

## Antimicrobial susceptibility testing of *Escherichia coli* strains isolated from urinary tract infections to fluoroquinolones and detection of gyrA mutations in resistant strains

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### ABSTRACT

Widespread uses of fluoroquinolones have resulted in increasing incidences of resistance against these agents all over the world. The aim of this study was to assess, susceptibility of *Escherichia coli* strains from patients with Urinary Tract Infection against common fluoroquinolones and detection of mutations in the gyrA gene. Antimicrobial susceptibility testing of 164 *E.coli* isolates from patients with UTI, was evaluated by disk agar diffusion (DAD) and MIC methods. Polymerase chain reaction of *E.coli* strains were performed by amplification of Quinolone Resistance Determining Region (QRDR) of gyrA gene. PCR products were tested by Conformational Sensitive Gel Electrophoresis (CSGE) and those with heteroduplexes were selected and examined by DNA sequencing. According to disc agar diffusion, 49.3% were resistant to nalidixic acid, 41.4% to norfloxacin, 44.5% to ofloxacin and 40.2 % to ciprofloxacin. By Minimal Inhibitory Concentration (MIC) testing a high-level of resistance (42.1%) to ciprofloxacin was observed. Mutations in codons 83 and 87 in all 81 isolates were positive by CSGE method.

**Keywords:** UTI, *E.coli*, Fluoroquinolones, Resistance, Mutation, PCR

### INTRODUCTION

Urinary Tract Infections (UTIs) affect 40 to 50 % of adult women and occur frequently (1). However its impact and frequency varies in different populations. It is estimated that about 20-30% of adult women have UTI at least once in their life (2). Almost 95% of cases of UTIs are caused by bacteria that typically multiply at the opening of the urethra and emerge to the bladder (3). Meanwhile most episodes of UTIs are caused by *Escherichia Coli* (up to 85%). Antibiotics which are used in UTI treatment usually achieve a high urinary concentration in urine and that is why they are clinically effective. Fluoroquinolones are preferred as initial agents for empirical therapy of UTI because they have a high bacteriologic and clinical care rates, as well as low rates of resistance to them (4). However, resistances to fluoroquinolones have increased markedly since the late of 1980s (5). European surveys show that frequency of resistance to fluoroquinolones among *E.coli* isolates are rising year by year (1). The primary target of fluoroquinolones on Gram-negative bacteria is DNA gyrase which is composed of two A and B subunits encoded by the gyrA and gyrB genes. In

*E.coli* resistance to fluoroquinolones it has been shown that resistance is mostly associated with alteration in gyrA (6-9). Most of mutations are located in a region of gyrA known as Quinolone Resistance Determining Region (QRDR). The QRDR includes the part of DNA gyrase that interacts with DNA (1). Several evidences from in-vitro studies have shown that *E.coli* with selective single mutations are typically associated with the low-level of resistance to fluoroquinolone resistance and that high -level resistance is due to accumulation of several mutations (10-12). In this study relationship between fluoroquinolone, MIC and numbers of mutations associated with *E.coli* isolated from patients with UTIs which were resistant to fluoroquinolones was investigated.

### MATERIALS AND METHODS

#### General

Fluoroquinolone discs and Ciprofloxacin powder were from MAST. Inc, UK. Primers, PCR buffer, Taq DNA polymerase, MgCl<sub>2</sub>, dNTPs, Agarose and Ethidium Bromide were purchased from Sina gene Inc, Iran. Acrylamide, Formamide, Taurine,

EDTA, Glycerol, Bromophenol Blue, Muller Hinton Broth and Xylene were from Merck Inc, Germany. Bis-Acryloyl Piperazin (BAP), Ethylene Glycol and TEMED were from Sigma, St. Louis, MO. Ammonium persulfate: (Boehringer Mannheim). ABI and PCR purification kit: (Macrogen, Inc, South Korea) were used in this study.

#### *Instruments*

Eppendorf thermal cycler 5330, Universal Mutation Detection System for CSGE (Dcode Bio Rad), and DNA sequencer: (capillary system ABI Applied Bio system 3730 DNA Sequencer) were used in this investigation.

#### **Procedures**

##### *Bacterial strains*

One hundred sixty four clinical isolates of *E.coli* were collected from Urine cultures of patients with UTI in Sina and Shariati hospitals in Iran and Children Medical Center in Tehran, IRAN, from May to September 2005. The colony count of these cultures was  $\geq 10^5$  bacteria/ml and  $\geq 10$  WBC/hpf. They were identified by routine laboratory methods.

##### *Antimicrobial susceptibility testing*

Antimicrobial susceptibility testing was determined by disk agar diffusion method (DAD) as described by the National Committee for Clinical Laboratory Standards (13). For susceptibility data collection, 30 $\mu$ g of nalidixic Acid, 5 $\mu$ g of ofloxacin, 10 $\mu$ g of norfloxacin and 5 $\mu$ g ciprofloxacin per disk were used. The minimal inhibitory concentrations (MICs) were defined as the lowest antimicrobial concentration which is able to inhibit bacterial growth with final inoculum's of  $10^6$  CFU and were determined by two fold serial dilution in cation-adjusted Muller Hinton Broth as described by National Committee for Clinical Laboratory Standards (13) for ciprofloxacin. All plates were incubated aerobically at 37 °C overnight.

Antibiotic concentrations in micro plates were 0.25 to 128 $\mu$ g/ml. *Escherichia coli* ATCC 25922 was used as standard strain according to NCCLS protocols.

##### *PCR amplification of QRDR of gyrA gene*

PCR amplification of *gyrA* was performed by using primers: 5'-TGCCAGATGTCCGAGAT-3' (nucleotide 12020 to 12004) and 5'-GTATAACGCATTGCC GC-3' (nucleotide 11753 to 11769) as previously described (5). A single colony of each bacterial isolate was used for DNA extraction and suspended in 100  $\mu$ l of double deionized H<sub>2</sub>O, boiled at 95° for 5 min and

then centrifuged at 13000 rpm for 10 min. Supernatant was transferred to a micro tube and used as template for PCR amplification. PCR reaction contained: 40 Pico mol of each of forward and reverse primers, 3  $\mu$ l of extracted DNA (approximately 0.1 $\mu$ g DNA), 0.2 mM of dNTPs, 10x PCR buffer 5 $\mu$ l, 1.5 mM MgCl<sub>2</sub>, 2.5 U Taq DNA polymerase in 50  $\mu$ l final volumes. PCR amplification was carried out by thermal cycler in 30 cycles at denaturation temperature of 94 °C for 30 sec, annealing temperature of 49 °C for 30 sec and extension of 72 °C for 30 Sec. Before and after PCR cycling, reactions were kept at 94 °C and 72 °C for 5 min respectively. PCR product was subjected to electrophoresis on 2% agarose gel, stained by ethidium bromide and visualized by UV transilluminator.

##### *Conformational Sensitive Gel Electrophoresis (CSGE)*

Mutation detection was carried out by CSGE method using Universal Mutation Detection System. PCR product of *E.coli* ATCC25922 (QRDR region) was used as control.

CSGE was performed by acrylamide gel which contained 10% acrylamide, 0.1% w/v bis acryloyl piperazin (BAP), 15% formamide, 10% ethylene glycol, 0.075% TEMED 0.1% w/v ammonium persulfate and 0.5 X TTE buffer of pH 9 (44.5 mM Tris/14.25 mM Taurine/ 0.1 mM EDTA). A 5.5  $\mu$ l of each sample case and 5.5  $\mu$ l of control PCR products were mixed as sample and mixed with equal volumes of sample loading buffer (30% Glycerol/ 0.25% w/v Bromophenol Blue / 0.25%w/v Xylene Cyanol FF). Sample was denatured at 98°C for 5 min, reannealed at 68°C for 30 min. and electrophoresed for 4 hrs at room temperature in 1X TTE buffer. Gels were stained by ethidium bromide and visualized under UV transilluminator (14-16).

##### *DNA sequencing*

The PCR products were purified using an ABI PCR purification kit. The DNA sequences were determined by a DNA Sequencer. Direct cycle sequencing in both directions were performed by forward and reverse primers. Computer analyses of the sequences were performed using the Bio-Edit (version 7.0.5.2) multiple sequence alignment program (Copyright 1997-2005. Tam Hall.)

## **RESULTS**

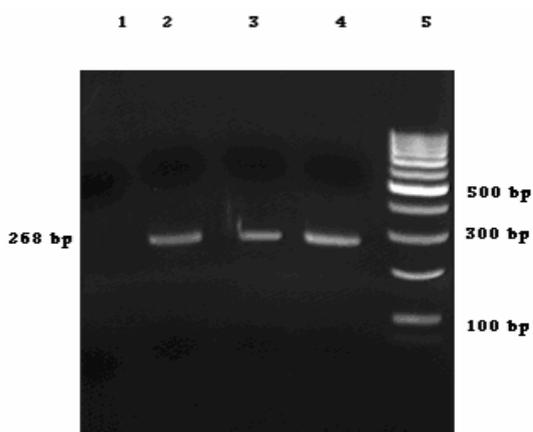
##### *Antimicrobial susceptibility testing*

47% of *Escherichia coli* isolates were from Shariati hospital, 25% were from Sina hospital and 28 % were from Children Medical Center of Tehran. Of all 164 samples 35.3% were from

males and 64.6% from females. The extent of resistant to nalidixic acid, ofloxacin, norfloxacin and ciprofloxacin, by disk diffusion method were 49.3%, 44.5%, 41.4% and 40.2 % respectively. Approximately similar trends were observed for sensitivity pattern of norfloxacin and ofloxacin. The extent of resistance to ciprofloxacin determined by MIC method revealed that 4.9% had reduced susceptibility and 53% were susceptible. The percentage of resistance in different hospitals is showed in the table 1.

**Table 1.** The percentage of resistance of the isolates from different hospitals

Antibiotic	Hospital	Sina	Children
	Shariati Hospital	Hospital	Medical Center
Nalidixic acid	61%	46.3%	32.6%
Ofloxacin	55.8%	43.9%	26.1%
Norfloxacin	55.8%	34.1%	23.9%
Ciprofloxacin	53.2%	34.1%	23.9%



**Figure 1.** 2% agarose gel electrophoresis of PCR amplified gyrA gene

Lane 1: *Staphylococcus aureus* as negative Control  
Lanes 2 and 3: Patient samples  
Lane 4: PCR product of *Escherichia coli* ATCC 25922 as positive Control. Lane 5: 100bp DNA ladder marker

#### PCR amplification

PCR amplification was performed and all 164 samples and *E.coli* ATCC25922 as control were amplified

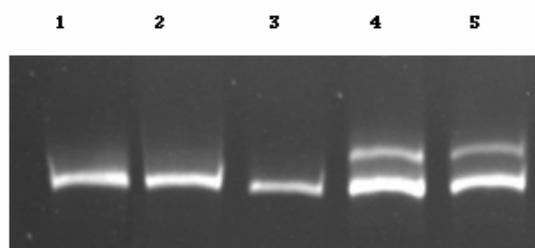
#### Conformational Sensitive Gel Electrophoresis

Sequenced and confirmed PCR product of *Escherichia coli* ATCC25922 was used as positive control. The PCR product of *E.coli* ATCC25922 (QRDR region) was used as control for comparison of other samples in CSGE method.

According to CSGE method, 81 samples showed hetrodublexes. These samples were considered as mutant strains and consequently selected for DNA sequencing.

#### DNA sequencing of QRDR region of gyrA

DNA sequencing of the 268-bp PCR product covering the entire QRDR of gyrA demonstrated presence of mutations at codons 83 and 87 in all 81 isolates (showed hetrodublexes in CSGE) when compared to wild-type *E.coli* K-12. In 79 cases, the mutations at codon 83 was C→T located in the codon TCG, by substitution of lucine for serine. In 70 cases, mutation at codon 87 (G→A located in codon GAC) resulted by substitution of asparagine for an aspartat. Sixty eight cases had both two mutations, 11 isolates had only one mutation in codon 83 and 2 isolates had only one mutation in codon 87. There were no alterations in codon 87 by substitution of tyrosine or other amino acids for asparagines.



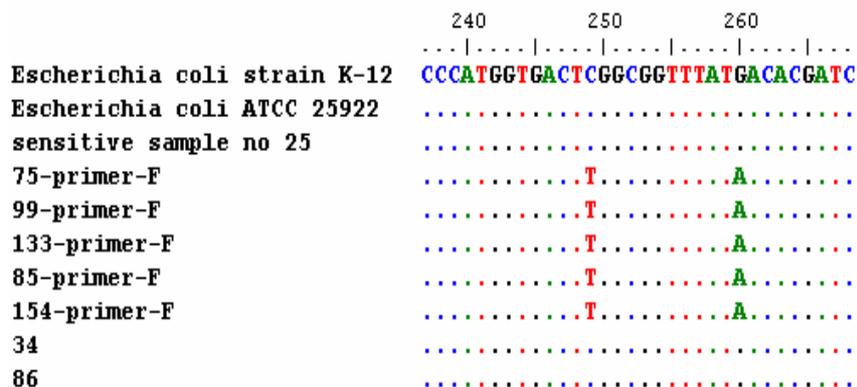
**Figure 2.** 10% acrylamide gel electrophoresis by CSGE method.

Lane 1: PCR product of *Escherichia coli* ATCC 25922 as negative control.

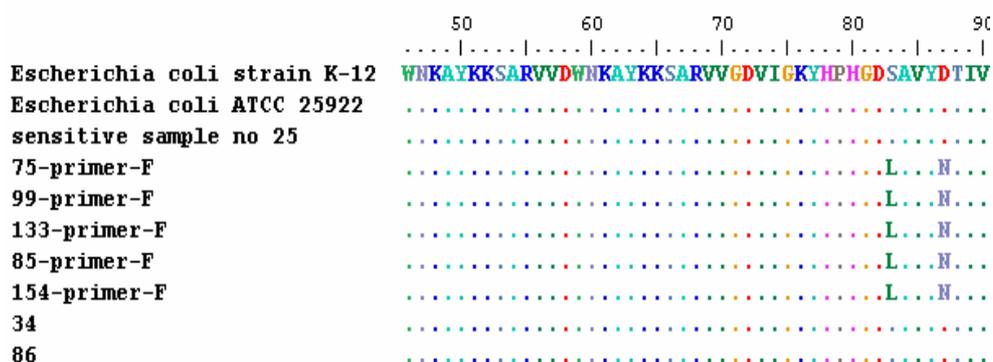
Lanes 2 and 3: PCR product without hetrodublexes formation. Lanes 4 and 5: PCR products with hetrodublexes formation.

#### DISCUSSION

Since the use of quinolones in clinical treatment was begun, it has been assumptions that in comparison with other antibiotics it has less resistance problems. The basis for this hypothesis is that the quinolones resistance mutations occurred in the bacterial chromosome not in plasmid or transposon. On contrary, the number of reports about occurrence of quinolone-resistance bacteria has been increasing all over the world. It has been shown that the incidence of resistance of *E.coli* of urinary cultures to quinolones have increased from 6.4% in 2000 to 21% in 2003 (17). Similar findings have been reported in other countries. For example in the year of 2003 in Kuwait the extent of resistance was 17.8% ( 2) and in Gaza stripe in the year of 2005 it was 17.5% ( 4). In this investigation 42.1% of *E.coli* isolates from UTIs in patients were resistant to ciprofloxacin and 4.9% of them had reduced susceptibility. Therefore, the finding of such large resistance population of *E.coli* (41.2% resistant and 4.9% with reduced susceptibility) isolates from Tehran suggests that the rate of resistance may rapidly increases further. This large resistant population of isolates increases the risk of clinical failure with fluoroquinolones.



**Figure 3.** DNA Sequencing of QRDR region of *gyrA* gene. Comparison of *Escherichia Coli* ATCC 25922 with *E.coli* K-12, mutant samples (samples No 75, 99, 133, 85,154) and Non mutant samples (samples No25, 34 and 86) after Multiple alignment



**Figure 4.** DNA Sequencing of QRDR region of *gyrA* gene. Comparison of *Escherichia Coli* ATCC 25922 with *E.coli* K-12, mutant samples (samples No 75, 99, 133, 85,154) and non mutant samples (samples No 25, 34 and 86) after Toggle Translation.

For example, it has been demonstrated that infections caused by *salmonella spp.* with reduced susceptibility have higher clinical failure rates than infections caused by fully susceptible strains when fluoroquinolones are used for treatment (17, 18).

In this study resistant isolates were in two groups. One of them was composed of 68 isolates and had two mutations in S 83 and D 87. Other group was composed of 13 isolates with one mutation. Of 13 isolates, 11 had one mutation in S 83 and two isolates had only one mutation in codon 87. All isolates with MIC > 4µg/ml had two mutations in QRDR region of *gyrA* gene and all isolates with one mutation had MIC < 4µg/ml except for one isolate with MIC of 4µg/ml which had one mutation in S 83. The relationship between MIC and numbers of *gyrA* mutations has previously been reported (1, 5, 17, 19-21). Usually, changes at positions 83 and 87 which cause the greatest reduction in quinolone susceptibility are located on the surface of the recognition helix where quinolone binding may occur (22).

In the case of one mutation and high MIC, other resistance mechanisms such as efflux system may be involved (12). Besides of target gene mutations, studies have shown that high level fluoroquinolone resistance can be influenced at least in part by mutations in one or more of the known global regulator loci (24), such as *marA* (25), *soxS* (26), and *robA* (27) which result in increase of multidrug efflux pump (12).

Also increase in MIC of the isolates with two mutations demonstrated a predictable association between single and double mutations in the *gyrA* and the MICs for fluoroquinolones.

In this study, almost 50% of the bacteria showed increase in tendency of resistance to commonly used fluoroquinolones. A possible cause of increased resistance might be widespread use of fluoroquinolones in patients with UTI in medical centers. Other reasons for this alarming phenomenon might be inappropriate prescribing, poor infection control strategies as well as incorrect administration of fluoroquinolones in blind cases. To overcome this problem unnecessary antibiotics therapy should be limited.

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