Prevalence of plasmid-mediated quinolone resistance determinants in *Citrobacter freundii* isolates from Anhui province, PR China

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This study was conducted to detect and analyse the presence of plasmid-mediated quinolone resistance (PMQR) determinants [*qnr*, *aac(6')-Ib-cr* and *qepA*] among *Citrobacter freundii* isolates from patients in Anhui province, PR China. During 2009–2010, 31 *C. freundii* strains were collected from various hospital units and patient specimens. Using PCR, *qnr* genes were detected in eight isolates, but *aac(6')-Ib-cr* and *qepA* genes were not found. The genes *qnrA1*, *qnrB1*, *qnrB2*, *qnrB4*, *qnrB10* and *qnrB24* were present in 6.5, 3.2, 6.5, 3.2, 3.2 and 3.2 % of *C. freundii* isolates, respectively. A new subgene of *qnrB* variant (*qnrB24*) was found and identified for what we believe to be the first time. PFGE after *Xba*I digestion of genomic DNA indicated that *qnr*-positive strains were not clonally related. Conjugation experiments were conducted to determine whether the *qnr*-carrying plasmids were self-transferable, and plasmids of transconjugants were extracted and analysed. The *qnr* genes were transferred from three clinical isolates to their transconjugants. Two *qnrA1* genes transferred quinolone resistance with a plasmid of ~11 kb, whilst the size of the plasmid carrying the *qnrB4* gene was ~64 kb. The susceptibility of positive isolates and transconjugants was tested using an agar dilution method according to Clinical and Laboratory Standards Institute guidelines, and the MICs of ciprofloxacin and levofloxacin were determined using Etest strips. Most isolates with *qnr* genes were resistant to fluoroquinolones and other antimicrobial agents. The MICs of transconjugants showed reduced susceptibility to fluoroquinolones.

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INTRODUCTION

Fluoroquinolones are broad-spectrum antimicrobial agents currently effective in the treatment of a wide range of infections (Hopkins *et al.*, 2005). Quinolones and fluoroquinolones are used extensively in both human and veterinary medicine and are therefore found as residues in the environment. This widespread use has been associated with increased levels of quinolone resistance, particularly in the last 10 years (Robicsek *et al.*, 2006a). However, resistance is emerging in both production (Webber & Piddock, 2001) and companion (Cohn *et al.*, 2003; Pomba *et al.*, 2009) animals. During these years, the extensive use and misuse of fluoroquinolones has

become a driver of both chromosome-encoded and plasmid-encoded mechanisms of quinolone resistance (PMQR), with a potential selective advantage for PMQR determinants. PMQR determinants also play a role in the tight association between quinolone resistance and resistance to other antimicrobial agents.

PMQR genes (*qnr*) have been reported worldwide (Nordmann & Poirel, 2005; Wang *et al.*, 2008). The first *qnr* determinant was reported in a clinical isolate of *Klebsiella pneumoniae* from the USA in 1998 (Martínez-Martínez *et al.*, 1998). *qnr* genes have now been identified widely in various enterobacterial species, in both community and nosocomial settings (Robicsek *et al.*, 2006a). Robicsek *et al.* (2006b) screened 313 ceftazidime-resistant *Enterobacteriaceae* isolates obtained in the USA from 1999 to 2004 for all three known *qnr* genes. A *qnr* gene was present in 20 % of *K. pneumoniae* isolates, 31 % of *Enterobacter* sp. isolates and

Abbreviations: PMQR, plasmid-mediated quinolone resistance; QRDR, quinolone resistance-determining region.

The GenBank/EMBL/DDBJ accession number for the *qnrB24* sequence of *Citrobacter freundii* is HM192542.

4 % of *Escherichia coli* isolates. PMQR genes were significantly more prevalent in *Enterobacteriaceae* (Robicsek *et al.*, 2006a; Ma *et al.*, 2009). Jacoby *et al.* (2008) found various *qnr* genes differing from each other by ≥ 40 % in nucleotide sequence, identified in different species of *Enterobacteriaceae*. Within each family, minor (≤ 10 %) variation in sequence has defined a growing number of alleles (Jacoby *et al.*, 2008). The Qnr proteins belong to the pentapeptide repeat family, which protects the DNA gyrase and topoisomerase IV from inhibition by quinolones. The aminoglycoside acetyltransferase variant AAC(6')-Ib-cr is capable of acetylating and subsequently reducing the activity of norfloxacin and ciprofloxacin. The recently described fluoroquinolone-specific efflux pump protein QepA is involved in pumping fluoroquinolones out of bacterial cells (Yamane *et al.*, 2007). In recent years, these PMQR genes have been found in various bacteria.

Citrobacter freundii, a pathogen that is increasingly frequently causing nosocomial infections, is becoming multiresistant. Park *et al.* (2007) found that the prevalence of *qnr* genes was 38.4 % in 138 *C. freundii* isolates, whilst Bae *et al.* (2010) found *qnrB22* and *qnrB23* genes in *Citrobacter werkmanii* and *C. freundii*. These studies found that *C. freundii* often has PMQR determinants and some confer resistance to multiple drugs, and that *qnr* genes and other determinants are widely distributed in human clinical isolates of the family *Enterobacteriaceae* around the world (Park *et al.*, 2007). The current study was thus designed to investigate the prevalence and transmission of PMQR determinants among *C. freundii* in Anhui province, PR China.

METHODS

Strains, plasmids and growth conditions. In total, 31 *C. freundii* strains were isolated from 2009 to 2010 at the First Affiliated Hospital of Anhui Medical University, PR China. The azide-resistant *E. coli* J53 strain (*E. coli* J53Az^R) was chosen as the recipient strain for conjugation experiments. Strains were routinely grown in Luria–Bertani broth. Culture plates contained Mueller–Hinton agar.

Susceptibility testing. Antimicrobial susceptibility to various antibiotics was determined by agar dilution on Mueller–Hinton agar plates, according to Clinical and Laboratory Standards Institute guidelines (CLSI, 2009a, b). Quality control for MICs was performed using the reference strain *E. coli* ATCC 25922. The MICs of ciprofloxacin and levofloxacin were determined using Etest strips according to the manufacturer's instructions.

PCR amplification and sequencing. All the isolates were screened for the genes *qnrA*, *qnrB*, *qnrS*, *qnrC*, *qnrD*, *aac(6')-Ib-cr* and *qepA* by multiplex PCRs, using the primers shown in Table 1. Positive and negative controls were included. Positive PCR products were confirmed by sequence analysis. Sequences were analysed using the DNASTAR software package and compared with sequences deposited in GenBank.

Transfer of PMQR genes and plasmid characterization. The mechanism of PMQR transfer from *qnr*-positive *C. freundii* isolates to *E. coli* J53Az^R was examined by conjugation experiments

(Martínez-Martínez *et al.*, 1998). To avoid the development of spontaneous chromosomal mutations to fluoroquinolone resistance in the recipients, transconjugants were selected on Mueller–Hinton agar plates containing ampicillin (64 µg ml⁻¹) with sodium azide (200 µg ml⁻¹). Conjugation experiments were carried out at 37 °C. The transconjugant isolates were also screened by PCR for *qnr* genes. Plasmid DNA was extracted from transconjugants and subjected to electrophoresis on a 0.9 % (w/v) agarose gel with ethidium bromide at 120 V for 1 h. The size of plasmid was estimated by comparison with plasmid molecular size standards from *E. coli* strain V517.

PFGE. PFGE analysis of *Xba*I-digested (Promega) genomic DNA was performed (Gautam, 1997). The results of PFGE were analysed according to Tenover *et al.* (1995).

RESULTS AND DISCUSSION

Screening for the PMQR gene

The prevalence of *qnr* genes among the screened isolates was 25.8 % (8/31) in this study. *qnrA1*, *qnrB1*, *qnrB2*, *qnrB4*, *qnrB10* and *qnrB24* were present in 6.5, 3.2, 6.5, 3.2, 3.2 and 3.2 % of *C. freundii* isolates, respectively. Other PMQR genes were negative in these isolates. Sequence analysis revealed that the *qnrB* gene of isolate 3 had a maximal nucleotide identity of 96.7 % compared with the *qnrB10* gene of *C. werkmanii* (GenBank accession no. EU136184). Three nucleotide changes were observed. The C→A change at nt 139 resulted in Leu→Met, C→T at nt 257 produced an Ala→Val change, and A→G at nt 670 produced an Ile→Val change. Hence, we propose that *qnrB* is designated *qnrB24*, a new variant of the *qnrB* gene, in accordance with the *qnr* nomenclature rules of Jacoby *et al.* (2008).

Results of PFGE

The PFGE patterns of the eight PMQR-positive isolates appeared to be different from each other, which suggested that these isolates were not clonally related.

Conjugation experiments and plasmid analysis

qnr genes were successfully transferred from three clinical isolates (isolates 4, 13 and 23) to their transconjugants. Transconjugants 4 and 13 with the *qnrA1* gene harboured a single plasmid of ~11 kb, whilst transconjugant 23 with the *qnrB4* gene contained a plasmid of ~64 kb. Among these *qnr*-positive isolates, *qnrA* genes could be transferred by conjugation to the recipient *E. coli* J53Az^R (2/2, 100 %). In contrast, *qnrB* genes were transferred successfully in only one isolate for *qnrB4* (1/6, 16.7 %).

Antimicrobial susceptibility testing

In addition to being resistant to quinolones, *qnr*-positive clinical isolates were also resistant to various antibiotics, including aminoglycosides and β-lactams. Isolates 4, 5, 7, 12, 13, 22 and 23 had a ciprofloxacin MIC of 8–64 µg ml⁻¹,

Table 1. Primers used for PCR detection of PMQR genes

Gene	Primer sequence (5'→3')*	Annealing temperature (°C)	Amplicon size (bp)
<i>qnrA1-6</i>	F: TCGGCAAAGGTCAGGTCACAGC R: GCAAGAGGATTTCTCACGCCAGGAT	50	479
<i>qnrB1</i> group	F: GGCTCTGGCACTGGCACTCGTT R: CCCTTTCTGGCTGGCTTTTCAC	50	157
<i>qnrB4</i> group	F: ATCACCACCCGCACCT R: TCTAAATCGCCAGTTCC	50	242
<i>qnrB5</i> group	F: GCCTTGGGCATTGAA R: CCTAACTCCGAATTGGTC	50	305
<i>qnrB8</i> group	F: AGCCAGAAAGGGTGAAT R: ATCGAACCTAACG	50	318
<i>qnrS1</i> and <i>qnrS3</i>	F: CACTTTGATGTCGCAGAT R: CAACACTACCCAGTGCTT	50	471
<i>qnrS2</i>	F: ATCGCTCCATGGGACTC R: AACAATACCCAACGCTTC	50	216
<i>qnrA-T</i>	F: TTGAGCGGTAAACGAGTGAG R: GCAACACCGACAGGGAT	50	823
<i>qnrB-T</i>	F: TTGAATTACGGTTTCAGCCCCTAC R: TTGGATCCAACGCACATTTGCAGATGTTAT	50	875
<i>qnrC</i>	F: GAATTATTCCATAAAACG R: GCTCCCAAAAGTCATC	55	509
<i>aac(6')-Ib-cr</i>	F: TTGCGATGCTCTATGAGTGG R: CTCGAATGCCTGGCGTGTTT	51	478
<i>qepA</i>	F: GCAGGTCCAGCAGCCGGTAG R: CTCGAATGCCTGGCGTGTTT	55	218
<i>qnrD</i>	F: CGAGATCAATTTACGGGAATA R: AACAAGCTGAAGCGCCTG	55	582

*F, Forward; R, reverse.

whilst isolate 7 had a ciprofloxacin MIC of $1 \mu\text{g ml}^{-1}$. The overall resistance rate of all *qnr*-positive isolates to ciprofloxacin was 87.5 %.

The percentages of isolates with intermediate resistance or resistance to cefotaxime, amikacin, ceftazidime, cefepime and gentamicin were 75, 37.5, 62.5, 37.5 and 87.5 %, respectively. All isolates were susceptible to imipenem (Table 2). All transconjugants showed reduced susceptibility to fluoroquinolones compared with the recipient *E. coli* J53Az^R. The *qnr*-containing transconjugants conferred decreased susceptibility to ciprofloxacin (MIC range 0.19–1.0 $\mu\text{g ml}^{-1}$) and levofloxacin (MIC range 0.19–0.75 $\mu\text{g ml}^{-1}$), representing 10–23-fold increases compared with those of the recipient, which had a ciprofloxacin MIC of 0.016 $\mu\text{g ml}^{-1}$ and a levofloxacin MIC of 0.032 $\mu\text{g ml}^{-1}$. Some were also resistant to other antimicrobial agents. The co-transferred plasmid-mediated resistance to non-fluoroquinolone antimicrobial agents is shown in Table 2.

This study examined the plasmid-mediated mechanisms of fluoroquinolone resistance in 31 *C. freundii* isolates obtained from Anhui, PR China. Our results found that the prevalence of *qnr* genes was 25.8 %. The *qnrA* gene found was *qnrA1*, whilst the *qnrB* genes comprised *qnrB1*, *qnrB2*, *qnrB4*, *qnrB10* and *qnrB24* in *C. freundii*. These results are

consistent with conclusions in previous studies (Park *et al.*, 2007). In this study, we found a novel *qnrB* variant, *qnrB24*, and this is also the first report, to our knowledge, of the presence of *qnrB10* in *C. freundii* in Anhui, PR China. We also found that the MICs of fluoroquinolones for clinical isolates were higher than those for their transconjugants. Many studies have indicated that chromosomal quinolone resistance-determining region (QRDR) mutations in *gyrA* and *parC* play an important role in mediating high-level quinolone resistance (Eaves *et al.*, 2004). Fang *et al.* (2009) found that *Enterobacteriaceae* harbouring *qnrB4* or *qnrB6* were highly resistant to all the fluoroquinolones tested. This high-level resistance was associated with multiple chromosomal substitutions in *gyrA* and *parC*. Alterations at codons Ser83 and Asp87 in GyrA and at codons Ser80 and Glu84 in ParC were observed in these isolates. In fact, some reports (Martínez-Martínez *et al.*, 2003; Tran *et al.*, 2005) have suggested that *qnr* confers only low-level resistance to quinolone and that its main contribution is to facilitate the development of QRDR mutations. The mechanism of action of the QRDR might cause various MICs of fluoroquinolone in *qnr*-positive isolates and transconjugants. Previous studies have also shown variable *qnr* expression in transconjugants, and variable promoter strengths can account for differences in MICs (Xu *et al.*, 2007). In this study, we found that one

Table 2. Resistance profiles of clinical *C. freundii* isolates, the recipient strain *E. coli* J53Az^R and transconjugants

CIP, Ciprofloxacin; LVX, levofloxacin; CL, chloramphenicol; PIP, piperacillin; CZ, cefazolin; CAZ, ceftazidime; CTX, cefotaxime; FEP, cefepime; IMP, imipenem; GEN, gentamicin; AMK, amikacin.

Strain (gene)	MIC ($\mu\text{g ml}^{-1}$)										
	CIP	LVX	CL	PIP	CZ	CAZ	CTX	FEP	IMP	GEN	AMK
Isolate 3 (<i>qnrB24</i>)	8	32	128	>128	128	>128	128	64	1	32	64
Isolate 4 (<i>qnrA1</i>)	32	32	8	64	128	128	>128	4	0.5	64	16
Isolate 5 (<i>qnrB1</i>)	8	1	128	128	128	2	2	4	0.5	16	16
Isolate 7 (<i>qnrB2</i>)	1	4	64	>128	128	16	8	0.0625	0.5	0.125	2
Isolate 12 (<i>qnrB2</i>)	32	64	16	64	128	16	32	16	0.5	16	16
Isolate 13 (<i>qnrA1</i>)	64	32	32	>128	>128	16	64	4	0.5	64	8
Isolate 22 (<i>qnrB10</i>)	8	4	32	128	8	4	8	2	2	128	32
Isolate 23 (<i>qnrB4</i>)	64	32	>128	64	>128	16	64	16	1	64	32
<i>E. coli</i> J53Az ^R	0.016*	0.032*	8	2	2	0.25	0.0625	0.0625	0.25	0.25	0.5
Transconjugant 4	0.38*	0.38*	8	64	128	64	128	4	1	2	16
Transconjugant 13	0.19*	0.19*	8	128	16	16	32	4	0.25	64	8
Transconjugant 23	1.0*	0.75*	128	64	>128	16	64	8	0.25	64	16

*Etest results.

isolate did not confer the ciprofloxacin resistance phenotype, indicating that using quinolone or fluoroquinolone break-points as markers for detection might underestimate the prevalence of PMQR. This finding could have clinical implications, as *qnr* genes might promote further selection from low- to high-level resistance when quinolones are used. Conjugation experiments suggested the occurrence of horizontal mobility of the *qnr* genes. Susceptibility testing of all the transconjugants demonstrated the transfer of quinolone resistance and hence plasmid-mediated resistance determinants. The *qnr* genes were located in conjugative plasmids, so the genes could increase bacterial resistance to fluoroquinolones through the transmitted plasmid. As a method for studying epidemics, the PFGE results showed that these isolates were not clonally related. The plasmids carrying *qnr* genes varied widely in size and associated multiresistance. The transconjugant susceptibility testing results showed they were not only resistant to fluoroquinolones but also to β -lactams, aminoglycosides and other antibiotics. Genes for *qnrA* and sometimes *qnrB* have been found as part of complex *sulI*-type integrons containing a presumed recombinase gene, *orf513* (Robicsek *et al.*, 2006a). The *qnr* gene is probably aided by its genetic linkage to resistance determinants for various other antibiotics. A similar result was found in the results of Corkill *et al.* (2005). Two possible causes for the resistance change are as follows: (i) *qnr*-positive isolates often carry extended-spectrum β -lactamase or *ampC* genes; and (ii) the *qnr* gene is often located on plasmids or integrons that include the genes *aac*, *cat*, *ampR*, *aar*, *dfr* or *aad* (Li, 2005).

In conclusion, we believe this to be the first description of plasmid-mediated fluoroquinolone resistance in *C. freundii* isolates from Anhui province, PR China. In this survey, *qnr* genes, especially *qnrB*, were prevalent in *C. freundii* isolates

from Anhui province, together with resistance to various other antimicrobial agents. We also found a new variant of *qnrB*, *qnrB24*. We intend to study the function of *qnrB24* and its genetic environment further in the future.

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