3]

and Endimiani et al. [4] reported the presence of plasmid-mediated quinolone resistance in *K. pneumoniae* isolates possessing *bla*_{KPC} in China and the USA, respectively, whilst Chmelnitsky et al. [5] reported the presence of *bla*_{KPC-2} and *qnrB2* on the same plasmid in *Enterobacter cloacae* isolates. The metallo- β -lactamase (MBL) IMP-8 has been associated with *qnrB* and *qnrS* in *E. cloacae* isolates in Taiwan, whilst *bla*_{VIM-1} was located on the same plasmids as *qnrS1* in multiple species of Enterobacteriaceae in Italy [6,7]. Very recently Miró et al. [8] showed a linkage between *bla*_{VIM-1}, *qnrS* and *aac(6')-Ib-cr* genes and between *bla*_{CTX-M-9} and *qnrA* genes identified in *Klebsiella* and *Enterobacter* spp.

We report the isolation of a *K. pneumoniae* and an *Escherichia coli* isolate, both ciprofloxacin-susceptible but nalidixic acid-resistant, possessing *bla*_{VIM-1} on the same conjugative plasmid as *qnrA* and *qnrB*, respectively.

2. Material and methods

The computerised database of the hospital's Infectious Diseases Research Laboratory was retrospectively searched for *E. coli* and *K. pneumoniae* strains that were nalidixic acid-resistant and ciprofloxacin-susceptible isolated during a 5-month period (February–June 2008) from inpatients and outpatients of Attikon University General Hospital (Athens, Greece). Only one isolate per patient was included in the study. Species identification of isolated bacteria and MIC determinations were performed using an automated system (BD Phoenix automated microbiology system; BD Diagnostic Systems, Sparks, MD). MICs of nalidixic acid were also evaluated by broth microdilution in accordance with Clinical and Laboratory Standards Institute (CLSI) guidelines [9], whereas MICs of ciprofloxacin, moxifloxacin, imipenem,

meropenem, doripenem, aztreonam and trimethoprim/sulfamethoxazole (SXT) were determined by Etest (AB BIODISK, Solna, Sweden) in accordance with the manufacturer's instructions. Results were interpreted applying CLSI criteria for in vitro susceptibility [9]. All isolates were screened for extended-spectrum β -lactamase (ESBL) production by the double-disk synergy test and the CLSI confirmatory test, for MBL production using the ethylene diamine tetra-acetic acid (EDTA)–imipenem disk synergy test, and for KPC production by the imipenem–boronic acid disk synergy test [9–11].

Isolates were screened for the presence of *qnrA*, *qnrB* and *qnrS* by multiplex polymerase chain reaction (PCR) using universal primers for each gene amplifying all related alleles [12]. Conjugation experiments were carried out by the filter method with the two *qnr*-positive clinical isolates as donors and a rifampicin-resistant *E. coli* K-12 RC85 laboratory strain as a recipient. Transconjugant clones were selected on brain–heart infusion agar (Bio-Rad, Marnes-la-Coquette, France) plates containing 150 μ g/mL rifampicin and 8 μ g/mL nalidixic acid.

qnr-positive isolates and their transconjugants were additionally tested by isoelectric focusing of sonic extracts on pre-cast polyacrylamide gels with a pH 3–10 gradient (PhastGel IEF 3–9; Amersham Biosciences, Freiburg, Germany) for β -lactamase detection. The presence of *bla*_{VIM}, *bla*_{CMY}, *bla*_{SHV} and *bla*_{TEM} was confirmed by PCR with specific primers shown in Table 1. The genes *aac(6')-Ib* and *qepA* were detected by PCR with specific primers (Table 1). QRDRs of *gyrA* and *parC* were amplified with primers shown in Table 1 and were sequenced to evaluate possible coexisting chromosomal mutations.

Sequencing of PCR products was performed by Eurofins MWG Operon (Ebersberg, Germany). For sequence analysis, the BLAST program from the National Center for Biotechnology Information website was used (<http://www.ncbi.nlm.nih.gov/BLAST>).

3. Results and discussion

Among 231 single-patient *E. coli* and 231 single-patient *K. pneumoniae* isolates found on the database during the study period, 13 and 11 isolates, respectively, were resistant to nalidixic acid and susceptible to ciprofloxacin and were studied further. Ciprofloxacin MIC values ranged from ≤ 0.125 $\mu\text{g}/\text{mL}$ to 1 $\mu\text{g}/\text{mL}$ for *E. coli* and ≤ 0.125 $\mu\text{g}/\text{mL}$ to 0.5 $\mu\text{g}/\text{mL}$ for *K. pneumoniae*.

Escherichia coli m4353, which was isolated from a faecal specimen of a patient hospitalised in the Intensive Care Unit of Attikon Hospital, was positive for *qnrB*. *Klebsiella pneumoniae* U8699, which was isolated from a urine specimen of a patient with a history of multiple hospitalisations in Attikon Hospital, was positive for *qnrA*. Sequencing revealed the existence of *qnrB2* and *qnrA1* alleles, respectively. No isolates from those collected were positive for *qnrS*. Both *qnrA1*- and *qnrB2*-positive isolates were ESBL-negative and MBL-positive by phenotypic tests and harboured the *bla*_{VIM-1} gene. *Klebsiella pneumoniae* U8699 in addition to VIM-1 carbapenemase coded for CMY-13 [isoelectric point (pI) 9.1] and the intrinsic SHV-11 (pI 7.6), whilst *E. coli* m4353 coded for VIM-1 and a TEM-1-like enzyme. *aac(6')-Ib-cr* variant and *qepA* were not detected in either isolate and no mutations were detected in *gyrA* and *parC*.

Results of conjugation experiments showed that *qnrA1* and *qnrB2* were located on conjugative plasmids of *K. pneumoniae* and *E. coli*, respectively. The plasmid containing *qnrA1* also contained *bla*_{VIM-1} and *bla*_{CMY-13} and was transferred to the *E. coli* recipient at a frequency of 10^{-5} transconjugants per recipient, conferring resistance to nalidixic acid, several β -lactams, inhibitor combinations and SXT (Table 2). The *qnrA1* transconjugant exhibited decreased susceptibility to carbapenems (imipenem MIC = 1 μ g/mL; meropenem MIC = 0.19 μ g/mL) and aztreonam (MIC = 0.2 μ g/mL).

The plasmid carrying *qnrB2* also contained *bla*_{VIM-1} and *bla*_{TEM-1-like} and was transferred to the *E. coli* recipient at a frequency of 10^{-4} transconjugants per recipient, conferring resistance to nalidixic acid, several β -lactams, inhibitor combinations, tetracycline and SXT (Table 2). The *qnrB2* transconjugant exhibited decreased susceptibility to carbapenems (imipenem MIC = 4 μ g/mL; meropenem MIC = 0.19 μ g/mL) and amikacin (MIC = 16 μ g/mL).

Vasilaki et al. [13] have reported that a considerably high proportion (10%) of quinolone-resistant *E. coli* isolates from northern and central Greece were found to carry *qnrS1* in addition to mutations in *gyrA* (S83L, D87N) and *parC* (mainly S80I). None of these isolates carried *qnrA* or *qnrB*, whilst only one was an ESBL-producer, carrying *bla*_{CTX-M-15}. Our findings indicate that *qnrA1* and *qnrB2* are also present in *E. coli* and *K. pneumoniae* clinical isolates in Greece and they are not always associated with the QRDR mutations.

The gene *bla*_{CMY-13}, which codes for a cephalosporinase with activity similar to that of CMY-2, has been previously reported to be located on an IncN plasmid from an *E. coli* isolate from Greece [14]. This plasmid carried *bla*_{VIM-1}, *aacA7*, *dhfrI* and *aadA* gene cassettes on a class I integron, but not a *qnr* determinant [15].

In conclusion, this is the first report of the presence of *qnrA1* and of *qnrB2* on the same transferable plasmid with *bla*_{VIM-1} and *bla*_{CMY-13} in *K. pneumoniae* and with *bla*_{VIM-1} in *E. coli*, respectively. Although the *qnr* genes are responsible for low-level resistance to quinolones, they are easily transferable and they provide a favourable background for higher level resistance to occur if other mechanisms coexist, such as secondary changes in DNA gyrase or topoisomerase IV, porin or efflux systems. The presence of mechanisms for broad-spectrum resistance, such as genes coding for carbapenemases on the same plasmid, highlights the clinical importance of these genes and the potential for selection and dissemination of resistance to various antimicrobials through improper quinolone use.

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Competing interests

None declared.

Ethical approval

Not required.

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Table 1

Primers used in this study

Primer ^a	Sequence (5'→3')	Gene	Size of PCR-amplified product (bp)
AAC-cr-F	TTGCGATGCTCTATGAGTGGCTA	<i>aac(6')</i> - <i>lb</i>	482
AAC-cr-R	CTCGAATGCCTGGCGTGTTT		
QEPA-F	CGTGTTGCTGGAGTTCTTC	<i>qepA</i>	403
QEPA-R	CTGCAGGTA CTGCGTCATG		
VIM-F	AGTGGTGAGTATCCGACAG	<i>bla_{VIM}</i>	509
VIM-R	ATGAAAGTGCCTGGAGAC		
SHV-F	ATGCGTTAT ATTCGCCTGTG	<i>bla_{SHV}</i>	865
SHV-R	GTTAGCGTTGCCAGTGCTCG		
CMY-F	CTGCTGCTGACAGCCTCTTT	<i>bla_{CMY}</i>	1108
CMY-R	TTTTCAAGAATGCGCCAGGC		
TEM-F	CATTCAAATATGTATCCGCTCATG	<i>bla_{TEM}</i>	745
TEM-R	CATCCATAGTTGCCTGACTCCCC		
GYR-A-F	TTAATGATTGCCGCCGTCGG	<i>gyrA</i>	648
GYR-A-R	TACACCGGTCAACATTGAGG		
PAR-C-F	AAACCTGTTTCAGCGCCGCATT	<i>parC</i>	395
PAR-C-R	GTGGTGCCGTTAAGCAAA		

PCR, polymerase chain reaction.

^a F, sense primer; R, antisense primer.

Table 2

Minimum inhibitory concentrations (MICs) of clinical and laboratory isolates used in the study

Antimicrobial agent	MIC (mg/L)				
	<i>Escherichia coli</i> K-12 RC85 recipient	<i>Klebsiella pneumoniae</i> U8699	<i>E. coli</i> U8699 transconjugant	<i>E. coli</i> m4353	<i>E. coli</i> m4353 transconjugant
Amikacin	≤8	16	≤8	32	16
Amoxicillin/clavulanic acid	≤4/2	>16/8	>16/8	>16/8	>16/8
Ampicillin	≤4	>16	>32	>16	>32
Aztreonam ^a	0.032	4	0.2	0.094	0.032
Cefepime	≤2	>16	>16	>16	>16
Cefotaxime	≤1	>32	32	>32	>32
Cefoxitin	≤4	>16	>16	>16	8
Ceftazidime	≤1	>16	>16	>16	>16
Cefuroxime	≤4	>16	>16	>16	>16
Cefalothin	8	>16	>16	>16	>16
Ciprofloxacin ^a	0.16	0.38	0,19	0,25	0,19
Colistin	≤0.5	N/D	≤0.5	N/D	≤0.5
Doripenem ^a	0.23	6	0.25	0.19	0.38
Gentamicin	≤2	8	≤2	4	≤2

Imipenem ^a	0.19	>32	1	4	4
Meropenem ^a	0.094	6	0.19	0.25	0,19
Nalidixic acid ^b	4	16	32	16	32
Nitrofurantoin	≤16	>64	≤16	≤16	≤16
Norfloxacin	≤2	4	≤2	≤2	≤2
Piperacillin	≤4	N/D	>64	N/D	>64
Piperacillin/tazobactam	≤4/4	>64/4	>64/4	>64/4	>64/4
Tetracycline	≤2	Sc	≤2	R ^c	>8
Trimethoprim/sulfamethoxazole ^a	0.032	>32	>32	>32	>32
PCR/sequencing	None	<i>bla</i> _{CMY-13} , <i>bla</i> _{SHV-11} , <i>bla</i> _{VIM-1} , <i>qnrA1</i>	<i>bla</i> _{CMY-13} , <i>bla</i> _{VIM-1} , <i>qnrA1</i>	<i>bla</i> _{VIM-1} , <i>bla</i> _{TEM-1-like} , <i>qnrB2</i>	<i>bla</i> _{VIM-1} , <i>qnrB2</i>

N/D, not determined; R, resistant; PCR, polymerase chain reaction.

^a MICs determined by Etest.

^b MICs evaluated by broth microdilution.

^c Susceptibility determined by disk diffusion test.