Molecular and physiological characterization of fluoroquinolone resistance in relation to uropathogenicity among *Escherichia coli* isolates isolated from Wenyu River, China

Jianxian Sun, Jianying Hu*, Hui Peng, Jiachen Shi, Zhaomin Dong

College of Urban and Environmental Sciences, Peking University, Beijing 100871, China

**Abstract**

Increasing antibacterial resistance and pathogenicity in the environment is of growing concern due to its potential human risk. In the present study, 236 *Escherichia coli* isolates were collected from Wenyu River in China on drugless (48 isolates) and quinolone-containing plates (189 isolates). Their minimum inhibitory concentrations (MICs) were determined ranging from 0.125 μg mL⁻¹ to 128 μg mL⁻¹. Mutation points related to fluoroquinolone resistance were observed at S83 to L and D87 to N or Y in the Gyra subunit and S80 to R or I and E84 to G in the ParC subunit. Generally, MICs of LEV and GAT are dependent on the patterns of these mutation points. The profile with three mutation points was related to LEV-resistant *E. coli* isolates, and the (S83L, D87N + S80I) mutation profile was most prevalent (65.7%) in LEV-resistant isolates, while a large proportion of isolates, even those with three mutation points, were susceptible to GAT. The incidence of virulence factors in LEV-resistant isolates (44.7%, 59/132) was much higher than in nonresistant isolates (23.1%, 24/104) \((\chi^2 = 7.843, 1^\text{df}, p = 0.0079)\). The profile with (S83L, D87N + S80I) mutation points in LEV-resistant isolates was more frequent (53.7%) in uropathogenic isolates than in non-uropathogenic isolates (20.4%) \((\chi^2 = 11.925, 1^\text{df}, p < 0.001)\) indicating that fluoroquinolone-resistant *E. coli* would pose a potential risk. A similar distribution was also found in isolates resistant to GAT \((\chi^2 = 7.843, 1^\text{df}, p = 0.0079)\).
relation between the phenotypes and genotypes of \textit{E. coli} resistant to fluoroquinolone in a natural river basin.

The molecular and physiological characterization of fluoroquinolone resistance in relation to their pathogenicity should be paid particular attention because of their direct hazard to human health. Among \textit{E. coli} strains, UPEC are most common fluoroquinolone-treated pathogens, and are responsible for more than 70\% of UTIs (Kahlmeter, 2000, 2003), most common and problematic global health issues resulting in considerable morbidity and expense (Russo and Johnson, 2003). High percentages of UPECs with various urovirulence factors (\textit{aer, pap, sfa, cnf1, afal}, and \textit{hly}) have been detected in river and lake environments polluted by urban municipal wastewater (Hamelin et al., 2006, 2007), suggesting potential adverse effects on human health. However, there have been no reports of a detailed study on fluoroquinolone resistance in UPEC from a natural river basin.

Our previous paper clarified the incidence (12.2\%) of levofloxacin (LEV)-resistant \textit{E. coli} in the Wenyu River Basin (Hu et al., 2008), where relatively high concentration of quinolones such as LEV and gatifloxacin (GAT) were detected (Xiao et al., 2008). In the present study, we collected 236 \textit{E. coli} isolates from water samples of the Wenyu River Basin in Beijing, China, and investigated their resistance to fluoroquinolones by determining the minimum inhibitory concentrations (MICs) of LEV and GAT. Mutation points related to fluoroquinolone resistance with different MICs were determined by amplifying and sequencing the QRDRs of 69 \textit{E. coli} isolates. Additionally, their uropathogenicities were detected by determining six urovirulence factors using multiplex polymerase chain reaction (PCR). The objectives of this study were (i) to clarify the dependence of fluoroquinolone resistance on the distribution of mutation points in \textit{E. coli} from a natural river and; (ii) fluoroquinolone resistance in uropathogenic \textit{E. coli} isolates. To our knowledge, this is the first study in which the phenotypes and genotypes of fluoroquinolone-resistant uropathogenic \textit{E. coli} isolates from a natural river basin were comprehensively characterized.

2. Materials and methods

2.1. Sample collection and \textit{E. coli} isolation

A total of 236 \textit{E. coli} isolates were isolated from eleven water samples collected along the Wenyu River (Fig. S1, Supporting Information), which flows for 47.5 km and has a catchment area of 2478 km$^2$. About 55\% of the total population of Beijing lives around Wenyu River Basin and its tributaries, the Qing, Ba, and Tonghui Rivers. While most of these areas have sewage treatment, there is discharge from untreated wastewater and livestock farms. A number of pig, sheep, and poultry farms are located in the Shunyi and Changping Districts of the Wenyu River Basin; however little is known on the effects of discharge from untreated wastewater and livestock farms.

Sample preparation and bacteriological tests for \textit{E. coli} isolation were performed by membrane filter (Parveen et al., 1997; Whitlock et al., 2002). Briefly, 500 to 1000 ml water samples were collected in sterile Whirl pack bags (Corning, America) along Wenyu River on 29 May, 2008. All samples were processed within 24 h of collection. Water samples were 10-fold serial diluted and 0.1 ml of each dilution was filtered through nitrocellulose filters (0.47 \textmu m pore-size, 47 mm diameter, Millipore Corporation, America) with the goal of obtaining 30 to 50 colonies per filter. The filters were placed onto \textit{E. coli} chromogenic agar (Chromagar Microbiology, France), and incubated at 44 \textdegree C for 24 h. After 24 h of incubation, colonies that turned blue on \textit{E. coli} chromogenic agar were chosen and streaked onto LB agar (BD, America) and incubated at 37 \textdegree C for 24 h. Approximately 25 isolates were collected with dilution method using 10 disks for each water sample. All isolates were randomly chosen from independent colonies growing on the disks and the number of selected isolates from each disk was less than four to avoid clones. The \textit{E. coli} were isolated as per previous research (Hu et al., 2008). To obtain \textit{E. coli} with different resistances, isolates were selected from duplicated fluoroquinolone-free plates and fluoroquinolone-containing plates (plates with 2 \mu g ml$^{-1}$ LEV, 2 \mu g ml$^{-1}$ GAT, 8 \mu g ml$^{-1}$ LEV and 8 \mu g ml$^{-1}$ GAT, respectively).

The pure cultures were then used to inoculate 1\% tryptone water (Oxoid, UK) and EC broth containing 4-methylumbelliferyl-D-glucuronide (Oxoid, UK) and incubated for 24 h at 37 and 44 \textdegree C, respectively. Isolates that produced indole from tryptophan and that were positive for gas production and fluorescence in EC broth containing 4-methylumbelliferyl-D-glucuronide were designated \textit{E. coli} isolates and used for subsequent studies. 16S rDNA sequences were analyzed to confirm all isolates using the universal eubacteria primers 27F (5' - AGA GTT TGA TCM TGG CTC AG-3') and 1492R (5' - TAC GGY TAC CTT GTT AGC ACT T-3') for amplification of 16S rDNA genes. Amplification was performed with a S320 Thermal Cycler (Beijing Botong Tech, China). The amplification was initiated by incubating the reaction mixture at 95 \textdegree C for 9 min, followed by 95 \textdegree C (1 min), 55 \textdegree C (1 min), and 72 \textdegree C (2 min) for 30 cycles and a final extension step at 72 \textdegree C (10 min). Products were sequenced by Applied Biosystem 3730XL sequencer according to the manufacturer’s instructions, and were subsequently compared for sequence identity using NCBI Blast.

2.2. Determination of fluoroquinolone susceptibility

LEV and GAT were used as inhibiting drugs since ofloxacin was detected with highest concentration among quinolones in environment (Nakata et al., 2005), and GAT was firstly found in this river (Xiao et al., 2008). Isolates were screened to determine their MICs to LEV and GAT on Mueller–Hinton agar (Oxoid) by a disk diffusion method, as described by the CLSI (Clinical and Laboratory Standards Institute) 2005 guidelines (CLSI, 2009). The MICs ranged from 0.125 to 128 \mu g ml$^{-1}$. All agar plates were incubated for 18 h at 38 \textdegree C, with \textit{E. coli} ATCC 25922 used as the reference strain. The MIC was recorded as the lowest concentration (in micrograms per milliliter of agar) of antibiotic at which the isolate was restrained (less than three colonies were left), as judged visually; a slight haze of growth was ignored. All tests were conducted in duplicate.

2.3. PCR amplification and DNA sequencing of QRDRs

Sixty-nine \textit{E. coli} isolates (32 resistant isolates and 37 sensitive isolates) at different resistance levels (each MIC) were randomly chosen for mutation point analysis, and their QRDRs of \textit{gyrA} and \textit{parC} were amplified and sequenced. The PCR assays and primers (Supporting Information, Table S1) used for the PCR amplification of QRDRs of \textit{gyrA} and of \textit{parC} were based on previous research (Everett et al., 1996). The PCR assays were conducted using a Takara Ex Taq kit (Takara, Japan) in a 50 \mu l volume reaction. The PCR mixture consisted of 5 \mu l 10× Ex Taq buffer (Mg$^{2+}$ Plus): 4 \mu l dNTPs (2.5 mM each); 0.2 \mu M of each primer; 2 \mu l of bacteria incubated on liquid LB at 37 \textdegree C for 24 h prior to use; and 0.25 \mu l 5 \mu M 1× Ex Taq DNA polymerase. Each PCR was performed with a S320 Thermal Cycler (Beijing Botong Tech, China). The PCR was initiated by incubating the reaction mixture at 94 \textdegree C for 5 min, followed by 94 \textdegree C (1 min), 55 \textdegree C (1 min), and 72 \textdegree C (1 min) for 30 cycles and a final extension step at 72 \textdegree C (10 min).

All PCR products were loaded on a 2% horizontal agarose gel and the bands were purified by an Asymprep DNA Gel Extraction Kit. The purified products were then sequenced by the Applied Biosystem...
3730XL sequencer according to the manufacturer’s instructions. Sequences of gyrA and parC were analyzed by Bioedit software.

2.4. PCR amplification procedures for virulence genes

Six urovirulence genes encoding for the following factors were identified by Multiplex PCR: extraintestinal E. coli attachment factors (pap, sfa, and afa); alpha-hemolysin (hly), a toxin often produced by UTI strains leading to cell lysis; cytotoxic necrotizing factor I (cnf1); and aerobactin determinant (aer), which competes with serum transferrin for iron uptake. The primers and the PCR procedure were based on published methods (Yamamoto et al., 1995), and the primer sequences were listed in Table S2 (Supporting Information). All PCR experiments contained a negative control (ATCC25922) and a blank control. Each PCR product (6 μL) was mixed with 1.2 μL 6× loading buffer dye (Takara, Japan) and loaded on a 1.5% horizontal agarose gel (agarose HT, Amresco, America) together with a 100 bp-size ladder (Takara, Japan). All gels were run in a 50× TAE buffer (Dingguo, China) for 30 min and 100 V, stained for 20 min in a 50 g of ethidium bromide per mL, and visualized by UV transillumination (Gel Doc 2000, Bio-Rad Laboratories; Milan, Italy).

2.5. Statistical analysis

A χ² test was used to test the differences between urovirulence rates in nonresistant and resistant E. coli, with difference considered significant if p < 0.01. Differences between MICs were evaluated by Mann–Whitney test. Statistical analyses were performed with SPSS 18.0.

3. Results and discussion

3.1. Isolation and fluoroquinolone resistance of E. coli isolates

Median values for E. coli in samples taken from Wenyu River ranged from 9 cfu mL⁻¹ (site 19) to 6.6 × 10⁶ cfu mL⁻¹ (site 20), similar with those in our previous investigation (Hu et al., 2008) but much higher than those in Kunming Lake where only two E. coli isolates were screened from 50 mL water. Totally 236 E. coli isolates were identified from Wenyu River samples and were further confirmed by 16S rDNA sequence analysis. Finally, 48 E. coli strains were obtained from drugless plates and 188 isolates from fluoroquinolone containing plates. MICs of LEV and GAT for all E. coli isolates were determined to characterize resistance to fluoroquinolones. According to CLSI (CLSI, 2009), MIC breakpoints for E. coli to LEV and GAT are resistant at ≥8 μg mL⁻¹, intermediate at 4 μg mL⁻¹, and susceptible at ≤2 μg mL⁻¹. In this study, among the 48 E. coli isolates isolated from the drugless plates, eight were resistant to LEV, with MICs of 8 μg mL⁻¹ for six isolates, 16 μg mL⁻¹ for one isolate, and 32 μg mL⁻¹ for one isolate. It should be noted that the LEV-resistant isolate with a MIC of 32 μg mL⁻¹ was isolated from a sample taken from the river, which contained no other antibiotics. The MIC of LEV for this isolate was 8 μg mL⁻¹, but it was resistant to GAT with a MIC of 32 μg mL⁻¹.

Thus, according to the results of MIC determination, the incidence of resistance to LEV and GAT in this river basin was estimated to be 16.7% (8/48) and 2.1% (1/48), respectively. The incidence of resistance to LEV is similar to our previous work (12.2%) in the same aquatic environment (Hu et al., 2008) suggesting a continuous input of fluoroquinolone resistant E. coli. More isolates with different resistance were obtained from drug-containing plates, and the highest MIC to LEV was up to 128 μg mL⁻¹. Since the fluoroquinolones are a group of synthetic antibiotics, the LEV and/or GAT resistant isolates in the environment should be originated from contamination of human activity or livestock rather than from naturally developed resistance. This was supported by the fact that no resistant E. coli isolates were isolated from Kunming Lake, which incurs little human activity.

Four GAT resistant E. coli isolates screened from the natural river water showed MICs up to 32 μg mL⁻¹, which were comparable to those reported in clinical or veterinary isolates (Boyd et al., 2009). Due to increasing resistance to fluoroquinolones in clinical and veterinary isolates (Goettsch et al., 2000), new drugs have been developed to fight resistance. Consequently, GAT was a new C₈-methoxy fluoroquinolone developed as a fourth generation quinolone antibiotic. It has been widely used in China and was first detected in the environment with a concentration of 16–42 ng L⁻¹ (Xiao et al., 2008). Although GAT is effective against some quinolone-resistant E. coli isolates and more potent than other fluoroquinolones (Lu et al., 1999), results from the present study showed that even for new antibiotics developed to combat increasing antibiotic resistance, their resistant isolates have spread to aqueous environments.

3.2. Molecular characterization of resistance and relation with MIC

A total of 69 isolates with MICs ranging from 0.125 μg mL⁻¹ to 128 μg mL⁻¹ were randomly chosen to determine the characteristics of resistance. Since mutations in gyrB and parE genes are seldom detected (Everett et al., 1996; Wang et al., 2001), we focused on the QRDRs of gyrA and parC genes in the 69 E. coli isolates and compared their sequences with E. coli ATCC25922. Silent nucleotide sequence mutation points with no amino acid alteration were ignored. In this study, mutation points at positions S83 to L and D87 to N or Y were observed in gyrA (Supporting Information, Fig. S2), and position S80 to R or I and E84 to G were observed in parC (Supporting Information, Fig. S3), which were less complicated compared with that reported in clinical E. coli (Everett et al., 1996; Lindgren et al., 2003) where, besides mutation points observed in this study, other patterns including S83 mutation to W (S83 W), A (S83 A), and V (S83 V), D87 to G (D87 G), H (D87 H), and V (D87 V) in gyrA, and E84 mutation to K (E84 K) and V (E84 V) in parC were also reported (Ruiz, 2003).

The relationship between the mutation points of the 69 isolates and their MICs were investigated to determine the potential mechanism of resistance to fluoroquinolones. As shown in Fig. 1a, MICs of LEV were generally dependent on the patterns of mutation points. It was expected that all isolates with a MIC of 0.125 μg mL⁻¹ to LEV carried no mutation in either subunit. A single mutation S83L in gyrA was detected in seven of the nine isolates with a MIC of 0.25 μg mL⁻¹. The S83L mutation was a preliminary step to fluoroquinolone resistance, and was the most prevalent mutation in both resistant and susceptible isolates and appeared in 54 of the 69 E. coli isolates. This phenomenon is similar to that shown in clinical and veterinary studies (Everett et al., 1996). Previous research shows E. coli with a single S83L mutation in the gyrA subunit are resistant to nalidixic acid, a first generation quinolone (Vila et al., 1994), therefore the wide appearance of S83L isolates in the river would pose a potential risk of quinolone resistance. While two mutation points emerged in three of eight isolates with a MIC of 0.5 μg mL⁻¹, and became the major profile in isolates with a MIC of 1 μg mL⁻¹, three mutation points appeared in one of three isolates with a MIC of 2 μg mL⁻¹. The two intermediate isolates possessed no wild type, and contained three mutation points, (S83L, D87N + S80I) and (S83L, D87Y + S80I), respectively.

Profiles with three mutation points explained the resistance mechanism for most resistant isolates with MICs of 8, 16, 32, 64, and 128 μg mL⁻¹, and MICs of isolates with three mutation points were significantly higher than those with less mutation points (p < 0.01, Mann–Whitney test). The (S83L, D87N + S80I) mutation profile was the most prevalent in the resistant isolates (65.7%) with MICs of 8 μg mL⁻¹ (8/9), 16 μg mL⁻¹ (7/8), and 32 μg mL⁻¹ (8/10).
This indicates that the D87N mutation point largely improved resistance to fluoroquinolones. This result is similar with resistant isolates from veterinary and clinical studies, where codon 87 was an important binding point in GyrA subunit in vivo (Everett et al., 1996; Madurga et al., 2008) and mutation D87N was much more popular than D87H or D87G. It should be noted that all 29 resistant isolates contained at least three mutation points, although E. coli resistant to ciprofloxacin with two mutation points, usually (S83L + D87N), (S83L + S80I), or (S83L + E84K), have been described in clinical isolates (Everett et al., 1996; Lindgren et al., 2003). Only three isolates with a MIC of 128 µg mL⁻¹, 32 µg mL⁻¹, and 16 µg mL⁻¹ carried four mutation points (S83L, D87Y + S80I, E84G), in which E84G was also sporadically observed in clinical or veterinary isolates as a third or fourth mutation (Everett et al., 1996; Vila et al., 1996). As for MICs of GAT (Fig. 1b), a significant percentage of isolates (58.8%, 20/34) with MICs lower than 1 µg mL⁻¹ carried one or two mutation points, while two or three mutation points were observed in isolates with MICs of 2 µg mL⁻¹, 4 µg mL⁻¹, 8 µg mL⁻¹ and 16 µg mL⁻¹. Isolates with the (S83L, D87Y + S80I) mutation profile showed a wide range of MICs (2–32 µg mL⁻¹) and explained the resistant mechanism for most resistant isolates with MICs of 16 and 32 µg mL⁻¹. While the mutation profile trend for GAT MICs was similar with LEV MICs, the same mutation patterns of GAT resistant E. coli were observed at lower MICs than LEV.

### 3.3. Uropathogenicity of E. coli isolates and relation with fluoroquinolone resistance

To better understand the potential risks of fluoroquinolone-resistant E. coli in environment, the uropathogenicity of all 236 isolates was investigated since fluoroquinolones are used with increasing frequency in the treatment of UTIs in many counties after increased resistance to trimethoprim-sulfamethoxazole (SXT) (Hooper, 1998; Talan et al., 2000), which was the standard antibiotic for UTI therapy in the past. Totally, 35.2% (83/236) of the screened E. coli from Wenyu River carried one or more urovirulence related factors (Table 1). The urovirulence factors, including aer, pap, sfa, cnfI, afaI, and hly are related to in vivo pathogenicity in animal models of extraintestinal infection, and therefore their detection can be used to infer actual virulence potential (Johnson, 1991; Picard et al., 1999). Twelve distinct patterns of these virulence factors were observed but were less diverse than that in clinical isolates (Birosova et al., 2004). Pathogenic E. coli isolates with single aer accounted for 25.0% of the 236 E. coli isolates, while those that presented with afaI, sfa, pap, pap + afaI, and pap + hly accounted for 2.1%, 1.7%, 2.5%, 0.4%, and 0.4%, respectively (Table 1). It should be noted that the aer gene accounted for 91.5% of the 83 pathogenic E. coli isolates, a relatively high occurrence compared with other virulence factors. This may be due to the relationship of aer genes to the aerobactin system, which effectively promotes E. coli growth in aquatic environments even at low soluble iron levels (Delorenzo and Martinez, 1988; Wandersman and Delepelaire, 2004). As limited iron availability in human and animal hosts is one of the first lines of defense against pathogenic bacteria infecting target cells, E. coli carrying aer can more easily grow in organisms due to the aerobactin system. E. coli isolates with the aer gene are of particular concern as they are frequently associated with UTIs, surgical prophylaxis, and enteric infections in humans and animals (Johnson, 1991).

The distribution of virulence factors in pathogenic E. coli isolates over LEV-resistance degree (expressed as the MIC of LEV) is shown in Fig. 2a. Of the 104 non-resistant isolates, 24 (23.1%) contained one or more virulence genes and eight profiles of virulence factors were observed. The single aer gene was most prevalent, accounting for 11.5% of the non-resistant (susceptive and intermediate to LEV) isolates, followed by aer + pap (2.9%), aer + sfa (2.9%), pap (0.96%), hly (0.96%), aer + afaI (1.9%), pap + cnfI (0.96%), and sfa + hly.
(0.96%). A total of 59 (44.7%) of the 132 LEV-resistant E. coli isolates carried virulence genes and nine profiles of virulence factors were detected, with aer again the most prevalent (35.6%). Two LEV-resistant isolates contained three virulence genes with aer + pap + afaI (0.8%), and aer + pap + hly (0.8%), respectively. The remaining six profiles included pap (0.8%), sfa (0.8%), afaI (0.8%), aer + pap (2.3%), aer + sfa (0.8%), and aer + afaI (2.3%). It should be noted that the rate of isolates containing virulence factors in LEV-resistant isolates (44.7%) was significantly higher than in non-resistant isolates (23.1%) ($\chi^2 = 11.925, 1^\circ$ of freedom, $p < 0.001$) (Fig. 2a), indicating that fluoroquinolone-resistant E. coli in this river would pose a human risk. A similar distribution was also found in isolates resistant to GAT ($\chi^2 = 7.843, 1^\circ$ of freedom, $p = 0.0079$) (Fig. 2b). This result may because quinolones are frequently used to inhibit pathogenic E. coli isolates in clinics, which may increase the opportunity for pathogenic E. coli isolates to form resistance.

UPEC, especially those resistant to fluoroquinolones, make people under exposure through various pathways such as swimming, fishing, having a barbecue or taking photographs around the river. UTIs caused by UPEC are very popular in women as reported that almost half of all women would experience at least one UTI during their lifetime (Foxman, 2003). The fluoroquinolone resistance of pathogenic isolates erodes the usefulness of fluoroquinolones in the effective management of UTIs. It has been reported that unsuccessful SXT – treatment of UTIs increased twice in SXT – susceptible pathogens (Talan et al., 2000). Since fluoroquinolones are used as an alternative to SXT with increasing frequency in the treatment of UTIs, 16.7% of resistance profiles included pap (0.8%), afaI (0.8%), respectively. The remaining six profiles were factors aer pap hly, aer pap, aer sfa, aer afaI, sfa, and pap.

References


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