ORIGINAL ARTICLE

High prevalence of fluoroquinolone-resistant Escherichia coli strains isolated from urine clinical samples

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Keywords

Antimicrobial resistance • Quinolone resistance-determining region • Urinary tract infection • Mutations

Summary

Background. *Fluoroquinolone resistant Escherichia coli isolates have become an important challenge in healthcare settings in Iran. In this study, we have determined Fluoroquinolone resistant E. coli isolates (from both outpatients and inpatients) and evaluated mutations of gyrA and parC within the quinolone resistance-determining regions (QRDR) of these clinical isolates.*
Materials and methods. *Clinical isolates were recovered from the urine sample of patients with urinary tract infections admitted at Alzahra hospital, Iran, between September and February 2013. We assessed antimicrobial susceptibility of all isolates and determined mutations in QRDR of gyrA and parC genes from 13 fluoroquinolone-resistant isolates by DNA sequencing.*
Results. *A total of 135 E. coli strains were obtained from 135 patients (91 outpatients and 44 inpatients). The resistance rate of fluoroquinolones (Ciprofloxacin, Norfloxacin and Ofloxacin) among our strains was 45.2%. Two E. coli isolates were shown just a single mutation, but other isolates possessed 2-5 mutations in gyrA and parC genes. Mutations in the QRDR regions of gyrA were at positions Ser83 and Asp87 and parC at positions Ser80, Glu84, Gly78.*
Conclusions. *Ciprofloxacin is the most common antimicrobial agent used for treating urinary tract infections (UTIs) in healthcare settings in Iran. Accumulation of different substitutions in the QRDR regions of gyrA and parC confers high-level resistance of fluoroquinolones in clinical isolates.*

Introduction

Urinary tract infections (UTIs) are one of the most frequent bacterial infections around the world that almost occurs in the healthcare setting [1]. UTIs are the second most common type of infections in the human that poses a serious health problem because of the antibiotic resistance and high recurrence rates. The available data shows that 150 million cases of UTIs occur on a global basis per year, resulting in more than 6 billion dollars in treatment costs [2]. Uropathogenic *Escherichia coli* (UPEC) is the essential cause of UTIs, including both cystitis and pyelonephritis and are responsible for more than 80% of these infections [3, 4]. It is supposed to, the pathogenic potential of UPEC isolates is dependent on a multitude of virulence factors (VFs) located on chromosome regions, referred to "pathogenicity islands" (PAIs) [3]. These different virulence factors promote colonization and infection of urinary tract [2].
Due to the complications of urinary tract infections, well-timed treatment of these infections has special importance and treatment often accomplish based on the most prevalent pathogenic bacteria [5]. First, quinolones were introduced with nalidixic acid in 1962 for the treatment of UTIs. In five decades, different generations of quinolones have introduced for clinical use. Since it was specified that fluoroquinolones have more potency than older quinolones, therefore use of these expanding classes of antimicrobial agents increased significantly [6]. Fluoroquinolones are essential antimicrobial agents used to treat UTIs [7]. Ciprofloxacin is the most frequently used fluoroquinolone for the treatment of UTIs in healthcare settings, because of its availability in oral and intravenous formulations [8, 9].
Quinolones act via inhibition of DNA synthesis by promoting cleavage of bacterial DNA in the DNA-enzyme complexes of DNA gyrase and type IV topoisomerase, resulting in rapid bacterial death [10]. Clinical experience has shown diverse antibiotic resistance among uropathogens [11-13]. The increased use of fluoroquinolones has caused a remarkable emergence of resistance that varies by both organism and geographic region [6]. This resistance commonly is the consequence of mutations involving genes encoding *gyrA* and *parC* [14]. In *E. coli*, alternation at positions Ser-83 or Asp-87 in *gyrA* and Ser-80 and Glu-84 in *parC* are the most frequent mutations [15]. The other substitutions are rare in clinical isolates [16].
The aim of this study was to determine the patterns of antimicrobial resistance and the presence of mutations in quinolone resistance coding regions in *gyrA* and *parC* in clinical isolates of *E. coli* from a hospital in Isfahan, Iran.

Methods

Data collection

In order to describe the demographic and clinical characteristics of patients with urinary tract infections, admitted patients were selected. Due to lack of access to inpatients, a permission was reached to access the inpatient files. The final results were summarized after careful examination of the files.

Bacterial isolates

All clinical isolates were recovered from 135 consecutive and not repetitive urine specimens of patients (91 outpatients and 44 inpatients) with urinary tract infections admitted at Alzahra hospital, Isfahan, Iran, between September and February 2013. Diagnosis of *E. coli* isolates have done according to Bailey & Scott's diagnostic microbiological and biochemical methods, including appearance of bacterial colonies on culture medium, Gram staining, shape, motility, catalase, oxidase, MR, VP, oxidative/fermentative (OF), indole, citrate, urease, nitrate reduction, H2S, Gas, PYR, CAMP, gelatin, coagulase, bile solubility, DNase, fermentation of Fructose, Glucose and Lactose tests [17].

Susceptibility testing

Susceptibility testing was determined by disk diffusion technique as described in the Clinical and Laboratory Standards Institute (CLSI) guidelines [18], using Mueller Hinton medium (Himedia Company). Antimicrobial disks used in this study (purchased from Himedia Company) were: Ciprofloxacin (5µg), Norfloxacin (10µg), Ofloxacin (5µg), Nalidixic acid (30µg), Amikacin (30µg), Ampicillin (10µg), Cefotaxime (30µg), Gentamicin (10µg), Nitrofurantoin (300µg), Trimethoprim/sulfamethoxazole (1.25/23.75µg), Cefoxitin (30µg), Meropenem (10µg), Cefepime (30µg), Ceftazidime (30µg), Cephalothin (30µg).

Escherichia coli ATCC25922 was used as a quality control strain. Then the data were entered into Whonet 5.6 (WHO, Geneva, Switzerland) software.

Preparation of bacterial DNA

Quinolone-resistant isolates (61 isolates) were cultured according to Baily & Scott's quantitative culture method [17] and cultured on Eosin Methylene Blue (EMB) and blood agar (BA) medium at the same time. DNA to be amplified was extracted from these isolates by boiling. In this method, cell pellets were transferred to 50µl of distilled water in an eppendorf tube and incubated at 100°C for 10 min. After centrifuging of the lysate at 6000×g for 10 min, the supernatant was stored at -20°C as a template DNA stock [19, 20].

PCR and DNA sequencing

Polymerase chain reaction (PCR) was performed by primer sequences designed for quinolone resistance-determining region (QRDR) of *gyrA* and *parC* genes of *E. coli* isolates. Oligonucleotide primers for the PCR amplification in this study have been shown in Table I.

DNA was amplified using an initial denaturation step of 5 min at 95°C, followed by 30 cycles consisting of 30 seconds at 94°C, 30 seconds at the annealing temperature of 55°C and 58°C (for *gyrA* and *parC,* respectively) and 45 seconds at 72°C, and a final extension step of 10 min at 72°C. PCR products were resolved by electrophoresis on 1.2% agarose gel containing ethidium bromide. Afterward, among fluoroquinolone resistant *E. coli* strains, 13 isolates randomly selected for genetic characterization of the QRDR of the *parC* and *gyrA* genes by sequencing process (Macrogene Company, Macrogen Inc., Seoul, Korea). *E. coli* ATCC25922 was used as a quality control for all PCR and sequencing reactions. After all, sequences were compared with the nucleotide sequence of the *gyrA* and *parC* genes in the GenBank database (accession numbers: FN554766.1 and CP003034.1respectively). These data were analyzed using MEGA4 and Gene Runner softwares.

Results

Distribution of fluoroquinolones resistant *E. coli* strains was determined in different age and gender groups. Most resistant isolates were observed among outpatient adult women (Tab. II). Based on the results of our study, meropenem, cefoxitin, amikacin, nitrofurantoin and gentamicin showed the best activity against *E. coli.* Percentage of resistance to antimicrobial agents is shown in Figure 1. No resistance has observed to meropenem while ampicillin has shown the least activity against *E. coli* isolates. Among the inpatients individuals, 50% had a history of catheter utilization, and 57% had a history of surgery.

A high resistance to three antibiotics ciprofloxacin, norfloxacin and ofloxacin was observed among strains (Fig. 1). Resistance rates to these three antibiotics were completely equal (45.2%). Out of the 135 *E.coli* isolates, 61 strains were resistant to fluoquinolones and all fluoquinolones- resistant strains include *gyrA* and *parC* genes (Fig. 2).

After sequencing process, two mutations were detected in the QRDR of *gyrA* gene, one at position 83

Tab. I. Oligonucleotide sequences of primer sets for PCR.

Primer	Sequence	PCR Product Size(bp)	Ref.
parC-F parC-R	5′- TTCAGCGCCGCATTGTGTAT -3′ 5′- GTTATGCGGTGGAATATCGGTC-3′	395	This study
gyrA-F gyrA-R	5′-TTACACCGGTCAACATTGAGG -3′ 5′- GACGACCGTTAATGATTGCC -3′	647	This study

Tab. II. Age category.

Patients	Sex	Adult	Pediatric	Newborn
Inpatient		12	1	1
Outpatient	Female	24	0	1
Inpatient	Male	10	1	0
Outpatient		11	0	0

(Ser_Leu) and another at position 87 (Asp_Asn). Between 13 isolates of fluoroquinolone-resistance strains, 11 isolates possessed these two mutations and 2 isolates showed a single mutation (Ser83Leu) in the *gyrA* gene. Also, five different mutations were detected in *parC* gene of *E. coli* isolates, encoding Ser80Ile, Ser80Val, Ser80Arg, Glu84Val, and Gly78Ser. One isolate showed three mutations in *parC*; three isolates showed two mutations and the rest (six isolates) showed a single mutation. On the other hand, three isolates showed no mutation in *parC* gene (Fig. 3). The mutations detected in the QRDR of the *gyrA* and *parC* genes are shown in Table III.

The demographics and clinical characteristics of inpatients are shown in Table IV. The number of inpatients in this study was 44, but due to a defect in the case of 4 patients, the information provided in this section is based on data from 40 inpatients.

Fig. 1. Antibiotic resistance pattern of *E. coli isolates* by CLSI disk diffusion methods.
CEP: Cephalothin, CTX: Cefotaxime, FOX: Cefoxitin, AMK: Amikacin, NAL: Nalidixic acid, NOR: Norfloxacin, STX: Trimethoprim/sulfamethoxazole, AMP: Ampicillin, CAZ: Ceftazidime, FEP: Cefepime, MEM: Meropenem, GEN: Gentamicin, CIP: Ciprofloxacin, OFX: Ofloxacin, NIT: Nitrofurantoin.

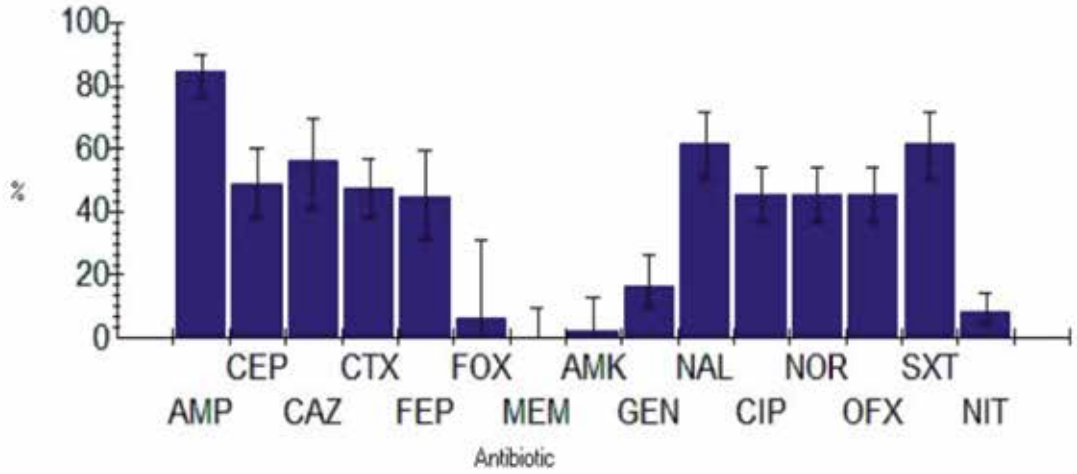


Fig. 2. a) PCR assay for the detection of *gyrA* gene. **b)** PCR assay for the detection *parC* gene.

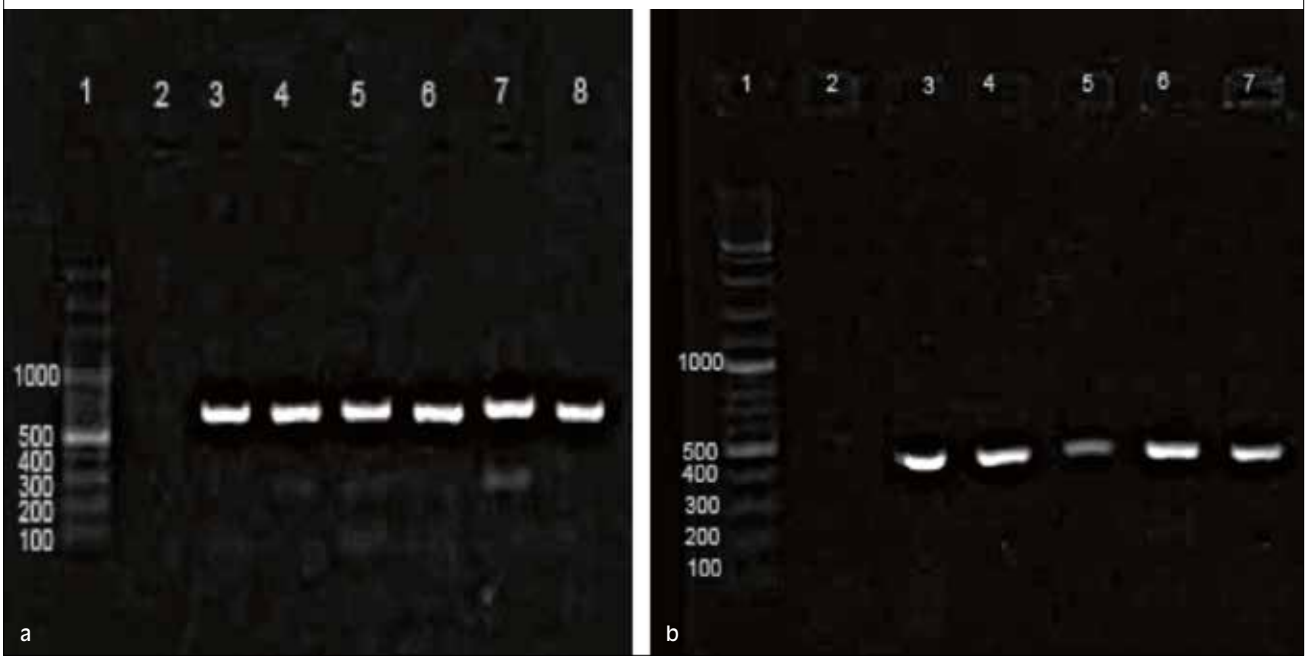
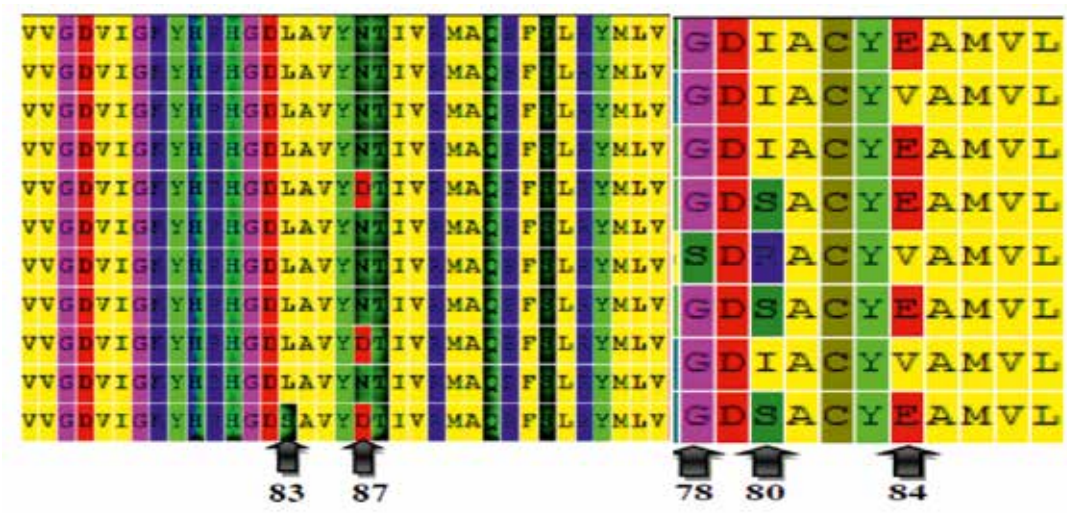


Fig. 3. Simultaneous alignment of partial *gyrA* and parC amino-acid sequences.



Tab. III. Location of mutations detected in *gyrA* and *parC* genes of *Escherichia coli* isolates.

Isolate	Mutation											
	gyrA					parC						
	83	84	85	86	87	78	79	80	81	82	83	84
	Ser	Ala	Val	Tyr	Asp	Gly	Asp	Ser	Ala	Cys	Tyr	Glu
E-1	Leu-	……-	……-	……-	-Asn	……-	……-	Ile-	……-	……-	……-	……
E-2	Leu-	……-	……-	……-	-Asn	……-	……-	Ile-	……-	……-	……-	……
E-3	Leu-	……-	……-	……-	-Asn	……-	……-	Ile-	……-	……-	……-	Val
E-4	Leu-	……-	……-	……-	-Asn	……-	……-	Ile-	……-	……-	……-	……
E-5	Leu-	……-	……-	……-	-Asn	……-	……-	Val-	……-	……-	……-	Val
E-6	Leu-	……-	……-	……-	-Asn	……-	……-	Ile-	……-	……-	……-	……
E-7	Leu-	……-	……-	……-	……-	……-	……-	……-	……-	……-	……-	……
E-8	Leu-	……-	……-	……-	-Asn	Ser-	……-	Arg-	……-	……-	……-	Val
E-9	Leu-	……-	……-	……-	-Asn	……-	……-	Ile-	……-	……-	……-	……
E-10	Leu-	……-	……-	……-	-Asn	……-	……-	Ile-	……-	……-	……-	……
E-11	Leu-	……-	……-	……-	-Asn	……-	……-	……-	……-	……-	……-	……
E-12	Leu-	……-	……-	……-	……-	……-	……-	……-	……-	……-	……-	……
E-13	Leu-	……-	……-	……-	-Asn	……-	……-	Ile-	……-	……-	……-	Val
E-14	……-	……-	……-	……	……-	……-	……-	……-	……-	……-	……-	……

E-14: quality control (*Escherichia coli* ATCC25922).

Tab. IV. Demographic and clinical characteristics for urinary tract infection in inpatients (n = 40).

Characteristics	No of inpatient (%)
Female	21 (52.5)
Male	19 (47.5)
History of previous urinary tract infection	3 (7.5)
Antibiotic use in the last 3 months	16 (40)
Catheter	23 (57.5)
Catheter history	20 (50)
Surgical history	23 (57.5)
History of admission	13 (32.5)
History of surgery in the last 12 months	11 (27.5)
Prostate enlargement	3 (7.5)

Discussion

Mutations in the *gyrA* gene are the main cause of the resistance to fluoroquinolones. The most mutations have been shown to near the start region of the *gyrA* gene, known as the "QRDR". This region encodes amino acid residues 67 to 106 in *gyrA* and the most common alterations occur at positions 83 and 87 [21, 22]. Topoisomerase IV is a secondary, less sensitive target for fluoroquinolone action in *E. coli* [23]. On the other hand, alterations in the *parC* gene, correlate with reduced susceptibility to quinolones [24].

In the present study, two isolates of *E. coli* possessed a single mutation (in *gyrA* gene) and consequently they were susceptible to fluoroquinolones (ciprofloxacin, norfloxacin and ofloxacin), but were resistant to nali-

dixic acid. These findings are in agreement with other studies, indicating that nalidixic acid could be used as a good marker for a single mutation by use of the disk diffusion method [25-28].

Another 11 isolates showed different mutations in *gyrA* and *parC* genes and were resistant to fluoroquinolones. This confirms that multiple mutations are necessary to a great extent for the high level of quinolone resistance [26]. Our study has several limitations, including; first, the number of sequencing isolates were too small for a definitive evaluation. Second, we have examined just two genes of *E. coli* isolates. Several studies have shown a correlation between *gyrB* and/or *parE* and FQ resistance in *E. coli* [29]. Also efflux pump genes can be a cause of FQ resistance.

In the present study, we determined antimicrobial resistance pattern (by focus on fluoroquinolones) of *E. coli* isolates from a university medical center, Alzahra Hospital, Isfahan, Iran. Generally, empirical therapy of patients with UTIs begins with extended-spectrum antibiotics (it often consists of FQ, especially ciprofloxacin). These treatments before the final microbiological results lead to the increased resistance and emergence of resistant strains. According to the type and method of taking antibiotics in each country, there is a considerable difference in susceptibility and resistance to antimicrobial agents in *E.coli* causing urinary tract infections [30]. For the hospitalized patients with urinary tract infections, 57% used the catheter during admission, 50% had a history of using the catheter and 57% had a history of surgery. It is possible that these factors can increase the risk of urinary tract infections, if validated by proper risk factors analyses.

This study has shown a significant high resistance to fluoroquinolones in respect of other surveys in different regions of Iran and different countries in Europe. These findings serve as a warning that resistance to fluoroquinolone is increasing quickly. As fluoroquinolones are the most important used antimicrobial agent in the treatment of UTIs in Iran, increasing resistance to these agents has caused concern to relevant treatment of these infections. There are different resistance mechanisms to FQ. One of them is mutations that alter the drug targets. We observed different mutations in the QRDRs of grain and pores that cause a great effect on FQ resistance. By doing more research on the molecular basis of FQ resistance, new therapeutic strategies will create for FQ-resistant *E. coli*. With regard to the continuous changing in antibiotic sensitivity pattern, we recommend a guideline for physicians, which could determine bacterial sensitivity in populations yearly and choose the correct empirical treatment according to these patterns.

Aknowledgements

The authors thank all staff of educational hospitals of Alzahra hospital, Isfahan, for cooperation in the presnt research. They also thank Ms. Aylin Esmailkhani for her helpful comments on manuscript.

Conflict of interest statement

None declared.

Authors' contributions

JF contributed to the conception and design of the work; the acquisition, analysis, and interpretation of data for the work. HK contributed to data collection and interpretation of data for the work. RD contributed to design of the work, data collection and final approval of the version to be published. MS contributed in data analysis, Drafting the work and revising it critically for important intellectual content. AZ and RD contributed in the revising the draft and agreement to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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■ Received on January 8, 2018. Accepted December 12, 2018.

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