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Molecular Epidemiology and Genetic Diversity of Fluoroquinolone-Resistant *Escherichia coli* Isolates from Patients with Community-Onset Infections in 30 Chinese County Hospitals

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The high frequency of fluoroquinolone resistance in *Escherichia coli* is a feature of clinical bacteriology in China, where the molecular epidemiology and genetic characteristics of this resistance in county hospitals remain unclear. A total of 590 nonduplicate *E. coli* isolates from 30 county hospitals located across seven Chinese regions were examined for plasmid-mediated quinolone resistance (PMQR) genes and mutations in quinolone resistance-determining regions (QRDRs). Multilocus sequence typing (MLST) and phylogenetic analysis of fluoroquinolone-resistant isolates were used to determine their genetic relatedness. The ciprofloxacin resistance rate of community-onset *E. coli* was 51.2%, and at least one PMQR gene was carried by 220 (37.3%) isolates. These included *qnr* (3.7%), *aac(6′)-Ib-cr* (19.7%), *qepA* (14.4%), and *oqxAB* (3.8%). Two novel *oqxB* mutants were identified and named *oqxB20* and *oqxB29*. From 60 sequence types (STs) isolated, 5 novel STs (ST4499 to ST4503) were identified. ST1193 (7.9%) was the second most abundant ST among fluoroquinolone-resistant isolates (ST131 was the most common, with 14.6%), and this is the first report of it in China. This is also the first report of ST2115 and ST3014 isolates from human samples. Ciprofloxacin-resistant *E. coli* isolates fell mainly into phylogroups B2 and D. The rates of fluoroquinolone resistance and the prevalence of PMQR genes in community-onset *E. coli* isolates from Chinese county hospitals were high. The wide-ranging molecular epidemiology of *E. coli* isolates from scattered locations across China indicates that fluoroquinolone resistance evolved from different sources.

The fluoroquinolones comprise one of the most widely used groups of antibacterial agents in China (1); however, concerns have developed about the emergence of bacteria resistant to these broad-spectrum antibiotics. In 1989, resistance of *Escherichia coli* to fluoroquinolones was about 0% to 5%; the resistance rose to 32% to 41% by 1992 and reached 50% to 60% in 1995 (2). Data from the Ministry of Health National Antimicrobial Resistance Surveillance Net (MOHNARIN) in China for tertiary hospitals revealed that 65.7% of clinical *E. coli* isolates in 2011 were resistant to fluoroquinolones (3), which was significantly higher than the resistance rate in the United States, Canada, Great Britain, France, Norway, and Sweden (4–6).

E. coli uses different mechanisms to provide resistance to quinolones, including mutations on quinolone targets, overexpression of efflux pump systems, and a reduction or absence of outer membrane porins. More recently, research focused on plasmid-mediated quinolone resistance (PMQR). An analysis of the literature up to the end of 2008 revealed the frequencies of the different PMQR genes *qnrA* (1.5%), *qnrB* (4.6%), *qnrS* (2.4%), and *aac(6′)-Ib-cr* (10.8%) among 20,960 global isolates (7). Several studies have highlighted the prevalence of antibiotic genes among isolates in Chinese tertiary hospitals. The frequencies of the *qnr*, *aac(6′)-Ib-cr*, and *qepA* genes in urinary tract *E. coli* isolates during 2007 to 2008 and clinical *E. coli* isolates from 2008 among tertiary hospitals were reported, and the rates were a similar magnitude to that of the global epidemic (8, 9). During 2010 to 2011, the incidence of detection for *aac(6′)-Ib-cr* in clinical *E. coli* isolates from a tertiary hospital in Shanghai was 11.6% (10). These studies highlight the characteristics of fluoroquinolone resistance in *E. coli* for China's tertiary hospitals; however, a shortfall exists in information about the molecular epidemiology in those of county status.

Much of China's population lives in rural areas and small towns, where county hospitals provide most of the medical services. It is particularly important to understand the prevalence and characteristics of fluoroquinolone resistance in county hospitals so that guidance can be provided on the clinical application of these antibiotics. The purpose of this study was to provide information about the molecular epidemiology and genetic characteristics of community-onset *E. coli* isolated from 30 county hospitals located in seven regions across China.

MATERIALS AND METHODS

Bacterial isolates. A total of 590 consecutive nonduplicate clinical *E. coli* isolates from community-onset infections were collected from 30 county hospitals in 12 provinces distributed in seven geographic regions of China during the period August 2010 to August 2011. Criteria used to decide the suitability of patients for sample collection were as follows: (i) outpatients,

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TABLE 1 Susceptibilities of 590 *E. coli* isolates to antimicrobial agents

Antibiotic	MIC[a] range (mg/liter)	MIC_{50} (mg/liter)	MIC_{90} (mg/liter)	Resistance rate (%)
Ampicillin	0.25 to >512	512	>512	84.9
Piperacillin≤	≤0.03 to >512	64	512	49.7
Ampicillin-sulbactam	≤0.03 to 512	16	64	39.1
Piperacillin-tazobactam	≤0.015 to 256	8	16	2.2
Cefazolin	0.125 to >128	32	>128	54.1
Cefuroxime	0.5 to >128	16	>128	48.6
Cefoxitin	0.125 to >256	4	32	14.9
Ceftriaxone	≤0.03 to >256	0.25	128	44.7
Ceftazidime	≤0.015 to >128	0.5	32	18.3
Cefepime	≤0.03 to >512	1	32	13.4
Imipenem	≤0.015 to 1	0.125	0.25	0
Meropenem	≤0.015 to 1	0.06	0.125	0
Gentamicin	0.125 to >256	16	128	51.9
Amikacin	≤0.03 to >256	2	8	6.1
Ciprofloxacin	≤0.015 to >128	4	64	51.2
Levofloxacin	≤0.015 to 256	4	32	47.9
Fosfomycin	≤0.03 to >128	1	32	4.8

[a] MIC results were interpreted according to CLSI breakpoint criteria 12.

emergency patients, or inpatients who were admitted with infections within 48 h of diagnosis; (ii) patients not hospitalized for the preceding 90 days; (iii) individuals with no long-term indwelling catheters; and (v) patients who received antimicrobial agents for ≤72 h. Among the 590 isolates, 516 (87.5%) originated from outpatients. The most common sources for isolates were urine (61.2% of isolates), blood (7.3%), sputum (6.4%), ascites (peritoneal cavity fluid, 6.1%), and bodily secretions (5.8%). The geographic distributions of isolates were north China (94 strains, 6 hospitals), northeast China (35 strains, 2 hospitals), northwest China (82 strains, 4 hospitals), east China (89 strains, 3 hospitals), central southern China (104 strains, 5 hospitals), south China (109 strains, 5 hospitals), and southwest China (77 strains, 5 hospitals).

Antimicrobial susceptibility testing. Antimicrobial susceptibilities of isolates were determined by Mueller-Hinton agar dilution in accordance with guidelines of Clinical and Laboratory Standards Institute (CLSI) document M07-A9 (11). The results were interpreted according to the recommended breakpoints of CLSI document M100-S23 (12). *E. coli* ATCC 25922 was used as a control.

Detection of PMQR genes. PMQR genes *qnr* (*qnrA*, *qnrB*, *qnrC*, *qnrD*, and *qnrS*), *aac(6′)-Ib-cr*, *qepA*, and *oqxAB* were detected by PCR (13–17) (see Table S1 in the supplemental material), then sequenced by Sanger dideoxy-mediated chain termination and analyzed using DNASTAR Lasergene v7.1 software. The BLAST program was used to compare DNA sequences against those in the NCBI database.

Screening for mutations in quinolone resistance-determining regions. Mutations in quinolone resistance-determining regions (QRDRs) were determined using 302 ciprofloxacin-resistant and 87 ciprofloxacin-sensitive isolates. Ciprofloxacin-sensitive isolates were selected according to their MICs and geographic location. PCR primers are listed in Table S1 in the supplemental material, amplification products were sequenced and analyzed as before, and amino acid mutations were determined using the control strain *E. coli* K-12(NZ_AKBV01000001.1) as a reference.

Multilocus sequence typing. Multilocus sequence typing (MLST) was performed on all ciprofloxacin-resistant isolates. Seven housekeeping genes were targeted: *adk*, *fumC*, *gyrB*, *icd*, *mdh*, *purA*, and *recA*. Alleles of these genes were termed as a sequence type (ST). The primers and protocols used are described on the MLST website (http://mlst.ucc.ie/mlst/dbs/Ecoli). Sequence data were analyzed using the MLST website. The distribution of STs across the regions was analyzed using BioNumerics software.

Determination of phylogroups. Multiplex PCR was used to segregate isolates resistant to ciprofloxacin into one of the four main phylogroups (A, B1, B2, and D) based on the amplification of the marker genes *chuA* and *yjaA* and the DNA fragment *TSPE4.C2* (18). Classification criteria for group inclusion were as follows: A, *yjaA* positive or *yjaA*, *chuA*, and *TSPE4-C2* negative; B1, *TSPE4-C2* positive; B2, *chuA* and *yjaA* positive or *chuA*, *yjaA*, and *TSPE4-C2* positive; and D, *chuA* positive or *chuA* and *TSPE4-C2* positive.

TABLE 2 Characteristics of PMQR genes among ciprofloxacin-resistant and -sensitive strains

PMQR gene	CIP-R[a] ($n = 302$) (n [%])	CIP-S[b] ($n = 288$) (n [%])	P value
qnrB	5 (1.7)	4 (1.4)	—[c]
qnrS	3 (1.0)	10 (3.5)	0.040
aac(6′)-Ib-cr	74 (24.5)	42 (14.6)	0.002
qepA	36 (11.9)	47 (16.3)	0.125
oqxAB	19 (6.3)	4 (1.4)	0.002

[a] CIP-R, ciprofloxacin-resistant strain.
[b] CIP-S, ciprofloxacin-sensitive strain.
[c] Two cells (50%) had expected counts of <5.

Statistical analysis. Differences in the prevalence of PMQR genes among ciprofloxacin-resistant versus ciprofloxacin-susceptible isolates were measured by Pearson's χ^2 test using SPSS software (SPSS, Chicago, IL), with a significance level (P) of <0.05.

Accession numbers. Two *oqxB* mutants were identified, and their complete sequences were submitted to NCBI with accession numbers KF414080.1 (OqxB20, G148N-G540S-D749E-Y783F) and KF414089.1 (OqxB29, H434Y-V612I-V635I).

RESULTS

Antimicrobial susceptibility. Antimicrobial susceptibilities for all *E. coli* isolates are presented in Table 1. Ciprofloxacin (51.2% isolates resistant) and levofloxacin (47.9% isolates resistant) had MIC_{50} values of 4 mg/liter and respective MIC_{90} values of 64 and 32 mg/liter.

Characterization of PMQR genes and QRDR mutations. PMQR genes were detected in 220 (37.3%) *E. coli* isolates. *qnr* genes were detected in 22 isolates (3.7%), and these comprised *qnrB* (9 isolates, 1.5%) and *qnrS* (13, 2.2%). Genes *qnrA*, *qnrC*, and *qnrD* were not detected. Three variant *qnrB* genes (*qnrB1*, *qnrB4*, and *qnrB6*) were identified, and all *qnrS* genes belonged to the *qnrS1* variant. Efflux pump genes *qepA* (85, 14.4%) and *oqxAB* (23, 3.8%) were detected. Both *oqxA* and *oqxB* genes were detected in 23 isolates, and a single isolate harbored *oqxB* only. All *aac(6′)-Ib* PCR positive isolates (195) were sequenced, among which 116 (19.7% of 590 isolates) had *cr* mutations (75R-152Y), 9 contained D152Y mutations, and 70 were *aac(6′)-Ib*. The PMQR gene combinations were detected in 24 isolates. Two PMQR genes were detected in isolates in the following combinations: 7 isolates, *aac(6′)-Ib-cr/qepA*; 5 isolates, *aac(6′)-Ib-cr/oqxAB*; 4 isolates, *qnrS/aac(6′)-Ib-cr*; 4 isolates, *qnrB/aac(6′)-Ib-cr*; 2 isolates, *qepA/oqxAB*; and 2 isolates, *qnrB/qepA*. One isolate contained three PMQR genes (*qnrB4/aac(6′)-Ib-cr/oqxAB*) (linkage between PMQR and ESBL is shown in Table S2 in the supplemental material). The detection of *aac(6′)-Ib-cr* and *oqxAB* genes was at a higher prevalence in ciprofloxacin-resistant than in ciprofloxacin-sensitive isolates (both $P = 0.002$). The frequency of the *qnrS* gene was lower in ciprofloxacin-resistant than in ciprofloxacin-sensitive isolates ($P = 0.040$), and the prevalence of *qepA* was no different between the two groups ($P = 0.125$) (Table 2). *qnrB* was not suitable for Pearson's χ^2 test (50% of the expected count was <5).

The most common point mutations in ciprofloxacin-resistant isolates were GyrA S83L-D87N (263 isolates, 87.1%) and S83L (21

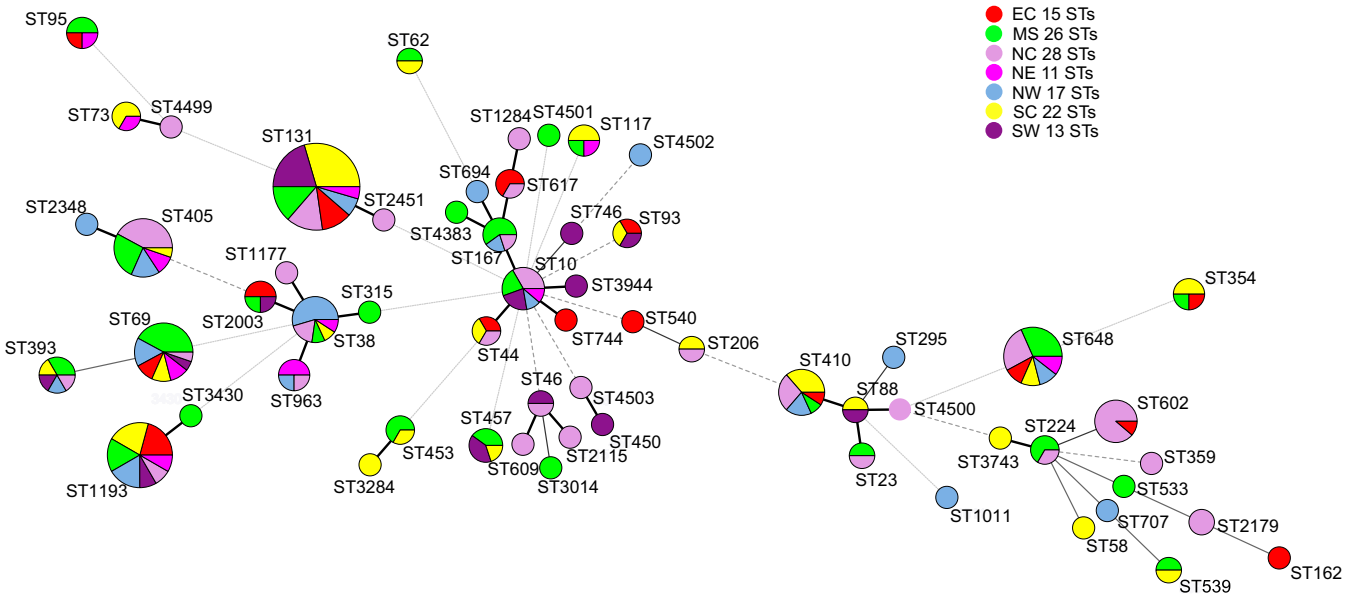

FIG 1 Minimum spanning tree of ciprofloxacin resistant *E. coli* isolates from seven Chinese regions. Solid line indicates one allele difference, dashed line indicates differences in two alleles, and dotted line indicates differences in three or more alleles. EC, east China; MS, central southern China; NC, north China; NE, northeast China; NW, northwest China; SC, south China; SW, southwest China.

isolates, 7.0%), and those for ParC were S80I (233 isolates, 77.2%) and S80I-E84V (35 isolates, 11.6%). Among *gyrB* genes, the most abundant mutation was S492N (32 isolates, 10.6%), and for *parE* genes, it was S458A (80 isolates, 26.5%). The most common mutation of *gyrA* genes among ciprofloxacin-sensitive isolates was S83L (44 isolates, 50.6%). The wild-type forms of the *gyrB* (78 isolates, 89.7%), *parC* (75, 86.2%), and *parE* (87, 100%) genes were the most dominant.

MLST and phylogenetic characterization. A total of 60 different STs were identified from 302 ciprofloxacin-resistant *E. coli* isolates. The most prevalent was ST131 (44 isolates, 14.6%), followed by ST1193 (24 isolates, 7.9%), ST405 (20 isolates, 6.6%), ST69 (19 isolates, 6.3%), and ST648 (19 isolates, 6.3%). The remaining 55 STs were all lower than 3.6% abundance. Five new STs were identified (ST4499 to ST4503). The genetic relationships of the new STs were analyzed with Mega 6.0 using the conserved housekeeping genes that were most closely related, i.e., ST73 (ST4499), ST23 (ST4500), ST453 (ST4501), ST746 (ST4502), and ST450 (ST4503). Ciprofloxacin-resistant *E. coli* isolates fell into each of the four main phylogroups: A, 19.9% strains; B1, 9.8%; B2, 30.5%; and D, 39.8%.

Variation in the regional distribution of resistance genes. *Aac(6′)-Ib-cr* was most abundant in the south (22 strains, 20.2%), central southern (21 strains, 20.2%), and southwest (33, 42.9%) regions of China. *qepA* was the most common in the east (49, 55.1%) and north (4, 11.4%) regions. *qnrS* was the most prevalent in northwest China (10, 12.2%), and *aac(6′)-Ib-cr* (25, 26.6%) and *qepA* (18, 19.1%) were most frequently detected in isolates from north China. Distributions of ciprofloxacin-resistant isolates in the different regions were analyzed using Prim's algorithm with BioNumerics software based on seven alleles. A diverse range of STs were identified from each of the regions. For example, 22 STs were identified from south China and 13 from southwest China. However, the sequences in these regions were dominated by ST131, comprising 26.5% (13 isolates) in south China and 33.3% (9 isolates) in southwest China. Between 11 and 28 STs were identified from the east, central southern, north, northeast, and northwest regions of China; however, no dominant type was isolated (Fig. 1).

DISCUSSION

In this study, we found that 51.2% of *E. coli* isolates associated with community-onset infections at 30 county hospitals across China were resistant to ciprofloxacin and that 37.3% of those strains contained genes associated with PMQR, including *qnr* (3.7% of the total isolates), *aac(6′)-Ib-cr* (19.7%), *qepA* (14.4%), and *oqxAB* (3.8%). A previous study reported that 75% of *E. coli* isolates from urinary tract infections in Chinese tertiary hospitals were resistant to ciprofloxacin. PMQR genes were present in 10.8% of all *E. coli* isolates, consisting of *qnr* (1.8%), *aac(6′)-Ib-cr* (7.2%), and *qepA* (1.8%) genes (8). In another study, 91.4% of clinical *E. coli* isolates from tertiary hospitals were resistant to ciprofloxacin, with 12.8% of all strains containing the PMQR genes, consisting of *qnr* (1.9%), *aac(6′)-Ib-cr* (7.1%), and *qepA* (4.8%) (9). The values reported here for ciprofloxacin MIC_{50} (4 mg/liter) were lower than previously reported (32 mg/ml) for clinical *E. coli* isolates from tertiary hospitals, although the MIC_{90} (64 mg/liter) was the same (19). These findings suggest that in tertiary hospitals, levels of ciprofloxacin resistance are greater and the prevalence of PMQR genes is lower (particularly *aac(6′)-Ib-cr* and *qepA*) than in the county hospitals. The mobile characteristic of PMQR genes allows them to move across plasmids or spread from one species to another via moving elements. Although they provide a low level of quinolone resistance, they can promote mutations of target enzymes such as DNA gyrase and topoisomerase IV. With the accumulation of QRDR mutations, higher-level resistance was present. Along with high-level resistance mediated by QRDR mutations, selection pressure from quinolones was absent,

and in this case PMQR genes may be lost (20). We suggest that evolution by natural selection explains the higher level of fluoroquinolone resistance and the relatively lower prevalence of PMQR genes in Chinese tertiary hospitals.

We identified 60 STs among 302 ciprofloxacin-resistant isolates. The range of STs was diverse; they were isolated from locations throughout the seven regions in China, and there was no apparent point of origin (Fig. 1). This is the first study to report the occurrence of ST2115 and ST3014 in *E. coli* isolates from human samples. ST2115 was initially identified from a bovine host found in Korea, and ST3014 was from rook hosts found in the Czech Republic and Poland. Our findings suggest that ST2115 and ST3014 may spread between animals and humans and that the *E. coli* strain may be copathogenic to humans and animals.

The most abundant MLST type among ciprofloxacin-resistant isolates was ST131 (14.6% of resistant isolates), followed by ST1193 (7.9%), ST405 (6.6%), ST69 (6.3%), and ST648 (6.3%). A recent Chinese hospital study of the main MLSTs associated with extended-spectrum β-lactamase (ESBL)-producing strains of *E. coli* harboring CTX-M-14, CTX-M-55, or CTX-M-15 genes reported that the most common STs were ST131 (14.6%), ST1193 (7.9%), ST405 (6.6%), ST69 (6.3%), and ST648 (6.3%) (21). Prior to this study, ST1193 was unreported in China and was not identified among ESBL (CTX-M-14 or CTX-M-15)-producing *E. coli* isolates from infections in Korea and uropathogenic *E. coli* strains in northwest England (22, 23). The incidence of ST1193 in many other countries remains unreported; however, 0.9% of ciprofloxacin-resistant *E. coli* clinical isolates from Veterans Affairs Medical Centers in the United States contain the MLST ST1193 (24). Our findings revealed that ST1193 was the second most abundant ST among ciprofloxacin-resistant *E. coli* strains across the seven regions in China (Fig. 1) and that this ST in *E. coli* from the virulent B2 phylogroup is a significant pathogen in Chinese clinics (25). This finding warrants further investigation in the future regarding its epidemiology.

Phylogenetic analysis of *E. coli* isolates revealed that 39.8% belonged in phylogroup D and 30.5% in phylogroup B2. Our findings are in general agreement with those of other phylotyping studies of tertiary hospitals (42.4% group D and 29.8% group B2) and a tertiary hospital in Shanghai (54.1% group D and 19.4% group B2) (8, 26). Phylogenetic typing from ESBL-producing clinical *E. coli* isolates in the United States indicated that 50% belonged in phylogroup B2 and 25% in phylogroup D (27). In Spain, 40.2% belonged in phylogroup A and 28.3% in phylogroup B1 (28).

The diversity of resistance genes across the country may have arisen because (i) China is a vast country with great regional economic and cultural diversity where self-medication in patients is evident (29), and (ii) antibacterial agents are widely used in animal breeding in different regions. The regionally diverse and wide distribution of fluoroquinolone-resistant *E. coli* in China suggest that resistance evolved independently on many occasions. However, the rational and controlled use of fluoroquinolone antibiotics is an effective way to control the development of higher levels of resistance in China.

In conclusion, there is a high degree of fluoroquinolone resistance among *E. coli* isolates in Chinese county hospitals, and the prevalence of PMQR genes is also higher. The frequency of fluoroquinolone resistance, the prevalence of PMQR genes, and the range of MLST types differed across the regions. Groups D and B2 were the dominant phylogenetic types found. Fluoroquinolone resistance among *E. coli* isolates from Chinese county hospitals probably emerged from multiple sources and is not spread by clonal strains.

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