Novel variants of the *qnrB* gene, *qnrB31* and *qnrB32*, in *Klebsiella pneumoniae*

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Quinolone resistance in the family *Enterobacteriaceae* is mostly attributed to the accumulation of mutations in the bacterial enzymes targeted by fluoroquinolones: DNA gyrase and DNA topoisomerase IV. Here we isolated the *Klebsiella pneumoniae* strains KP3606 and KP4707 from different specimens from 2008 to 2010 in Taizhou Municipal Hospital of China, and discovered a new subtype *qnrB31*, for which the GenBank accession number is HQ418999, and another new subtype *qnrB32*, for which the GenBank accession number is HQ704413. Susceptibility testing showed that KP3606 had a reduced susceptibility ($\text{MIC} \geq 0.5 \mu\text{g ml}^{-1}$) to quinolones, while KP4707 was resistant to quinolones. Of all *qnrB* alleles, the novel variants the *qnrB32* gene and *qnrB31* gene have the highest amino acid identity. The results suggested that of all the various genes involved in resistance to quinolones, the *qnrB* gene is the most likely to be mutated, and plasmids might play a role in the dissemination and evolution of *qnrB* genes.

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INTRODUCTION

Quinolones are synthetic broad-spectrum antibiotics used widely to treat a variety of infectious diseases. Bacterial resistance to quinolones emerged rapidly after their widespread use. In Gram-negative bacteria, quinolone resistance was for a long time considered to be entirely mediated by mutations in chromosomal genes encoding quinolone targets (that is, DNA gyrase and topoisomerase IV) and/or in regulatory genes of outer-membrane proteins or efflux pumps (Jacoby, 2005).

The first plasmid-encoded quinolone resistance gene (*qnr*) was discovered in a *Klebsiella pneumoniae* isolate from Birmingham, Alabama, USA, collected in 1994. It occurred in a multi-resistance plasmid, pMG252, in an integron-like structure near *orf513*. Qnr, the gene product, is a member of the pentapeptide repeat protein (PRP) family, and has been shown to block the action of ciprofloxacin on purified DNA gyrase and topoisomerase IV (Tran & Jacoby, 2002; Tran *et al.*, 2005). Qnr plasmids have been found in clinical isolates of *Citrobacter freundii*, *Enterobacter* spp., *Escherichia coli*, *K. pneumoniae*, *Providencia stuartii* and *Salmonella* spp., from the United States, Europe, and the Near and Far East

(Cheung *et al.*, 2005; Nordmann & Poirel, 2005). Another *qnr* gene, *qnrS*, has also recently been found in a plasmid from a strain of *Shigella flexneri* isolated in Japan (Kim *et al.*, 2010). Since then, *qnr* alleles have been discovered worldwide in clinical strains of Gram-negative bacilli. The Qnr proteins confer quinolone resistance, and belong to the PRP family (Guo *et al.*, 2010).

Plasmid-encoded quinolone resistance has been described along with three mechanisms: (i) a quinolone-protective mechanism encoded by the *qnr* genes; (ii) a modifying enzyme, *aac(60)-Ib-cr* (Robicsek *et al.*, 2006a); and (iii) an efflux pump encoded by the *qepA* gene (Périchon *et al.*, 2007).

Recently, plasmid-mediated quinolone resistances (PMQRs) have been reported worldwide in unrelated enterobacterial species associated with mobile elements (Robicsek *et al.*, 2006b). Quinolones and β -lactams are among the most commonly used antimicrobials in human therapeutics. PMQR determinants confer low-level resistance, but their presence could potentially facilitate the evolution of the bacterial host toward higher levels of resistance by mutational alterations in type II topoisomerases. PMQR determinants are often combined with extended-spectrum β -lactamases suggesting the potential for selection of PMQR by the use of β -lactams and of β -lactam resistance by the use of quinolones (Strahilevitz *et al.*, 2009; Guillard *et al.*, 2010).

Abbreviations: PMQR, plasmid-mediated quinolone resistance; PRP, pentapeptide repeat protein.

The GenBank/EMBL/DDBJ accession numbers for the genomic DNA sequences of *qnrB31* and *qnrB32* are HQ418999 and HQ704413, respectively.

Here, we researched the distribution and characteristics of *K. pneumoniae* PMQR *qnrB* genes, and characterized *qnrB* genes, *qnrB31* and *qnrB32*.

METHODS

Strains. A total of 72 isolates of *K. pneumoniae* were isolated from different specimens from 2008 to 2010 in Taizhou Municipal Hospital, China. Two isolates, KP3606 and KP4047, were found to exhibit resistance to quinolone and cephalosporin. The isolates were further used for susceptibility testing.

Conjugation and susceptibility testing. Conjugation experiments were carried out in Luria–Bertani (LB) broth with *E. coli* J53 Azide^R (a strain with resistance to sodium azide) as the recipient, a PCR positive strain was used as the donor strain (Wang *et al.*, 2003). Cultures of donor and recipient cells in exponential phase (0.5 ml of each) were added to 4 ml fresh LB broth and incubated overnight without shaking. Transconjugants were selected on trypticase soy agar plates supplemented with 300 mg sodium azide l⁻¹ and 0.03 mg ciprofloxacin l⁻¹, and incubated for 18–24 h at 35 °C. The susceptibility was assessed by MIC values. To determine if quinolone resistance was co-transferred, the MICs for antibiotics for the donor, recipient and transconjugant strains were compared (Kim *et al.*, 2010). The MICs were determined by broth dilution and interpreted according to Clinical and Laboratory Standards Institute (formerly the National Committee for Clinical Laboratory Standards) guidelines (NCCLS, 2003).

PCR amplification and sequencing. To carry out the genetic characterization of the *qnrB* allele gene in *K. pneumoniae* and its transconjugants (KP3606–ECJ53 Azide^R and KP4707–ECJ53Azide^R transconjugants), *qnrB31* and *qnrB32* were amplified by PCR, using primers F 5'-ATGTTGTCATTACTGTATA-3' and R 5'-CTAA CCAATCACAGCGATG-3', and sequencing was carried out using primers F 5'-ATG ACGCCATTACTGTATAAAAAA-3' and R 5'-CTAGCCAATAATCGCGATGCCA-3'. Transconjugants (KP3606–ECJ53 Azide^R and KP4707–ECJ53Azide^R transconjugants) were amplified and sequenced by PCR using primers F 5'-ATGACTC-TGGCGTTAGTTGG-3' and R 5'-TTAACCCATGACAGCGATAC-CAA-3', then the sequencing results were assembled to obtain the full-length nucleotides of *qnrB31* and *qnrB32* in *K. pneumoniae* and its transconjugants. Three pairs of primers were designed internal to the *qnrB* ORF, which allowed easy detection of the mutant alleles.

Gene mapping. To study the plasmids carrying the *qnrB31* and *qnrB32* genes, plasmid DNA was extracted and separated by 0.6% agarose gel electrophoresis, and then different sized fragments were cut out of the gel and purified. PCR was conducted to amplify *qnrB* gene using this plasmid DNA as the template, where the initial position of the plasmid *qnrB* gene was determined, and size of the plasmid DNA estimated (Wang *et al.*, 2003). The presence of PMQR determinants and quinolone resistance-determining region mutations

was investigated by PCR-based detection and sequencing (Cano *et al.*, 2009; Eaves *et al.*, 2004; Jeong *et al.*, 2004).

RESULTS

Susceptibility testing

The conjugation experiments of *qnrB31* gene were carried out nine times while *qnrB32* experiments were carried out six times until successful. The susceptibility testing results showed that the MIC values for quinolones for the transconjugants (KP3606–ECJ53 Azide^R and KP4707–ECJ53 Azide^R transconjugants) were higher than that of *E. coli* J53 Azide^R. Furthermore, the MIC values for quinolones for the transconjugant carrying *qnrB32* (KP4707–ECJ53 Azide^R transconjugant) was higher than that for the transconjugant harbouring *qnrB31* (KP3606–ECJ53 Azide^R transconjugant). Conjugation experiments were successful and indicated that quinolone resistance was caused by the plasmid-encoded gene. Characterization of the *qnrB*-harbouring plasmids is shown in Fig. 1.

The transconjugate isolates of *K. pneumoniae*/E. coli showed resistance to ofloxacin, levofloxacin, ciprofloxacin and nalidixic acid. *K. pneumoniae* KP3606 showed a reduced susceptibility (MIC ≥ 0.5 µg ml⁻¹) to quinolones (nalidixic acid, ofloxacin, levofloxacin and ciprofloxacin) (Table 1), while *K. pneumoniae* KP4707 was resistant to quinolones (Table 1).

qnrB gene alleles and gene mapping

Sequence analysis identified two novel *qnrB* variants, *qnrB31* (GenBank accession number HQ418999) and *qnrB32* (GenBank accession number HQ704413), in *K. pneumoniae*. The new variant *qnrB31* allele was assigned according to the *qnr* numbering scheme shown on the Lahey website (<http://www.lahey.org/qnrStudies>), and the new variant *qnrB32* allele sequence was released to the public on 22 February 2011 by GenBank. Based on gene mapping, there were three different length plasmids in isolates of KP3606 and KP4707. The *qnrB31* and *qnrB32* genes were separately located in an about 23.1 kb length plasmid.

Amino acid point mutation analysis

The amino acid point mutations associated with quinolone resistance were detected in the two isolates (Table 1). Six

Fig. 1. Characterization of the *qnrB*-harbouring plasmids. The -10 promoter element is indicated; the +1 start site is represented by an arrow. The start of the *qnrB* coding sequence is indicated by dashed lines and the consensus sequence of the LexA-protein-binding site is boxed. The LexA-protein-binding site was the determining feature of fluoroquinolone resistance. If the LexA-protein-binding site was present then strains were sensitive to fluoroquinolone, and vice versa.

Table 1. Characteristics of the two *K. pneumoniae* isolates and of the *E. coli* J53 Azide^R clone harbouring the *qnrB31* and *qnrB32* genes

Strain	Amino acid point mutations*	PMQR determinant	MIC (µg ml ⁻¹)†			
			NAL	OFL	LVN	CIP
<i>K. pneumoniae</i>						
KP3606	Arn27→Leu Ser79→Ala Arg87→Ser Ile142→Met Gly188→Arg Val212→Ile	<i>qnr31</i>	>256	16	8	4
KP4707	Ser79→Ala Val129→Ala Ile142→Met Gly188→Arg Val212→Ile	<i>qnr32</i>	>256	32	16	8
Transconjugants						
KP3606-ECJ53Azide ^R transconjugant		<i>qnr31</i>	16	0.5	0.125	0.0625
KP4707-ECJ53Azide ^R transconjugant		<i>qnr32</i>	32	2	0.25	0.125
<i>E. coli</i> J53 Azide ^R		—	2	0.0039	0.00195	0.00195

*Amino acid point mutations are compared with *qnrB31* and *qnrB32* (each other).

†NAL, Nalidixic acid; OFX, ofloxacin; LVX, levofloxacin; CIP, ciprofloxacin.

amino acid mutations were observed in the *qnrB31* gene, they were Arn27→Leu, Ser79→Ala, Arg87→Ser, Ile142→Met, Gly188→Arg and Val212→Ile when compared with the amino acid sequence of *qnrB1*. Five point mutations were observed in the *qnrB32* gene (Ser79→Ala, Val129→Ala, Ile142→Met, Gly188→Arg and Val212→Ile) compared with the amino acid sequence of *qnrB1*.

The *qnrB31* gene had 91.6 % nucleotide identity with *qnrB1*. The *qnrB32* gene had 90.3 % nucleotide identity with *qnrB1*. The *qnrB31* gene had 97.1 % nucleotide identity with *qnrB32*. The *qnrB* allele base sequence indicated that *qnrB32* (HQ704413) and *qnrB31* (HQ418999) were homogeneous, with 100 support value.

DISCUSSION

This study was carried out to determine the prevalence of *qnr* genes among 72 consecutive non-repetitive *Enterobacteriaceae* of patient origin and to characterize the positive isolates. The presence of PMQR determinants and quinolone resistance-determining region mutations was investigated, and the *qnrA*, *qnrS*, *qnrC*, *qnrD*, *aac(6')-Ib-cr* and *qepA* genes were not found. Two isolates of *K. pneumoniae*, KP3606 and KP4707, were found to carry *qnr*-like genes that were not found in *E. coli*. Sequence analysis identified two novel *qnrB* variants, named *qnrB31* (HQ418999) and *qnrB32* (HQ704413).

Plasmid-encoded genes, such as *qnr*, may facilitate the spread and increase of the prevalence of quinolone-resistant strains. To date, *qnr* genes have been widely evaluated in southern and eastern Asia, North and South America, and Europe (Martínez-Martínez *et al.*, 1998; Tran & Jacoby, 2002), and the *qnr* gene has been shown to block the action of ciprofloxacin on purified DNA gyrase and topoisomerase IV (Tran & Jacoby, 2002; Cattoir *et al.*, 2008; Tran *et al.*, 2005). *qnrB* provides low-level resistance to quinolones and belongs to the PRP family, one member of which has recently been shown to have a DNA-like structure that would allow it to mimic DNA as a substrate for DNA gyrase (Jiang *et al.*, 2008; Hegde *et al.*, 2005). For a protein in which overall structure is important rather than catalytic activity, considerable amino acid variability may be permissible.

An integrated *qnrB* gene sequence included three parts as follows: a promoter sequence (−35 to −10 region), the consensus sequence of the LexA-protein-binding site and the *qnrB* coding sequence. If any antibiotic can open the consensus sequence of the LexA-protein-binding site, promoter sequence and *qnrB* coding sequence this will result in quinolone resistance. Therefore, of these three parts, the presence of the consensus sequence of the LexA-protein-binding site was the key to quinolone resistant (Da Re *et al.*, 2009; Wang *et al.*, 2009). The LexA-protein-binding site was determining fluoroquinolone resistance. If the LexA-protein-binding site was present then those

strains were sensitive to fluoroquinolone, and vice versa. In our analysis, the strains were quinolone resistant, which might indicate the consensus sequence of the LexA-protein-binding site was subject to autoproteolytic cleavage leading to quinolone resistance.

On induction of the SOS response, by ciprofloxacin for example, ssDNA is produced and the co-protease activity of the RecA protein is activated by binding to ssDNA. The interaction between LexA and the nucleoprotein filament RecA/ssDNA results in autoproteolytic cleavage of LexA and subsequently in *qnrB* derepression. Induced expression of *qnrB* leads to an increase in the ciprofloxacin MIC (Da Re *et al.*, 2009).

Mutation in the gene sites induced bacterial quinolone resistance. Abuse of quinolone antibiotics could transmit to the next generation and lead to large numbers of quinolone-resistant bacteria. Therefore, clinical quinolone antibiotics could not be applied to infection. However, these phenomena might be temporary. If the use of quinolone antibiotics was reduced, the mutated genes could self-repair after several generations, then the rate of quinolone-resistant bacteria would be reduced.

In summary, the quinolone resistance genes *qnr31* and *qnr32* led to multi-drug resistance in *K. pneumoniae* KP4707 and KP3606. The abuse of broad-spectrum antibiotics would induce the emergence of resistant genes easily, resulting in multi-drug resistant bacteria, and attention should be paid to this.

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