ORIGINAL ARTICLE

Prevalence of plasmid-mediated quinolone-resistance determinants in *Shigella flexneri* isolates from Anhui Province, China

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Quinolones are used extensively to treat *Shigella flexneri* infection, and plasmid-mediated quinolone resistance (PMQR) determinants were recently reported. A total of 26 *S. flexneri* isolates collected from Anhui province, China, in 2005 were screened for PMQR determinants, *bla* gene, *gyrA* and *parC* genes, by PCR and sequencing. PMQR determinants were present in 53.8% (14 of 26) of isolates and *qnrA*₁, *qnrS*₁, *qnrS*₂, *aac(6′)-Ib-cr* and *qepA* were present in 30.8, 11.5, 3.8, 19.2 and 11.5% of those isolates, respectively. All PMQR determinants' positive isolates exhibiting 8 different genetic clones had mutations in *gyrA* and *parC* genes, and 11 carried *bla* genes, including 7 *bla*_{CTX-M} with resistance to cefotaxime. These isolates were resistant to nalidixic acid and 57.1% (8 of 14) of them were resistant to ciprofloxacin and levofloxacin. Our data show that there was a high prevalence of PMQR determinants in *S. flexneri* isolates from China.

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INTRODUCTION

Shigella flexneri is a causative agent of bacillary dysentery, especially in China, and quinolones are among the most important antibacterial agents used extensively for treating this infection.^{1–3} Recently, quinolone resistance has been increasing markedly in these isolates.^{2,3} The common mechanisms for resistance to quinolone in pathogenic bacteria including *S. flexneri* are mutations in the chromosomal genes of DNA gyrase and DNA topoisomerase IV, the targets of quinolones.⁴ Efflux pumps and alteration in outer membrane proteins are also among the effects of chromosomal quinolone resistance.⁴

However, since 1998,⁵ three kinds of plasmid-mediated quinolone resistance (PMQR) determinants have been described: *Qnr*, *AAC(6′)-Ib-cr* and *QepA*.^{6–8} Several families of *qnr* (*qnrA*, *qnrB*, *qnrS*, *qnrC*, *qnrD*) have been reported and each family has different subtypes.^{6,9} *qnr* encoded a protein of the pentapeptide family, protecting DNA gyrase directly from quinolone inhibition.¹⁰ *AAC(6′)-Ib-cr* is a variant of aminoglycoside acetyltransferase *AAC(6′)-Ib* with enzymatic modification of ciprofloxacin or norfloxacin.⁷ *qepA* encoded an efflux pump belonging to the major facilitator subfamily and quinolones were the specific substrates of *QepA*.⁸

To investigate the prevalence of PMQR determinants in *S. flexneri* isolates from China, we screened the prevalence of *qnr*, *qepA* and *aac(6′)-Ib-cr* genes by PCR and sequencing of positive PCR products in *S. flexneri* clinical isolates collected from Anhui province, China in 2005.

MATERIALS AND METHODS

Bacteria

A total of 26 consecutive clinical isolates of *S. flexneri* were collected from nine hospitals in Anhui province, China in 2005: *Escherichia coli* ATCC (American Type Culture Collection, Manassas, VA, USA) 25922, PMQR determinants' positive control strains and *bla*-positive control strains.

Antimicrobial agents

The following antimicrobial agents were used: ciprofloxacin, levofloxacin *E*-test strip (AB Biodisk, Solna, Sweden), cefotaxime, ceftazidime (National Institute for Control of Pharmaceutical and Biological Products, Beijing, China) and nalidixic acid (Sigma, St Louis, MO, USA).

Screening of *qnr*, *qepA*, *aac(6′)-Ib-cr*, *gyrA*, *gyrB*, *parC*, *parE* and *bla* genes

Amplification of *qnr*, *qepA* and *aac(6′)-Ib-cr* was performed using the methods described previously;^{8,9,11,12} different primers (shown in Table 1) were used for the amplification of *qnrB* and *qnrA* subtypes in this study. Amplification of *bla* genes (β -lactamase genes) was also performed with the methods described previously for *bla*_{TEM}, *bla*_{SHV} and *bla*_{CTX-M}.^{13–15} The mutations in the quinolone resistance-determining regions of the *gyrA*, *gyrB*, *parC* and *parE* genes were amplified by PCR.¹⁶ All purified PCR products were directly sequenced using an ABI3100 genetic analyzer and continued using primers walking on both DNA strands. For sequence comparison, the NCBI BLAST program and facilities of the TIGR Comprehensive Microbial Resource

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Table 1 Primers for *qnrB* and *qnrA* used in this study

Target	Primer	Sequence (5'–3')	Length of product (bp)
<i>qnrB</i> 1-3,6,7,9,10,13,15–18,20	<i>qnrB</i> 1F	GGCTCTGGCACTCGTT	157
	<i>qnrB</i> 1R	CCCTTTCTGGCTTTCAC	
<i>qnrB</i> 4,10	<i>qnrB</i> 4F	ATCACCACCCGCACCT	242
	<i>qnrB</i> 4R	TCTAAATCGCCCAGTTCC	
<i>qnrB</i> 5,19	<i>qnrB</i> 5F	GCCTTGGGCATTGAA	305
	<i>qnrB</i> 5R	CCTAACTCCGAATTGGTC	
<i>qnrB</i> 8,11,12,14	<i>qnrB</i> 8F	AGCCAGAAAGGGTG(T/C)AAT	318
	<i>qnrB</i> 8R	ATC(C/T)GAACCT(G/C)AACG	
	<i>qnrA</i> F	TTGAGCGGTAACGAGTGAG	
<i>qnrA</i> 1-6	<i>qnrA</i> F	TTGAGCGGTAACGAGTGAG	823
	<i>qnrA</i> R	ATCTGCCTTGTGTAGCG	

Table 2 Characteristics of the 14 PMQR determinants positive isolates

Isolate number	PFGE type	Resistance genes	Mutations in QRDRs		MIC (mg l ⁻¹)				
			<i>gyrA</i>	<i>parC</i>	NAL	CIP	LVFX	CTX	CAZ
05ChZ60	A	<i>qnrA</i> 1, <i>qepA</i> , <i>aac</i> (6')-Ib-cr, <i>bla</i> _{TEM-1}	S83L	S80I	>32	2	2	0.125	0.125
05ShC15	B	<i>qnrA</i> 1, <i>bla</i> _{CTX-M-14}	S83L	S80I	>32	0.25	0.25	>128	2
05ShC18	B	<i>qnrA</i> 1, <i>bla</i> _{TEM-1} , <i>bla</i> _{CTX-M-14}	S83L	S80I	>32	0.38	0.5	>128	2
05ShC36	C	<i>qnrA</i> 1, <i>qepA</i>	S83L	S80I	>32	0.38	0.38	≤0.0625	0.125
05ShC42	D	<i>qnrA</i> 1, <i>bla</i> _{CTX-M-14}	S83L	S80I	>32	0.25	0.5	>128	2
05FuY15	E	<i>qnrA</i> 1	S83L	S80I	>32	0.38	0.5	0.125	1
05FuY2	F	<i>qnrA</i> 1, <i>qnrS</i> 1, <i>bla</i> _{TEM-1} , <i>bla</i> _{CTX-M-14}	S83L	S80I	>32	6	4	128	2
05FuY17	G	<i>qnrA</i> 1, <i>qnrS</i> 1, <i>bla</i> _{TEM-1} , <i>bla</i> _{CTX-M-14}	S83L	S80I	>32	4	8	>128	2
05HN73	E	<i>qnrS</i> 1	S83L	S80I	>32	6	8	0.125	2
05ChY30	H	<i>qnrS</i> 2, <i>aac</i> (6')-Ib-cr, <i>bla</i> _{TEM-1} , <i>bla</i> _{SHV-1} , <i>bla</i> _{CTX-M-15}	S83L, D87N	S80I	>32	>32	>32	128	32
05ChY64	H	<i>qepA</i> , <i>bla</i> _{TEM-1}	S83L, D87Y	S80I	>32	>32	>32	≤0.06	0.125
05ShC24	I	<i>aac</i> (6')-Ib-cr, <i>bla</i> _{TEM-1}	S83L	S80I	>32	4	0.5	0.5	1
05ShC25	I	<i>aac</i> (6')-Ib-cr, <i>bla</i> _{TEM-1}	S83L	S80I	>32	>32	16	0.5	1
05GZ24	H	<i>aac</i> (6')-Ib-cr, <i>bla</i> _{TEM-1} , <i>bla</i> _{CTX-M-14}	S83L	S80I	>32	>32	>32	128	128

Abbreviations: CAZ, ceftazidime; CIP, ciprofloxacin; CTX, cefotaxime; LVFX, levofloxacin; MIC, minimum inhibitory concentration; NAL, nalidixic acid; PFGE, pulsed-field gel electrophoresis; PMQR, plasmid-mediated quinolone resistance; QRDR, quinolone resistance-determining region.

(<http://www.tigr.org>, National Center for Biotechnology Information, Bethesda, MD, USA) were used.

Antimicrobial susceptibility tests

Minimum inhibitory concentrations (MICs) of ciprofloxacin and levofloxacin were determined using *E*-tests. Susceptibility testing for cefotaxime, ceftazidime and nalidixic acid was performed by the agar dilution method according to the guidelines recommended by the Clinical and Laboratory Standards Institute.¹⁷ *E. coli* ATCC 25922 was used as the quality control strain.

Molecular typing of bacterial DNA

To elucidate the clonality of PMQR determinants' positive isolates, genomic DNA of each PMQR determinants' positive strain was analyzed by pulsed-field gel electrophoresis (PFGE) in a CHEF-MAPPER apparatus (Bio-Rad Laboratories, Hercules, CA, USA) after digestion with *Xba*I.^{15,18} Genetic clones were identified according to criteria established by Tenover *et al.*¹⁸

RESULTS AND DISCUSSION

PMQR determinants were present in 53.8% (14 of 26) of isolates with *qnr*, *aac*(6')-Ib-cr and *qepA* detected alone or in combination (Table 2). *qnrA*1, *qnrS*1, *qnrS*2, *aac*(6')-Ib-cr and *qepA* were present in 30.8% (8 of 26), 11.5% (3 of 26), 3.8% (1 of 26), 19.2% (5 of 26) and 11.5% (3 of 26) of those isolates, respectively. Five isolates carried two or three types of PMQR determinants. In 14 PMQR determinants'

positive isolates, all had the mutations in *gyrA* (S83L and/or D87N) and *parC* (S80I) genes, and the ciprofloxacin MICs of these strains were relatively high, 0.25 to >32 µg ml⁻¹; of these, nine strains had MICs 2 to >32 µg ml⁻¹. A total of 11 carried *bla* genes (most of the strains carried more than one type), including 7 *bla*_{CTX-M} (6 *bla*_{CTX-M-14} and 1 *bla*_{CTX-M-15}), 9 *bla*_{TEM-1} and 1 *bla*_{SHV-1}, in all 14 PMQR-positive strains simultaneously. All PMQR determinants' positive isolates were resistant to nalidixic acid and 57.1% (8 of 14) of them were resistant to ciprofloxacin and/or levofloxacin. Seven *bla*_{CTX-M}-positive strains were resistant to cefotaxime, only two of seven were resistant to ceftazidime according to CLSI standards.¹⁷ Results of PFGE show that 14 PMQR determinants' positive isolates exhibited eight different genetic clones by Tenover *et al.*¹⁸ criteria.

In 2005, *qnrS*1 was first described in *S. flexneri* isolates from Japan.¹⁹ The *qnrS*1 gene has ~60 and ~50% homology to the gene of *qnrA* and *qnrB*, respectively. There are 17 amino-acid substitutions between *QnrS*1 and *QnrS*2, but only one amino-acid substitution between *QnrS*1 and *QnrS*3.⁶ *qnrC* and *qnrD* are novel *qnr*-like genes and encoded 221 amino-acid and 214 amino-acid pentapeptide repeat proteins, respectively, with similarities to *qnrA*, *qnrB* and *qnrS*.^{9,11} *aac*(6')-Ib-cr and *qepA* genes are the second and third most recently detected PMQR determinants differing from the *qnr* family and are being increasingly identified worldwide among various clinical isolates

of *Enterobacteriaceae*.^{7,8,12,20} To our knowledge, this study is the first report of *qnrA*₁, *qnrS*₂, *aac(6')-Ib-cr* and *qepA* genes in *S. flexneri* (the nucleotide sequence reported herein has been registered with the EMBL database under accession nos. FJ177519, FJ177521, FJ949571, FJ842530, respectively). The emergence of PMQR determinants in *S. flexneri* may affect the use of quinolones in the treatment of dysentery, and this study provides some new and useful information.

Most of the PMQR determinants provide only low-level resistance to fluoroquinolones, but can supplement to other quinolone-resistance mechanisms, such as the selection of chromosomal mutations in the presence of a quinolone.^{4,5,7,8} PMAR determinants confer low-level resistance to quinolones with ciprofloxacin MICs 0.1–1.0 µg ml⁻¹ in *E. coli* J53. For high-level ciprofloxacin- or levofloxacin-resistant strains, additional mechanisms such as target mutations and/or active efflux pump frequently coexist with PMQR determinants.⁷ In this study, mutations in *gyrA* and *parC* genes were identified in all PMQR determinants' positive isolates with resistance to nalidixic acid, and more than half of which were resistant to ciprofloxacin by CLSI criteria.¹⁷ S83L, D87Y mutations in *gyrA* and S80I in *parC* are the most common point mutations identified to cause quinolone resistance in several previous studies,^{4,16,21} and mutants with a single *gyrA* mutation had ciprofloxacin of MIC 0.32 µg ml⁻¹.²¹ PMQR determinants are usually associated with additional resistant determinants and have often been reported to be coassociated with ESBL (extended-spectrum β-lactamase) genes and plasmid-mediated AmpC genes.⁴ More than half of the PMQR determinants associated with *bla* genes in this study, especially *bla*_{CTX-M}, which conferred coreistance to the third generation cephalosporins. CTX-M-ESBLs (especially CTX-M-14) prefer hydrolyzing cefotaxime than ceftazidime and are the main type of ESBLs in *Enterobacteriaceae* isolates, including *Shigella* spp., in China.^{3,13,14}

Results of PFGE show clonal spread in a few of these strains, which poses a serious threat to clinic. Some isolates (05ShC15/05ShC42 and 05FuY2/05FuY17) carry the same resistance genes, but the strains are in different PFGE types. It is possible that they were invaded by the same plasmid, which we will study in the future. Strains 05ShC24 and 05ShC25 are both in PFGE group I, and both carry *aac(6')-Ib-cr* and *bla*_{TEM-1}, but are different in CIP and LVFX. There may be other forms of chromosomal resistance, such as alteration in the outer membrane proteins.⁴ Of three PFGE H types, two strains had two mutations at both 83 and 87 positions in *gyrA*, whereas one strain only had a mutation at 83. The explanation might be that PFGE detects the molecular typing of bacterial DNA only by big DNA fragment mappers¹⁸ and those strains isolated from different hospitals.

Our data show that PMQR determinants were prevalent and *qnrA*₁, *qnrS*₂, *aac(6')-Ib-cr* and *qepA* genes were first reported in *S. flexneri* from China. Most of those isolates are coresistant and clone spread exists in a few of them. It is necessary to monitor these isolates closely.

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