Brief Original Article

Detection of plasmid-mediated quinolone resistance determinants and *qnrS* expression in *Enterobacteriaceae* clinical isolates

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Abstract

Introduction: Rapid dissemination of plasmid-mediated quinolone resistance (PMQR) has been reported in clinical isolates.
Methodology: A total of 149 clinical isolates of *Enterobacteriaceae* were collected in Beijing and screened for PMQR genes usingpolymerase chain reaction (PCR). Real-time quantitative PCR was used to study the expression of *qnrS*.
Results: The rates of *qnr* and *aac(6')-Ib-cr* genes were 7.4% and 8.1%, respectively. The higher basal expression of *qnrS* was observed in transconjugant strains, which had higher minimum inhibitory concentrations (MICs) of quinolones. Furthermore, *qnrS* expression levels increased in all three isolates when a quinolone was present.
Conclusions: Our data suggest that the level of *qnrS* expression was associated with quinolone resistance.

Key words: *Enterobacteriaceae*; PMQR; *qnrS*; expression.

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Introduction

Resistance to quinolones has increased globally in recent years, particularly in members of the*Enterobacteriaceae* [1-2]. Two main resistance mechanisms to quinolones are recognized: mutations in the quinoloneresistance-determining regions (QRDRs) of the chromosomal *gyrA* and *parC* genes, and decreased accumulation inside the bacteria due to impermeability of the membrane and/or an overexpression of efflux pump systems [3,4]. However, plasmid-mediated quinolone resistance (PMQR) has been reported since 1998 [5]. The plasmid-borne *qnr* genes currently comprise five families:*qnrA*, *qnrB*, *qnrC*, *qnrD*, and *qnrS*, which encode Qnr proteins that protect the DNA gyrase from the activity of quinolones [6]. Additionally, the *aac(6')-Ib-cr* gene encodes a variant aminoglycoside acetyltransferase that can modify quinolones with a piperazinyl substituent [7]. Quinolone induction of *qnr* genes independent of the SOS system has been observed in previous studies [8-9]. Although PMQR determinants confer low-level resistance to quinolones, they are a favorable background for selection of additional chromosome-encoded quinolone resistance mechanisms, which makes the clinical therapy more difficult. In this study, we investigated the presence of PMQR genes in clinical isolates of *Enterobacteriaceae*, identified mutations within the QRDRs, and examined the expression of *qnrS* in transconjugants obtained from different donor isolates.

Methodology

One hundred and forty-nineclinical isolates were obtained from two hospitals in Beijing, China, between March and July 2011.These included 86 strains of *E. coli*, 35 strains of *P. mirabilis*, and 28 strains of *K. pneumoniae*. All isolates were screened for the *qnr* (*qnrA*, *qnrB*, *qnrC*, *qnrD*, and *qnrS*) and *aac(6')-Ib* genes by polymerase chain reaction (PCR) amplification using the primers described previously [10-13]. Mutations in the *gyrA* and *parC* genes were identified by DNA sequencing of their PCR products [14].The transfer of quinolone resistance was studied by performing conjugation experiments with *Escherichia coli* J53Az^r (resistant to sodium azide) as the recipient, as described previously [10]. Transconjugants wereselected on trypticase soy agar

(TSA; Land Bridge Technology Co. Ltd., Beijing, China) plates containing sodium azide (150 μg mL^{-1}; Sigma-Aldrich, St. Louis, USA) and ciprofloxacin (0.25 μgmL^{-1}; Sigma-Aldrich).Minimum inhibitory concentrations (MICs) for the donor, recipient, and transconjugant strains were measured by agar dilution, according to Clinical and Laboratory Standards Institute (CLSI) guidelines.

Total RNA was extracted from 1.5 mL of exponentially growing cellsusing TRIzol (Invitrogen, Carlsbad, USA),according to the manufacturer'sinstructions.Real-time quantitative PCR (qPCR) was used to study gene expression of *qnrS*. Using the Reverse Transcription System (Promega, Madison, USA), 200 ng of total RNA were reverse transcribed in a final volume of 20 μL. Primers for *qnrS* were 5'-CTCTTCCAAGTGTAGGGAGATAT-3' and 5'-ATTGGGATAGATTTCGCTTATTG-3'. Using primers as described previously [15], 16S rRNA was used as the reference gene. PCR reactions were performed using 1 μL of cDNA with RealMasterMix (Tiangen Biotech Co. Ltd., Beijing, China).

Results

The *qnr* genes were present in 11 (7.4%) of 149 isolates, and among them, three isolates carried *qnrA* (2.0%), two isolates carried *qnrB* (1.3%), and six isolates carried *qnrS* (4.0%) (Table 1). The *qnrC* and *qnrD* genes were not detected in the isolates examined in this study. Twenty-two (14.8%) of the 149 isolates were positive for *aac(6')-Ib*, of which 12 (8.1% of all) isolates carried the *-cr* variant. Notably, the *aac(6')-Ib-cr* gene was detected in 63.6% (7/11 isolates) of *qnr*-positive isolates, compared with only 3.6% (5/138 isolates) of *qnr*-negative isolates.

Quinolone resistance could be transferred by conjugation from nine of the eleven *qnr*-positive donors, and one of the five donors carried the *aac(6')-Ib-cr* gene alone. PCR experiments confirmed that the transconjugants harbored the same PMQR determinants as their donors, and that the *qnr* and *aac(6')-Ib-cr* genes could be cotransferred from different donors (Table 2). The 10 transconjugants showed 32- to 128-fold increases in the MICs of ciprofloxacin and 16- to 32-fold increases in the MICs of levofloxacin, relative to those of the recipient. All transconjugants showed 4- to 128-fold increases in the MICs of ceftazidime, and three showed more than a 256-fold increase in the MICs of gentamicin. Thus, ciprofloxacin resistance was cotransferred with resistance to other antimicrobial agents, such as cephalosporins, aminoglycosides, and sulfamethoxazole.

DNA sequencing of the PCR products covering the entire QRDRs of *gyrA* demonstrated the presence of mutations at codon 83 in the 10clinical isolates examined (Table 2). Point mutations in *gyrA* were found in *E. coli*and *K. pneumoniae*, and the resulting amino acid substitutions were Ser83Leu and Ser83Ile, respectively. Four *E. coli* isolates had an additional mutation, Asp87Asn. In *parC*, a Ser80Ile substitution was found in seven of ten clinical isolates. There were no mutations in the target genes among the transconjugants and the recipient.

There were fourfold and twofold differences in the MICs for ciprofloxacinand levofloxacin, respectively, among the three transconjugants that only harbored *qnrS*. The basal expression level of *qnrS* in H483T was 4.9-fold, much higher than in the transconjugants H707T and K337 (Table 3). The effect of ciprofloxacinand levofloxacin on *qnrS* transcription was investigated, and maximal transcription was observed at 0.4×MIC with both ciprofloxacinand levofloxacin (data not shown). The *qnrS* transcript levels increased in all three transconjugants when ciprofloxacin was present, with 1.9-fold, 2.7-fold, and 5.4-fold increases for H707T, K337T, and H483T, respectively (Table 3). The increases over baseline observed when levofloxacin was added to the medium were 1.7-fold, 3.0-fold, and 5.1-fold for the three transconjugants, respectively (Table 3).

Table 1. Prevalence of *qnr* and *aac(6')-Ib-cr* genes in selected *Enterobacteriaceae* isolates

Organism	No. of isolates with *qnr*/total no. of isolates (%)				
	qnrA	*qnrB*	*qnrS*	*aac(6')-Ib*	*aac(6')-Ib-cr*
E. coli	2/86 (2.3)	0/86 (0.0)	6/86 (7.0)	17/86 (19.8)	8/86 (9.3)
P. mirabilis	1/35 (2.9)	0/35 (0.0)	0/35 (0.0)	2/35 (5.7)	1/35 (2.9)
K. pneumoniae	0/28 (0.0)	2/28 (7.1)	0/28 (0.0)	3/28 (10.7)	3/28 (10.7)

Table 2. Plasmid-mediated quinolone resistance genes and MICs of antimicrobial agents for donors, recipients, and transconjugants

Isolate	QRDRs mutation(s)		PMQR genes		MIC (μg/mL)								
	GyrA	ParC	*qnr*	*aac(6')-Ib-cr*	CIP	LVX	GEN	AMK	CAZ	CFP	FEP	AMP	SMZ
E. coli													
H560	S83L	S80I	*qnrA*	+	8	2	128	4	4	16	8	>128	>256
H560T	WT	WT	*qnrA*	+	0.5	0.25	0.5	2	2	8	1	>128	>256
H982	S83L	WT	*qnrA*	–	1	1	>128	>128	64	128	32	>128	>256
H982T	WT	WT	*qnrA*	–	0.25	0.25	>128	>128	32	128	1	>128	>256
H707	S83L	WT	*qnrS*	–	4	2	2	8	4	128	32	>128	>256
H707T	WT	WT	*qnrS*	–	0.25	0.25	1	4	1	32	2	>128	>256
K337	S83L, D87N	WT	*qnrS*	–	16	4	128	4	4	16	16	>128	256
K337T	WT	WT	*qnrS*	–	0.25	0.25	0.5	2	2	4	1	>128	128
H483	S83L, D87N	S80I	*qnrS*	–	32	16	32	4	64	128	128	128	>256
H483T	WT	WT	*qnrS*	–	1	0.5	1	2	2	128	1	128	>256
K280	S83L, D87N	S80I	*qnrS*	+	>32	>32	128	2	128	128	64	>128	>256
K280T	WT	WT	*qnrS*	+	1	0.5	1	2	4	128	1	>128	>256
K203	S83L, D87N	S80I	*qnrS*	+	>32	>32	32	4	64	256	128	>128	>256
K203T	WT	WT	*qnrS*	+	1	0.5	1	2	2	64	1	>128	>256
H943	S83L	S80I	–	+	8	4	128	64	4	16	8	128	256
H943T	WT	WT	–	+	0.25	0.25	128	64	2	8	1	64	256
K. pneumoniae													
J400	S83I	S80I	*qnrB*	+	>32	8	>128	64	32	32	2	>128	>256
J400T	WT	WT	*qnrB*	+	1	0.25	>128	64	32	32	2	64	128
K072	S83I	S80I	*qnrB*	+	>32	32	>128	64	256	128	0.5	>128	>256
K072T	WT	WT	*qnrB*	+	0.5	0.25	>128	32	16	64	0.125	>128	>256
J53	WT	WT	–	–	0.008	0.016	0.5	1	0.25	2	0.016	16	16

J53: recipient; T: transconjugant; WT: wild type; CIP: ciprofloxacin; LVX: levofloxacin; GEN: gentamicin; AMK: amikacin; CAZ: ceftazidime; CFP: cefoperazone; FEP: cefepime; AMP: ampicillin; SMZ: sulfamethoxazole

Table 3. Relative expression data for *qnrS* in transconjugants

Transconjugant	MIC (μg/mL)		Basal expression level	Increase in expression level	
	CIP	LVX		CIP	LVX
H707T	0.25	0.25	1	1.9 ± 0.4	1.7 ± 0.1
K337T	0.25	0.25	1.4 ± 0.7	2.7 ± 0.5	3.0 ± 0.3
H483T	1	0.5	4.9 ± 0.1	5.4 ± 0.2	5.1 ± 0.3

Gene expression was measured in the presence of ciprofloxacin and levofloxacin relative to the drug-free condition. The results are expressed in terms of the mean ± standard deviation. The value obtained from non-quinolone-exposed cells of strain H707T was considered as 1.

Discussion

The results of our study demonstrated a proportion of 7.4% for the PMQR genes among clinical isolates of *Enterobacteriaceae*. The rate of *aac(6')-Ib-cr* carriage among *qnr*-positive isolates was much higher than that among *qnr*-negative isolates, which was agreement with previous studies [11,16]. Conjugation experiments proved that the plasmid-mediated quinolone resistance was transferable. However, six other *qnr*- and/or *aac(6')-Ib-cr*-bearing isolates failed to produce transconjugants. The result showed that *qnr* and *aac(6')-Ib-cr* genescould be cotransferred from different donors. The enzyme encoded by the *aac(6')-Ib-cr* gene could inactivate quinolones (ciprofloxacin and norfloxacin) by N-acetylation of the amino nitrogen on its piperazinyl group [17]. Transconjugants carrying *aac(6')-Ib-cr* aloneshowed a 32-fold increase in ciprofloxacin with that of the recipient, suggesting that *aac(6')-Ib-cr*itself causes low-level ciprofloxacin resistance, as reported by other studies [14,16,17]. Furthermore, another study reported that *qnr* and *aac(6')-Ib-cr*could act additively to generate the ciprofloxacin resistance [17]. It was reported that the *qnr* and *aac(6')-Ib-cr* genes were always co-resident on the same plasmids and cotransferred to the recipient, which may have contributed to the rapid increase in resistance to quinolones among bacteria [14].Resistance to ciprofloxacinand levofloxacinwas lower in the transconjugants than in their donors. The DNA sequencing results indicated that chromosomal QRDR mutations in GyrA and ParC played an important role in mediating high-level quinolone resistance among the donor isolates.

Xu *et al*. reported that the differences in *qnrA* expression levels may account for the variations in the ciprofloxacin MICs of different transconjugants [15]. In our study, *qnrS* was found most frequently among the isolates examined, and the expression of *qnrS* was investigated to understand the relationship between *qnrS* expression level and MICs of ciprofloxacin. Our results showed that the basal expression level of *qnrS* in H483T was much higher than in H707T and K337. The higher basal expression of *qnrS* in H483T may explain, at least in part, the higher MICs of quinolones for this transconjugant. The *qnrS* transcript levels increased in all three isolates when ciprofloxacin or levofloxacin was present, suggesting that the *qnrS*expression was induced by quinolones, which is consistent with previous reports [9,18]. It can therefore be proposed that the level of *qnrS* expression was associated with the quinolone resistance in the transconjugants analyzed. A higher level of *qnrS* transcript could provide the cell with a rapid response to the damaging effects of antimicrobial agents that inhibit DNA replication by protecting the topoisomerases;this wouldpromote bacterial survival [19].

Conclusions

In summary, PMQR genes are implicated in low-level quinolone resistance, and may play an important role in the generation of resistant mutants and the rapid increase in bacterial resistance. The level of *qnrS* expression appeared to contribute to quinolone resistance in the transconjugants analyzed.

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