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Full Length Research Paper

Detection of mutations in quinolone-resistant determining regions in clinical isolates of *Escherichia coli* from Saudi Arabia

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Since the use of fluoroquinolone antibiotic in clinical practice was introduced about two decades ago, quinolone-resistant *E. coli* strains are being isolated with increasing frequency. This study devotes to determine the resistance rate of fluoroquinolones for 112 *Escherichia coli* isolates from Prince Salman Hospital, Riyadh and to detect the mutations in the quinolone resistance-determining region (QRDR) of *gyrA* and *parC* in the fluoroquinolones resistant isolates. The resistance rate of ciprofloxacin for *E. coli* isolates was 37.5%. We have determined QRDR of the *gyrA* and *parC* genes of ciprofloxacin resistant *E. coli* (MIC 2-64 mg/L). A single mutation in *gyrA* (Ser TCG-83→Leu TTG) was detected in the isolate with a reduced susceptibility to ciprofloxacin (MIC 2 mg/L). Three mutations were detected in *E. coli* isolates with ciprofloxacin MIC ≥16 mg/L. Double mutations in *gyrA* (Ser-83→Leu and Asp-87→Asn) and a single mutation in *parC* (Ser-80→Ile). This study suggests, that in clinical isolates of *E. coli*, DNA gyrase is a primary target of quinolones, that only a single amino acid change at Ser-83 in GyrA is sufficient to generate high-level resistance to nalidixic acid and to decrease susceptibility to ciprofloxacin, and that the accumulation of amino acid changes in GyrA and the simultaneous presence of the ParC alterations play a central role in developing high-level resistance to ciprofloxacin.

Key words: Fluoroquinolones resistance, *gyrA*, parC, Saudi Arabia.

INTRODUCTION

Fluoroquinolones are powerful broad-spectrum antibacterial used for the treatment of a wide variety of community-acquired and nosocomial infections (Acar and Goldstein, 1997). Fluoroquinolones inhibit DNA gyrase and topoisomerase IV activities (Wang et al., 2009). DNA gyrase is a tetrameric enzyme composed of two GyrA and GyrB subunits, encoded by *gyrA* and *gyrB* genes respectively and topoisomerase IV is tetrameric enzyme composed of two ParC and ParE subunits, encoded by *parC* and *parE* genes respectively. The main function of DNA gyrase is to catalyze the negative supercoiling of DNA (Hawkey, 2003) while the main action of topoisomerase V is to decatenates or removes the interlinking of daughter chromosomes at the completion of a round of DNA replication and allows their segregation into daughter cell (Ambrozic et al., 2007). Until now three mechanisms of fluoroquinolones resistance were reported: (1) Chromosomal mutations in the *gyrA* and *gyrB* of DNA gyrase and in *parC* and *parE* of topoisomerase IV, (2) Mutations causing reduced drug accumulation, either by a decreased uptake or by an increased efflux are also involved, (3) Recently, plasmid-mediated quinolone resistance (PMQR) mechanisms such as *qnr*, aac(6')1b-cr and *qepA* confer low levels of resistance but provide a favorable background in which selection of additional chromosomally encoded quinolone resistance mechanisms can occur (Martínez-Martínez et al., 1998; Wang et al., 2009). The most important mechanism of quinolone resistance is alteration in the *gyrA* and *gyrB* of DNA gyrase and in *parC* and *parE* of topoisomerase IV. Alterations in *gyrA* are reported more

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Abbreviations: QRDR, Quinolone resistance-determining region; **MIC,** minimum inhibitory concentration.

Table 1. Resistance pattern of 112 *E. coli* isolates toward quinolones.

Quinolones	Resistance pattern of 112 *E. coli* isolates					
	Susceptible		Intermediate		Resistance	
	Number	Percentage (%)	Number	Percentage (%)	Number	Percentage (%)
Ciprofloxacin	66	58.93	42	37.5	66	58.93
Enoxacin	68	60.71	38	33.93	68	60.71
Norfloxacin	70	62.5	38	33.93	70	62.5
Ofloxacin	70	62.5	42	37.5	70	62.5
Pefloxacin	72	64.28	36	32.14	72	64.28
Sparafloxacin	54	48.21	44	39.28	54	48.21
Pimpedic acid	74	66.07	38	33.93	74	66.07
Nalidixic acid	0	0	112	100	0	0

often than alteration in *gyrB* (Frank et al., 2011). Alterations described in the GyrA are predominantly in the so-called quinolone resistant determining region (QRDR) between positions 67 and 106 (Ambrozic et al., 2007; Vranakis et al., 2010). The presence of a single mutation in the QRDR of *gyrA* usually results in high level resistance to nalidixic acid, but to obtain high levels of resistance to fluoroquinolones, the presence of additional mutation(s) in *gyrA* and/or in another target such as *parC* is required. Thus, it has been proposed that the MIC of nalidixic acid could be used as a genetic marker of resistance for quinolone family in Gram-negative bacteria (Ruiz, 2003).

Quinolone resistance genes associated with plasmids have been also described, that is, the qnr gene that encodes a pentapeptide, which blocks the action of quinolones on the DNA gyrase and topoisomerase IV; the aac(6')-Ib-cr gene that encodes an acetylase that modifies the amino group of the piperazin ring of the fluoroquinolones and efflux pump encoded by the qepA gene that decreases intracellular drug levels (Fàbrega et al., 2009).

To our knowledge, there is no study aimed to understand the molecular resistance mechanism to quinolones in *Escherichia coli* strains isolated from Saudi Arabia. Therefore, this study was undertaken to determine the mechanism of quinolone resistance in *E. coli* isolates from Riyadh.

MATERIALS AND METHODS

Bacterial strains

One hundred and twelve non-duplicate, non-consecutive urinary clinical *E. coli* isolates were collected between January to March, 2010 from Prince Salman Hospital, Riyadh. The laboratory strain; *E. coli* ATCC 29525, was used as standard control in sensitivity testing. *E. coli* isolates were identified according to a commercial identification system of Micronaut-IDS (MERLIN Diagnostika, Germany).

Antimicrobial susceptibility testing

MICs for 112 *E. coli* isolates were determined in a broth microdilution test, using unsupplemented Mueller Hinton broth, according to the recommendation of clinical and laboratory standards institutes (CLSI, 2006) using Micronaut microtiter plates containing dehydrated antibiotics (Merlin Diagnostika, Germany) in two–fold dilution. The following quinolones were tested: ciprofloxacin, enoxacin, norfloxacin, ofloxacin, pefloxacin, pimpedic acid and sparfloxacin.

DNA template preparation

DNA templates were prepared from 200 µl volume of overnight culture of the test isolates in Luria Beratni broth (Difco, USA) by centrifugation using microfuge tubes and resuspending the bacterial pellet to the initial volume with distilled water. The template DNA was prepared by boiling for 10 min and directly used in the PCR assay.

Amplification of QRDR in *gyrA* and *parC* gene subunits

Amplification of QRDR in *gyrA* and *parC* gene subunits in quinolone-resistant clinical isolates of *E. coli* was done by polymerase chain reaction (PCR) according to methods described by Grigges et al. (1996) and Heisig (1996). Oligonucleotides used in this study are listed in Table 1. Reactions were run with Techne thermocycler (Techne, UK). The PCR products were electrophoresed in 0.7% agarose gels containing ethidium bromide 0.5 mg/L, the DNA fragments were photographed with video documentation system.

Sequencing of *gyrA* and *parC* gene subunits

Amplicons of *gyrA* and *parC* were purified with a QIAquick PCR purification kit (Qiagen, Hilden, Germany) and were subjected to direct sequencing reactions on both strands using the primers listed in Table 1 by dideoxy chain terminator method of Sanger et al. (1977) on an API310 automatic sequencer analyzer using API PRISM dye Terminator Cycle Sequencing Ready Reaction kit with AmpliTaq DNA polymerase FS (Perkin-Elmer/ Applied Biosystems, Foster City, CA, USA).

RESULTS

Antimicrobial susceptibility testing

The results of MICs for 112 *E. coli* isolates to ciprofloxacin are shown in Table 1. The susceptibility of

Table 2. Ciprofloxacin MIC distributions of 112 *E. coli* isolates.

MIC (µg/ml)	Isolates (n = 112)	
	Number	Percentage (%)
<0.0625	42	37.5
0.0625	12	10.71
0.125	0	0
0.25	0	0
0.5	2	1.78
1	10	8.93
2	4	3.75
4	0	0
8	4	3.57
16	2	1.78
32	16	14.28
64	20	17.85

Table 3. Alterations in *gyrA* and *parC* subunits in four tested *E. coli* isolates compared to *E. coli* K12 strain.

Tested organism	MIC (mg/L) of ciprofloxacin	Mutations in QRDR			
		GyrA		ParC	
		83	87	80	84
E. coli K12	<0.0325	Ser (TCG)	Asp (GAC)	Ser (AGC)	Glu (GAA)
E. coli E_2	2	Leu (TTG)	Asp (GAC)	Ser (AGC)	Glu (GAA)
E. coli E_{43}	16	Leu (TTG)	Asn (AAC)	Ile (ATC)	Glu (GAA)
E. coli E_8	32	Leu (TTG)	Asn (AAC)	Ile (ATC)	Glu (GAA)
E. coli E_5	64	Leu (TTG)	Asn (AAC)	Ile (ATC)	Glu (GAA)

isolates to ciprofloxacin showed that 42 (38.5%) of 112 *E. coli* isolates were resistant while 66 (58.92%) *E. coli* isolates showed susceptibility to ciprofloxacin and four isolates (3.57%) showed intermediate susceptibility to ciprofloxacin.

The results of MIC distribution for ciprofloxacin are shown in Table 2. 42 (37.5%) and 12 (10.71%) out of 112 *E. coli* isolates represented a lowest ciprofloxacin MIC (<0.0625 and 0.0625 mg/L respectively) whereas, 16 (14.28%) and 20 (17.85%) out of 112 *E. coli* isolates showed a highest ciprofloxacin MIC (32 and 64 mg/L respectively). However, the remaining 20 (17.85%) isolates showed ciprofloxacin MIC values ranging from 0.5 to 16 µg/ml. The MIC resistance breakpoints to ciprofloxacin are ≥ 4 mg/L. Four *E. coli* isolates with different ciprofloxacin MICs, 2, 16, 32 and 64 mg/L respectively were selected in this study to perform *gyrA* and *parC* sequence.

Determination of mechanisms of *E. coli* resistance to quinolones

The results of mutations in *gyrA* and *parC* subunits are shown in Table 3. The QRDR of *gyrA* subunit of three quinolone resistant *E. coli* isolates (Ciprofloxacin MIC≥ 16 mg/L) and one quinolone moderately resistant isolate (ciprofloxacin MIC 2 mg/L) was amplified. The purified QRDR of *gyrA* subunit for four *E. coli* isolates, with different ciprofloxacin MIC values 2, 16, 32 and 64 mg/L, were sequenced on both strands with specific primers. The results of nucleotide sequence of the QRDR of *gyrA* subunit for the *E. coli* isolates using the forward primer, P1 and reverse primer, P2BIO, revealed that single mutation in *gyrA* at codon 83 (Ser TCG→Leu, TTC) was observed in isolate with a reduced susceptibility to ciprofloxacin (MIC 2 mg/L). A nucleotide number 248 of *gyrA* exchanged from C to T, which subsequently exchanged amino acid from serine into leucin. Whereas, double mutations in *gyrA* at codon 83 (Ser T**C**G→Leu, T**T**C) and 87 (Asp **G**AC→Asn, **A**AC) were recorded in *E. coli* isolates with full resistance to ciprofloxacin. A nucleotide number 259 of *gyrA* exchanged from G to A, which subsequently exchanged amino acid from aspartic acid into asparagine.

The QRDR of *parC* subunit of topoisomerase IV of ciprofloxacin resistant *E. coli* isolates (MIC ≥16 mg/L) and of ciprofloxacin moderately resistant isolate with MIC equal to 2 mg/L was amplified. The purified QRDR of *parC* subunit for the tested four *E. coli* isolates were

sequenced on both strands with specific primers. The results of nucleotide sequence of QRDR of *parC* subunit of three isolates resistant to ciprofloxacin using the forward primer, ParC-S and reverse primer, parC-U, revealed that no mutation in *parC* was observed in the isolate with a reduced susceptibility to ciprofloxacin (MIC 2 mg/L) whereas a single mutation in *parC* at codon 80 (Ser A**G**C→Ile A**T**C) were recorded in *E. coli* isolates with full resistance to ciprofloxacin. A nucleotide A was exchanged into G at codon 80, which subsequently exchanged amino acid from serine into isoleucine. The codon number 91, CAA, of parC subunit, which translated into glutamine of E_2 was exchanged into CAG in *parC* of E_5 but this mutation was silent.

DISCUSSION

Increasing bacterial resistance has followed this widespread use that specifically target bacterial DNA gyrase and topoisomerase IV. However, resistance to fluoroquinolones has increased markedly since introduction in the late 1980s (Aguiar et al., 1992).

In the present study ciprofloxacin resistant *E. coli* was 38.5%, while the previous studies in Saudi Arabia, reported high levels of resistance to ciprofloxacin (60 to 80%) in Enterobacteriaceae (Al Johani et al., 2010; Khanfar et al., 2009) and other studies showed low level of ciprofloxacin resistance in *Klebsiella pneumoniae* from Al-Qassim (9.1%) and Riyadh (11%), Saudi Arabia (Tawfik et al., 2011; Al-Agamy et al., 2009). The difference in the resistance pattern could be due to differences in isolation dates, localities, rate of consumption of fluoroquinolones in the different hospitals…etc. The rate of *E. coli* resistance to fluoroquinolones is increasing worldwide. In USA the rate of resistance in *E. coli* isolates was 3.5% and 1.9 to 2.5% for nalidixic acid and fluoroquinolones respectively (Karlowsky et al., 2003).

The association of DNA gyrase and topoisomerase IV mutations with fluoroquinolones resistance has been established for both Gram-negative and Gram-positive organisms (Frank et al., 2011). Accumulation of alterations in *gyrA* and the simultaneous presence alterations in *parC* play fundamental role in developing high level of resistance to ciprofloxacin in clinical isolates. Point mutations in *gyrA* of Ser-83 to either Try or Leu, both of which convert the polar amino acid serine to non-polar amino acid tyrosine (Conrad et al., 1996) or double mutations of Ser-83→Leu and Asp-87→Gly (Vila et al., 1994), are the most frequent bases for the fluoroquinolones resistant in *E. coli* isolates. In addition, mutations in *parC* at Ser-80, Gly-78, and Glu-84 have been also been noted (Kumagai et al., 1996).

In the present study, four *E. coli* isolates with different ciprofloxacin MIC values ranged from 2 to 64 mg/L were selected [E_2 (MIC 2 mg/L), E_{43} (MIC 16 mg/L), E_8 (MIC 32 mg/L), and E_5 (MIC 64 mg/L)] to amplify and sequence of QRDR of *gyrA* and *parC*. A single mutation was detected in *gyrA* (Ser-83→Leu) of *E. coli* isolate E_2 (Ciprofloxacin MIC 2 mg/L) and this mutation led to decrease the susceptibility to ciprofloxacin. In the *E. coli* isolates E_{43}, E_8 and E_5 with ciprofloxacin MIC values of 16, 32, and 64 mg/L respectively; three mutations were detected, two mutations in *gyrA* (Ser-83→Leu and Asp-87→Asn) and a single mutation in *parC* (Ser-80→Ile). These mutations were responsible for high resistance of the isolates to fluoroquinolones. Wiegel et al. (1998) reported in *E. coli*, a C-to-T substitution at the second position of the codon, resulting in a Ser-83-to-Leu mutation, was consistent for all fluoroquinolone-resistant isolates. The present study agreed with the study reported by Wiegel et al. (1998). In the present study, a nucleotide number 248 of *gyrA* exchanged from C to T, which subsequently exchanged amino acid from serine into leucin. In the isolates with double mutations, the second alteration involved the codon for Asp-87 and the amino acid substitution was Gly, Tyr, or Asn. A single mutation in codon 83 of *gyrA* was associated with decreased susceptibility or low levels of resistance to fluoroquinolones, and double mutations (codons 83 and 87) were associated with high levels of resistance.

The highest level of resistance to fluoroquinolones (> 64 mg/L) was not detected in this study because this resistance generally results from four mutations: two in *gyrA* and two in *parC* (Vila et al., 1994; Sáenz et al., 2003) and this was not detected in the present study.

In summary, the rate of *E. coli* resistance to fluoroquinolones is increasing in Saudi Arabia and this finding indicates alarmingly that the fluoroquinolones will become useless within the next few years in Saudi Arabia. This data extends our understanding of the molecular mechanisms of fluoroquinolones resistance associated with *gyrA* and *parC* mutations in *E. coli* isolated from Saudi Arabia.

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