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Original article

Expression of *marA* is remarkably increased from the early stage of development of fluoroquinolone-resistance in uropathogenic *Escherichia coli*

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ABSTRACT

Background: Analyses of efflux pumps overexpression and mutations in quinolone resistance determining region (QRDR) in early stage of development of resistance to fluoroquinolones (FQs) are valuable to discuss countermeasures against them. We induced levofloxacin (LVFX)-resistant strains from susceptible uropathogenic *Escherichia coli* in vitro to analyze the mechanisms of development of FQs-resistance.
Methods: 89 strains were exposed to discontinuous elevation of LVFX dose, and mRNA level of efflux pumps and their regulators as well as mutations developed in QRDR of LVFX-resistant strains were analyzed.
Results: In 5 strains, a stepwise increase in MIC to LVFX (up to >128 μg/ml)was observed. Compared to the parent strains, additional mutations in QRDR were observed in the strains developing high MIC. Remarkable increase of *marA* expression was observed even in the early stage of LVFX-resistance development, and it lasted until high-level resistance was developed. On the other hand, moderate increase in *acrB* expression but only low increase in *yhiU*, *yhiV*, *mdfA*, *tolC* and *sdiA* were observed.
Conclusions: These results suggested that *marA* expression is a sensitive marker for early detection of development of LVFX-resistance.

1. Introduction

Since *Escherichia coli* (*E. coli*) is the bacteria most commonly isolated from patient with urinary tract infection (UTI), the emergence of *E. coli* isolates that are resistant to some of the most commonly used antimicrobial drugs has become a major problem in the management of UTI [1–3]. Fluoroquinolones (FQs) are the most widely used antibiotics worldwide, and are the drugs of choice for empirical therapy for UTI. Increasing emergence of FQs-resistant *E. coli* has been reported worldwide [3–5]. It has been reported that >10% of *E. coli* isolated from uncomplicated UTI and 30–40 % of *E. coli* isolated from complicated UTI were resistant to FQs [1,2]. It is not hard to understand that these high frequencies of FQs-resistant strains affect therapeutic strategy for UTI.

FQs can directly bind to DNA gyrase and topoisomerase IV, and inhibit the activity of these enzymes that are essential for DNA replication and transcription. The mechanism of resistance to FQs mainly consists of two separate strategies: a decrease in drug accumulation in the bacterial cells through an induction of efflux pumps overexpression and mutations in the genes encoding DNA gyrase (*gyrA* and *gyrB*) and topoisomerase IV (*parC* and *parE*). Previous reports demonstrated that multi-step process is responsible for the development of high-level resistance to FQs [6]. In the early step, low-level resistance is developed by overexpression of efflux-pumps which facilitate active extraction of FQs [7]. Five superfamilies of efflux pumps (RND (resistance-nodulation-division), MF (major facilitator), ABC (ATP binding cassette), MATE (multidrug and toxic efflux) and SMR (small multidrug resistance)) that

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promote resistance to various antimicrobial drugs had been described in *E. coli* [8,9]. And, AcrA-AcrB-TolC and YhiU-YhiV-TolC pumps belonging to RND superfamily and MdfA-Cmr pump belonging to MS superfamily were reported to be involved in excretion of FQs [9,10]. In addition, it has been reported that overexpression of *sdiA* and *marA*, both of them are the positive regulator for AcrA-AcrB-TolC pump expression, were associated with the resistance to FQs in *E. coli* [11,12]. SdiA is a quorum-sensing regulated transcription factor, and is involved in the regulation of cell division. There is increasing evidence that molecules involved in quorum-sensing participate in development of multidrug resistance [13,14]. Deletion of *sdiA* led to decreased level of the AcrB expression, which resulted in the decreased level of drug resistance [13]. MarA is the global activator, which modulates the expression of many genes including *acrA*, *acrB* and *tolC* [12]. It is known that expression of *marA* is affected by the presence of salicylate through the inactivation of transcription repressor MarR, and by the redox-inducible SoxS which is the positive regulator of *marA* expression. Although these efflux pumps expressions are rapid response observed in early stage of drug-resistant development, it conferred only 2- to 8-fold elevation in MIC [7] while, in the late step, high-level resistance is developed by the mutation in quinolone resistance determining regions (QRDRs) of *gyrA*, *gyrB*, *parC* and *parE*. Recently, we and others has reported that mutation at Ser83 and Asp87 in *gyrA* and Ser80 in *parC* are frequently observed in FQs-resistant strains isolated from UTI patient although single point mutation at Ser83 or Asp87 in *gyrA* was not sufficient to develop resistance to FQs [6,15]. Further, we have also reported that there is a phylogenetic preference among FQs-resistant strains [15]. Phylogenic group B2 was dominant among FQs-resistant strains (49.4%) although the dominancy was lower than that in FQs-susceptible strains (77.5%), and phylogenic group D which was second most prevalent phylogenic group among FQs-resistant strains (34.8%) is more observed among FQs-resistant strains than among FQ-susceptible strains (12.4%), suggesting that the acquisition of FQs-resistance might be frequent in the limited strains such as phylogenic group B2 or D rather than other phylogenic groups.

The mechanisms of FQs-resistance development have been mostly discussed based on statistical comparison between FQs-sensitive and FQs-resistant clinical isolates. Whereas the status during resistance development process has been much less investigated [7,11]. In the present study, we induced drug-resistant *E. coli* strains in vitro by exposure to levofloxacin (LVFX), which is the drug commonly used for treating UTIs, cystitis and pyelonephritis. The expressions of efflux pumps and their transcriptional regulators, and mutation in QRDR in early and late stage of resistance development were analyzed.

2. Materials and methods

2.1. Bacterial strains

Eighty nine FQ-susceptible *E. coli* strains, including 5, 4, 69, and 11 strains in phylogenic groups A, B1, B2, and D, respectively, were isolated from the patient with cystitis as described in previous study [15]. MICs for LVFX and mutation profiles in QRDR of these FQ-susceptible *E. coli* strains are summarized in Table 1.

2.2. LVFX-resistant strain development

FQ-susceptible *E. coli* strains were inoculated onto LB agar plate containing 0.5, 1, 2, 4 and 8 μg/ml LVFX (DAIICHI SANKYO PROPHARMA), and incubated at 37 °C for 7 days (first selection). Low-resistant isolates obtained in first selection were inoculated onto LB agar plate containing 4, 8, 16, 32 and 64 μg/ml LVFX and incubated at 37 °C for 7 days (second selection). Further, intermediate-resistant isolates obtained in second selection were inoculated onto LB agar plate containing LVFX of up to 128 μg/ml. All the developed strains were maintained on the agar containing LVFX at the same concentration with MICs of each strain until further analysis.

Table 1
MICs for LVFX and mutation profiles in QRDR of FQs-susceptible *E. coli* strains used in this study.

MIC for LVFX (μg/ml)	Mutation profiles in QRDR	No. of parent strain	Phylogenic				No. of resistant strains developed
			A	B1	B2	D	
0.016	None	9	1	0	7	1	0
	gyrA: S83L	1	0	0	1	0	0
0.03	None	25	1	2	20	2	0
	parE: D475E	1	0	0	0	1	0
0.06	None	34	2	0	30	2	0
	parE: D475E	1	0	0	0	1	0
0.13	None	2	0	1	1	0	0
	gyrA: S83L	2	0	1	1	0	0
	gyrA: D87G	2	0	0	2	0	0
	gyrA: D87G, *parE*: D475E	1	0	0	0	1	0
0.25	None	1	1	0	0	0	0
	gyrA: S83L, *parE*: D475E	1	0	0	0	1	1
0.5	None	1	0	0	1	0	0
	gyrA: S83L	6	0	0	5	1	3
1	None	1	0	0	0	1	1
	gyrA: S83L	1	0	0	1	0	0
Total		89	5	4	69	11	5

2.3. Sequence analysis of QRDR

Sequences of QRDRs of *gyrA*, *gyrB*, *parC* and *parE* in strains that developed LVFX-resistance were analyzed as described previously [15].

2.4. Quantitative analysis of mRNA expression of efflux pumps and their regulators

From the overnight cultures of *E. coli* strains in liquid LB medium containing LVFX at the same concentration with MICs of each strain, the total RNA were isolated using RNeasy Mini Kit (QIAGEN) according to the manufacture's instructions. Quality of isolated total RNA were analyzed by electrophoresis in 1.3% NuSieve3:1 Agarose (Lonza)/1 × TAE gel following visualization by LAS-1000 (Fujifilm). The samples for electrophoresis were prepared by using RNA loading buffer AG+ (BioDynamics Laboratory Inc.). The cDNA were prepared from the obtained total RNA by using PrimeScript RT reagent Kit (Perfect Real Time) (Takara Bio, Inc.) according to the manufacture's instructions.

The expression level of mRNA for components of efflux pumps (*yhiU*, *yhiV*, *mdfA*, *acrB* and *tolC*), transcription regulators of efflux pumps (*marA* and *sidA*) and a housekeeping gene, *gapA* (as an internal control) were analyzed by quantitative real-time PCR using SYBR® Premix Ex Taq™ II (Tli RNaseH Plus) (Takara Bio, Inc.) in 7500 Real-Time PCR System (Applied Biosystems). The gene-specific primers for *yhiU*, *yhiV*, *mdfA*, *marA* and *gapA* were designed as described by Yasufuku et al. [16] and the gene-specific primers for *acrB*, *tolC* and *sidA* were designed as described by Tavio et al. [11]. The PCR reaction mixture for *gapA*, *yhiU* and *yhiV* were subjected to the following PCR program: 30 s at 95 °C, and then 40 cycles of denaturation at 95 °C for 5 s and annealing/extension at 62 °C for 35 s. The PCR reaction mixture for *marA*, *mdfA*, *sidA*, *acrB* and *tolC* were subjected to the following PCR program: 30 s at 95 °C, and then 40 cycles of denaturation at 95 °C for 5 s, annealing at 55 °C for 30 s and extension at 72 °C for 35 s. The specificities of PCR