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Coexistence of *qnrB4* and *qnrS1* in a clinical strain of *Klebsiella pneumoniae*[1]

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Key words

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Abstract

Aim: To identify the location and the relationship, and to analyze the genetic background of 2 plasmid-mediated quinolone resistance genes, *qnrB4* and *qnrS1*, carried by a clinical strain of *Klebsiella pneumoniae* (*K pneumoniae*). **Methods:** The plasmids carrying *qnrB4* or *qnrS1* were identified by Southern blotting. A *Hin*dIII fragment containing *qnrB4* or *qnrS1* was cloned into plasmid puc18 and sequenced. **Results:** *qnrB4* and *qnrS1* were located on 2 different plasmids, pHS7 and pHS8, and were 180 and 45 kb in size, respectively. A transconjugant carrying plasmid pHS7 bearing *qnrB4* and another transconjugant carrying pHS9 bearing *qnrB4* and *qnrS1* were obtained by conjugation. Plasmid pHS8 bearing *qnrS1* was also transferred to J53 by transformation. The ciprofloxacin minimal inhibitory concentrations (MIC) for J53 transconjugants or the transformant carrying *qnrB4* only, *qnrS1* only, and both *qnrB4* and *qnrS1* were 0.19, 0.25, and 0.25 mg/L, respectively, while the parent clinical strain of *K pneumoniae* had a MIC of 0.75 mg/L. *qnrB4* was located in a *sul1*-type integron with $bla_{\text{DHA-1}}$, *ampR* and *psp* genes in upstream and insertion sequence IS26, and *sap* genes in downstream of *qnrB4*. *qnrS1* was not located in an integron, but IS26 was found both upstream and downstream, and IS2 was found directly upstream of *qnrS1*. **Conclusion:** *qnrB* and *qnrS* can be harbored simultaneously by a single clinical strain of *K pneumoniae*. These 2 genes are carried by 2 different plasmids and have different genetic environments in plasmid DNA structure.

Introduction

Three plasmid-mediated quinolone resistance genes, *qnrA*[1], *qnrB*[2], and *qnrS*[3], have been discovered since 1998. At least 6 *qnrA*, 6 *qnrB*, and 2 *qnrS* variants have been described[4]. *qnrA* was found in most common Enterobacteriaceae, including *Escherichia coli* (*E coli*), *Klebsiella* spp, *Enterobacter* spp, *Citrobacter freundii*, and *Providencia stuartii* [4], and it was located in complex *sul1*-type class 1 integrons[5]. *qnrB* and *qnrS* were identified in clinical strains of *Klebsiella pneumoniae* (*K pneumoniae*), *E coli, Enterobacter* spp, and *Salmonella* spp [4,6–9]. *qnrB* and *qnrS* were also detected in *Citrobacter koseri*[2]and *Serratia marcescens*[7], respectively.

Interestingly, *qnrB* and *qnrS* could be detected simultaneously in a few clinical strains[8, 9]. Among 28 *qnrA* positive clinical strains of Enterobacteriaceae, 7 strains also harbored *qnrS*[10]. A further study was only carried out on a single strain of *Enterobacter cloacae* carrying 2 *qnr* genes simultaneously[9].

In China, *qnrA* has been detected in 8% of 78 clinical isolates of *E coli*[5], but *qnrB* and *qnrS* have not yet been reported in clinical strains. We found a clinical strain of *K pneumoniae* carrying both *qnrB4* and *qnrS1*. The aim of this study was to identify the location and the relationship of the 2 genes, to analyze the genetic background of the *qnrB4* and *qnrS1* genes, and to evaluate their respective roles in the production of quinolone resistance.

Materials and methods

Bacterial strains A clinical strain of *K pneumoniae* carrying *qnrB4* and *qnrS1* was found during the study on extended-spectrum β-lactamases. The strain was isolated from sputum of an inpatient with acute exacerbation of chronic bronchitis at a teaching hospital in Shanghai in 2005. The patient was treated with cefradine, cefotaxime and levofloxacin prior to the isolation of *K pneumoniae*. Additional strains used were *E coli* V517[1]; *E coli* J53, containing plasmid R27[5] as standards for plasmid size; *E coli* J53Az^R (resistant to azide)[5] as a recipient for conjugation; and *E coli* DH5α, which was used in cloning.

PCR detection The *qnr* genes (*qnrA, qnrB, and qnrS*), class A β-lactamase genes (*bla*$_{TEM}$, *bla*$_{SHV}$, *bla*$_{PER}$, *bla*$_{VEB}$, *bla*$_{SFO}$, and *bla*$_{CTX}$), and class C plasmid-mediated *ampC* β-lactamase genes were detected by PCR with specific primer sets in the clinical strain, transconjugants, and transformant. The primers used for *qnrA*, *qnrB*, and *qnrS* were 5'-GGG TAT GGA TAT TAT TGA TAA AG-3' and 5'-CTA ATC CGG CAG CAC TAT TA-3', 5'-ATG ACG CCA TTA CTG TAT AA-3' and 5'-GAT CGC AAT GTG TGA AGT TT-3', 5'-ACG ACA TTC GTC AAC TGC AA-3' and 5'-TAA ATT GGC ACC CTG TAG GC-3', respectively. The PCR conditions were 94°C for 45 s, 56°C for 45 s, and 72°C for 1min, and cycled 30 times for the detection of *qnr* genes. The primers used for CTX-M were 5'-AGT GCA AAC GGA TGA TGT-3' and 5'-GGC TGG GTA AAA ATA GGT C-3'. The primers for the *ampC* genes were previously described by Perez-Perez and Hanson[11] . All positive results were confirmed by direct sequencing of the PCR products on both strands.

Transfer of quinolone resistance Conjugation experiments were carried out in Luria-Bertani broth with azide-resistant *E coli* J53 as the recipient, as previously described[5] . Transconjugants were selected on Trypticase soy agar (TSA) plates containing sodium azide (200 mg/L) for counterselection and ampicillin (100 mg/L) or cefotaxime (8 mg/L) to select for plasmid-encoded resistance. Four hundred colonies were picked from the selection plates and detected by PCR for *qnrB4* and *qnrS1*.

Transformation was performed for the *qnrS1*-bearing plasmid, which could not be transferred by conjugation. Plasmid DNA was extracted from the parent *K pneumoniae* strain using the QIAGEN plasmid midi kit (QIAGEN GmbH, Hilden, Germany) and introduced into electrocompetent *E coli* DH5α by electroporation. Colonies were selected on ciprofloxacin (0.06 mg/L). The colony carrying only 1 plasmid and harboring *qnrS1* was confirmed with PCR. The *qnrS1*-bearing plasmid DNA was extracted again from the colony and introduced into *E coli* J53 by electroporation. Colonies were selected on plates containing 50 mg/L ampicillin, and were also confirmed carrying *qnrS1* with PCR.

The plasmid size was estimated by agarose gel electrophoresis, as previously described[5] ; the presence of *qnrB4* and *qnrS1* were confirmed with Southern blot hybridization using the DIG nucleic acid detection kit (Roche Applied Science, Mannheim, Germany).

***In vitro* susceptibility testing** Minimal inhibitory concentrations (MIC) for the donor, recipient, transconjugant, and transformant strains were measured by agar dilution in accordance with the guidelines of the Clinical Laboratory Standards Institute (CLSI)[12] for ciprofloxacin, amikacin, ampicillin, cefepime, cefotaxime, ceftazidime, gentamicin, nalidixic acid, levofloxacin, sulfamethoxazole, and trimethoprim. The Etest (Biodisk AB, Solna, Sweden) was used to detect minimal changes in ciprofloxacin and levofloxacin susceptibility.

Cloning and nucleotide sequence analysis Plasmid DNA extracted from transconjugant or transformant strains harboring *qnrB4* or *qnrS1* were digested with *Hin*dIII, ligated to pUC18, and introduced into *E coli* DH5α with selection on TSA plates containing 50 mg/L ampicillin. Sequencing was carried out with an ABI Prism 3730 genetic analyzer (Applied Biosystems, Foster City, CA, USA), and was continued by primer walking on both DNA strands. For the sequence comparisons, the NCBI BLAST program (www.ncbi.nlm.nih.gov/blast/Blast.cgi) was utilized.

Nucleotide sequence accession numbers The nucleotide sequences in pHS7 and pHS8 containing *qnrB4* and *qnrS1*, respectively, have been submitted to GenBank and have been assigned accession numbers EF683583 and EF683584, respectively.

Results

PCR detection and MIC determination of a clinical strain of *K pneumoniae* The clinical strain of *K pneumoniae* was identified as containing *qnrB4*, *qnrS1*, *bla*$_{CTX-M-14}$, and *bla*$_{DHA-1}$ genes by PCR and DNA sequencing. The clinical strain was susceptible to quinolones according to the CLSI criteria, with ciprofloxacin and levofloxacin MIC of 0.75 and 1.0 mg/L, respectively; but highly resistant to ampicillin, cefotaxime, ceftazidime, gentamicin, amikacin, sulfamethoxazole, and trimethoprim, and intermediate to cefepime (MIC 16 mg/L; Table 1).

Transfer of quinolone resistance and plasmid characterization *qnrB4* and *qnrS1* were located on 2 different

plasmids, pHS7 and pHS8, and were 180 and 45 kb in size, respectively, by Southern blot hybridization. The *qnrB4*-bearing plasmid pHS7 could be transferred by conjugation to produce a transconjugant *E coli* J53 pHS7. Another transconjugant J53 pHS9 carrying plasmid pHS9 with a size similar to pHS7 bearing both *qnrB4* and *qnrS1*, was also obtained by conjugation. *qnrB4* was detected alone from 380 of 400 (95%) colonies picked from the selection plates by PCR; no *qnrS1* alone was detected; *qnrB4* and *qnrS*1 in combination were detected in 9 of 400 colonies (2.25%). The non-conjugative plasmid pHS8 bearing *qnrS1* was transferred to J53 by transformation.

The ciprofloxacin MIC for J53 transconjugants or transformant carrying *qnrB4* only (J53 pHS7), *qnrS1* only (J53 pHS8), and both *qnrB4* and *qnrS1* (J53 pHS9) were 0.19, 0.25, and 0.25 mg/L, respectively. The MIC of levofloxacin for transconjugant J53 pHS8 and transformant J53 pHS9 were 0.38 and 0.5 mg/L, respectively, which were higher than that of J53 pHS7 (0.19 mg/L; Table 1).

Transconjugant J53 pHS7 was resistant to β-lactam antibiotics (ampicillin, cefotaxime, and ceftazidime), and pHS7 was found harboring $bla_{\text{CTX-M-14}}$ and $bla_{\text{DHA-1}}$ β-lactamase genes. Transformant J53 pHS8, also harboring the $bla_{\text{CTX-M-14}}$ gene, was resistant to ampicillin and cefotaxime, but not to ceftazidime. Comparing the MICs of antimicrobials other than quinolones, we found that the resistance pattern in pHS9 was similar to that of pHS8, except that resistance to gentamicin and amikacin in pHS9, and resistance to sulfamethoxazole, trimethoprim, and ceftazidime in pHS7 was lost in pHS9 (Table 1).

Analysis of plasmid structures The DNA sequences of plasmid pHS7 *Hin*dIII fragment showed that the upstream of *qnrB4* included *sapA* and partial *sapB*, coding for putative peptide transport system permease, and *aphA1*. Notably, insertion sequence IS26 was located between *aphA1* and *sapB*; downstream of *qnrB4* included *psp* operons, coding for putative phage shock proteins, and *ampC* and *ampR* genes located between orf1 and partial *qacEΔ1*. The plasmid structure adjacent to *qnrB4* was similar to that in plasmids, pRBDHA and pMPDHA[13] , 2 *qnrB4* and $bla_{\text{DHA-1}}$-bearing plasmids (GenBank accession numbers AJ971343 and AJ971344, respectively), but found *aphA1* and IS26 in the upstream of *qnrB4* in pHS7 (Figure 1).

The sequence of the immediate region surrounding *qnrS1* in pHS8 was nearly identical to the 3 reported *qnrS1*-bearing plasmids: pAH0376, pINF5, and pK245. Insertion sequence IS26 was found both upstream and downstream of *qnrS1*, and an IS2 was directly located upstream of *qnrS1* (Figure 2).

Discussion

Table 1. Profiles of transconjugants and/or transformants and quinolone resistance recipient *E coli* J53.

Strains	Resistance gene		MIC (mg/L)										
	qnr type	*bla* type	CIP	LEV	NA	AMP	CTX	CAZ	FEP	GEN	AMK	SMZ	TMP
Recipient *E coli* J53	NA	NA	0.008	0.023	4	4	≤0.06	≤0.06	≤0.06	0.125	0.25	16	0.25
Transconjugant J53 pHS7	*qnrB4*	CTX-M-14, DHA-1	0.19	0.19	8	>128	32	32	4	32	>128	>128	>128
Transformant J53 pHS8	*qnrS1*	CTX-M-14	0.25	0.38	16	>128	8	0.5	1	0.25	0.25	16	0.125
Transconjugant J53 pHS9	*qnrB4*, *qnrS1*	CTX-M-14, DHA-1	0.25	0.5	16	>128	16	1	2	32	>128	16	0.125
Donor *K pneumoniae*	*qnrB4*, *qnrS1*	CTX-M-14, DHA-1	0.75	1.0	16	>128	>128	>128	16	>128	>128	>128	>128

AMK, amikacin; AMP, ampicillin; CAZ, ceftazidime; CIP, ciprofloxacin; CTX, cefotaxime; FEP, cefepime; GEN, gentamicin; LEV, levofloxacin; NA, nalidixic acid; SMZ, sulfamethoxazole; TMP, trimethoprim. NA, not applicable.

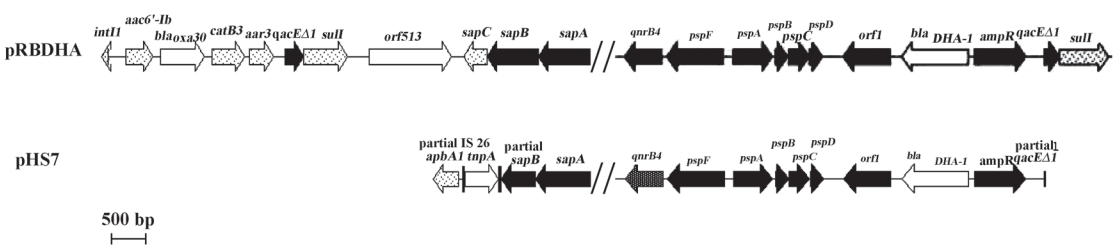

Figure 1. Regions containing the *qnrB4* gene in the conjugative plasmid pHS7 and plasmid pRBDHA.

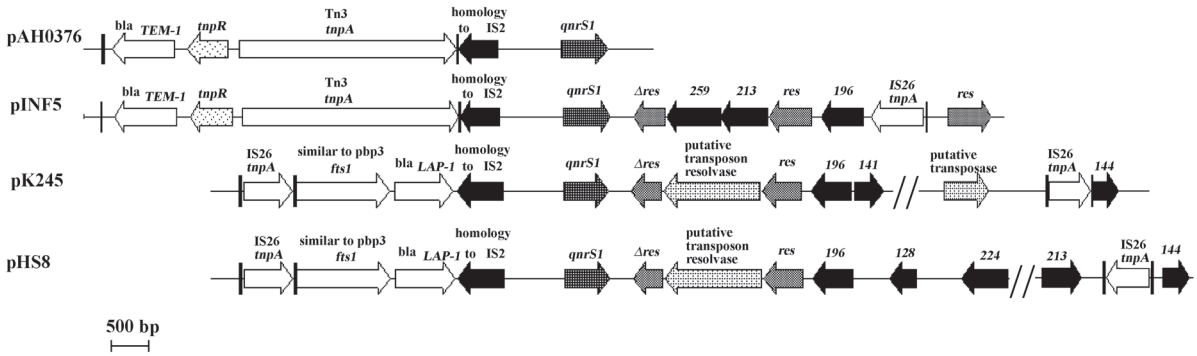

Figure 2. Regions containing the *qnrS1* gene in the non-conjugative plasmid pHS8 and other three plasmids. The arrows marked as 259, 213, 196,141, 144 , 128 and 224 in the map indicate reading frames for hypothetical proteins of 259, 213, 196, 144, 141, 128 and 224 amino acids, respectively.

A clinical strain of *K pneumoniae* was identified as carrying both *qnrB4* and *qnrS1*, and was isolated from a patient with acute exacerbation of chronic bronchitis. *qnrB4* and *qnrS1* were located on 2 separated plasmids, pHS7 and pHS8, respectively. pHS7 could be transferred to *E coli* J53 by conjugation. pHS8 could not be transferred alone by conjugation, but was successfully transferred to J53 by transformation. A recent study on 526 clinical strains isolated in Taiwan indicated that *qnr* genes were highly prevalent in *E cloacae* with a positive rate of 16% (86/526). Both *qnrB2* and *qnrS1* were detected simultaneously in 4 of the 86 *qnr*-positive strains, but there was no further study on the relationship, transferability, and location of the 2 *qnr* genes on these strains[8]. The only study was on an *E cloacae* clinical isolate co-expressing QnrB4 and QnrS1 determinants, isolated in France[9]. *qnrB4* and *qnrS1* were also located on 2 different plasmids, 100 and 160 kb in size, respectively, and could be transferred to *E coli* TOP10 by conjugation or transformation. The ciprofloxacin MIC of transconjugant or transformant carrying *qnrB4* or *qnrS1* were 0.06 and 0.12 mg/L, respectively. In our study, transformant J53 pHS8 carrying *qnrS1* had higher MIC values for ciprofloxacin or levofloxacin (0.25–0.38 mg/L) than that of transconjugant J53 pHS7 carrying *qnrB4* (0.19 mg/L). *qnrS1* conferred higher quinolone MIC than *qnrB4*.

qnrA was located on complex *sul1*-type class 1 integrons, according to several reports[4,5,8]. *qnrB* was also located on *sul1*-type class 1 integrons from 2 reports[6,13]. A genetic environment analysis of *qnrB2* in a *Salmonella enterica* Serovar Keurmassar showed that *qnrB2* was located in a complex *sul1*-type integron which contained 2 class 1 integrons surrounding 2 common regions separated by a partial 3′ conserved segment[6]; the structure was similar to pMG252, the first *qnrA*-bearing plasmid[4]. During the study on the genetic organization of the *ampC* and *ampR* genes in *Morganella morganii* (*M morganii*), a *qnrB4* gene was found to be located upstream of *psp* operons, and this was a complex *sul1*-type integron (*qnrB4* was not labeled in the original figure, as it had not been reported at that time)[13]. In our study, the genetic environment of *qnrB4* was similar to that of *M morganii*, but found *aphA1* and IS26 upstream of *qnrB4*. Partial *qacESΔ1* was found downstream of *ampR*, and *intl1* gene and orf513 were detected by PCR (data not shown), so we supposed that *qnrB4* was also located in a *sul1*-type class 1 integron in *K pneumoniae*.

Unlike *qnrA* and *qnrB*, *qnrS* was not located on any integrons according to 3 reported *qnrS*-bearing plasmid structure analyses[3,14,15], but *qnrS* was directly downstream of IS2. The PCR amplification was negative for *intl1* and orf513 in *qnrS*-bearing plasmid pHS8 (data not shown), indicating that pHS8 did not carry any class 1 integron. Like in pK245, the *fts1* and a $bla_{\text{LAP-1}}$ genes were found upstream of *qnrS1* in this study, and notably, insertion sequence IS26 was found both upstream and downstream of *qnrS1*. *qnrS* could also be located on a mobilizable incQ-related plasmid[16].

A transconjugant was obtained carrying plasmid pHS9, of a similar size to pHS7, but bore both *qnrB4* and *qnrS1*. We supposed that there was an integration between pHS7 and pHS8 to produce pHS9 during the conjugation experiment. There was a possibility that a segment of plasmid pHS8 containing *qnrS1* was integrated to pHS7. The $bla_{\text{LAP-1}}$ gene that was located upstream of *qnrS1* in pHS8, but not harbored by pHS7, was detected in pHS9 by PCR (data not shown). The movement of *qnrS1* might be related to the insertion sequences of IS26 located upstream and downstream of *qnrS1*, and IS2 directly upstream of

qnrS1. The resistance to sulfamethoxazole, trimethoprim, and ceftazidime in pHS7 was lost in pHS9, indicating that the *qnrS1* segment might replace part of plasmid DNA in pHS7 or interrupt the expression of some genes in pHS7. However, the MIC of ciprofloxacin in pHS9 was not shown to be augmented from *qnrB4* to *qnrS1*. Further study is ongoing on the analysis of the structure of pHS9 to understand the mechanism of the integration between the 2 plasmids.

This is the first report of *qnrB* and *qnrS* from China and these 2 genes are harbored by a single clinical strain of *K pneumoniae.*

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