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https://doi.org/10.31925/farmacia.2024.4.12 **ORIGINAL ARTICLE**

BACTERIOLOGICAL AND MOLECULAR DETECTION OF FLUOROQUINOLONE RESISTANCE IN *ESCHERICHIA COLI* ISOLATED FROM WOMEN PATIENTS WITH URINARY TRACT INFECTIONS

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Abstract

Fluoroquinolone (FQ)-resistant strains are the main reason for outbreaks of urinary tract and hospital-acquired infections (HAIs). FQ-resistant *Escherichia coli* is becoming frequent in patients with urinary tract infections (UTIs), and UTI patients' widespread abuse of antibiotics has led to an increase in FQ resistance in *E. coli*. Mutations of *gyrA* gene, which encodes DNA gyrase, are the main cause of fluoroquinolone resistance in *E. coli*. In this study, 63 clinical isolates were recognised as *E. coli*, of which 33 showed resistance to all fluoroquinolones. Direct sequencing revealed 9 mutations in the *gyrA* gene at different locations which were distributed into 2 silent mutations, 2 deletion mutations, 4 missenses, and 1 non–sense mutation. The findings obtained in the current study indicate that the high prevalence of mutations in resistance *gyrA*gene is responsible for the resistance to fluoroquinolones group in local isolates of *E. coli* resistance towards five types of antibiotics that are related to fluoroquinolones group.

Rezumat

Tulpinile de *Escherichia coli* rezistente la fluorochinolone sunt tot mai întâlnite la pacienții cu infecții urinare nosocomiale. Mutațiile genei gyrA, care codifică ADN giraza, sunt principala cauză a acestei rezistențe. În cadrul acestui studiu, s-au utilizat 63 de izolate, în care a fost identificată *E. coli*, dintre care 33 au fost rezistente la toate fluorochinolonele. Secvențierea directă a evidențiat 9 mutații în gena *gyrA*, incluzând 2 mutații silențioase, 2 mutații de deleție, 4 mutații de sens greșit și 1 mutație nonsens. Aceste constatări indică faptul că mutațiile frecvente în gena *gyrA* sunt responsabile pentru rezistența la fluorochinolone în izolatele locale de *E. Coli.*

Keywords: gyrA, Fluoroquinolone Resistant, Urinary tract Infection, *Escherichia coli*.

Introduction

The most prevalent diseases of bacteria are Urinary tract infections (UTIs), affecting a lot of individuals each year throughout the world [1, 2]. Between 50% and 80% of female have experienced a single UTI throughout their life [3, 4]. *Escherichia coli* is the most frequent cause of UTI, which represents 75% to 95% of cases [5, 6]. Fluoroquinolones (FQs) are broad spectrum antimicrobial agents that are clinically employed to prevent and cure *E. coli* infections in the urinary tract [7-10].
Recently an increase in the rates of fluoroquinolone resistance among *E. coli* isolates has been reported [9, 10]. In bacteria, fluoroquinolones bind to the active sites of Topoisomerase IV and DNA gyrase enzyme which principally play a role in the replication of DNA, compaction and chromosomal separation [11, 12, 13, 14]. The fundamental mechanism of fluoroquinolone resistance in *E. coli* is mutations mainly in *gyrA* gene, which encodes for subunit A of DNA gyrase [13-17]. This study is conducted to identify mutations linked with fluoroquinolone resistance (*gyrA* gene) among *E. coli* isolates from urine samples using direct sequencing.

Materials and Methods

Specimens collection
Urine specimens were collected in a clean container from women suffering from urinary tract infections in Baghdad medical hospital, educational laboratories, Ghazi al-Hariri hospital, Iraq, and Private Nursing Home hospital from 1st November 2020 to 1st March 2021 in Baghdad, Iraq.
Bacterial isolation and characterization
All specimens were cultivated immediately on Selective media for Gram Negative Bacteria (MacConkey and EMB agar plates). The plates were incubated aerobically

at 37°C according to the manufacturer's instructions [16]. Gram stain was performed for microscopic examination [17]. Several diagnostic tests were used to make the diagnosis (Catalase test, Urease Test, Methyl Red test, Indol test, Simmon's Citrate test and Voge's Proskauer test) APE20 test was employed in parallel to Vitek compact test utilizing Gram-negative card kits (GN ID) to confirm the identification results [18-21].

Antibiotic sensitivity test

The Kirby-Bauer test and the Agar Disc Diffusion Method using Mueller-Hinton medium were performed to assess isolates antibiotic sensitivity (AST), as approved by the Clinical Laboratory Standards Institute (CLSI, 2020, 30th). The following 5 fluoroquinolone antibiotics were used with Disc Content (ug) (Ciprofloxacin CIP 5 ug, Nalidixic Acid NA30 1 ug, Ofloxacin OFX 5 ug, Levofloxacin LEV 5 ug, Norfloxacin NOR 10 ug provided by BioAnalyse company Turkey [22-25].

Minimum inhibitory concentration test (MIC)

The MICs were carried out using VITEK compact 2 devices. Three mL of normal saline was used to dilute the bacterial broth to 0.5 McFarland. The VITEK 2 instrument was automatically filled, sealed and loaded with cassete to readout automatically. The AST-GN74 cassete included Ciprofloxacin, Levofloxacin, while the AST-GN222 cassete had Norfloxacin, Ofloxacin and Naldixic acid. The duration to obtain MICs ranged from 6 to 17 hours.

DNA Extraction and PCR Reaction

According to the manufacturer's user manual, a specialist kit was employed for DNA extraction from the 20 isolates obtained. A specific procedure for Gram negative bacteria provided fromWizard kit from Promega company was followed [18]. The *gyrA* genes Sequence (Forword Primer 5`-TGTCCGAGATG GCCTGAAGC-3`, Reverse Primer 5`-TACCGTCA TAGTTATCAACG-3) with Product size of 347 (bp) were amplified using PCR with specified primers [20]. The components of the mixture were listed in (Table I) to reach 20 µL as a final volume. The PCR product was electrophoresed on 1.5% agarose gel. The *gyrA* genes were identified using a UV transilluminator.

Table I

Component of PCR Reaction applied in the experiment

Master Mix Components	Stock / Unit	Final / Unit	Volume	
			1 Sample	20 Sample
Master Mix	2X	1X	10	200
Forward Primer	10 uM	1	1	20
Reverse Primer	10 uM	1	1	20
Nuclease Free Water				
DNA	10 ng/mL	10 ng/mL	2	
Total Volume			20	
Aliquot Per Single rxn	18 µL of Master mix per tube and add 2 µL of Template			

The main PCR Conditions were: temperature of initial denaturation was 95°C for 5 minutes, 1 cycle, temperature of Denaturation 95°C for 30 seconds, 30 cycles, Annealing temperature was 54°C for 30 seconds, Extension temperature 72°C for 30 seconds, Final Extension temperature 72°C for 7 minutes, 1 cycle and Hold temperature 10°C for 10.1 minutes with 1 cycle.

DNA sequencing

To establish the fundamental nucleotide sequences for the genes, PCR results for the 20 isolates (18 for *gyrA*) were sent to the Macrogen Corporation company in South Korea. The NCBI engine was used to evaluate the sequencing findings using BioEdit and geneious programs.

Results and Discussion

Results

A total of 320 clinical samples from UTI Women patients were processed. Based on a combination of laboratory methods including cultural, biochemical, APIE20 kits and VITEK2 Compact; the results revealed that 63 of isolates were identified as *E. coli* Using (Table II).

Table II

Biochemical test of *Escherichia coli* and Results

Test	Results
Gram Stain	Negative
Indol	Positive
Methyl Red	Positive
Voges-Proskuer	Negative
Simmon Citrate	Negative
Catalase	Positive
Oxidase	Negative
Urease	Negative

Antibiotic sensitivity test

Out of the 63 isolate, 33 (52%) isolate were resistant, 16 isolates (25%) were sensitive and 14 isolates (22%) showed different modes of sensitivity to resistance for one or more antibiotics tested (Table III). These results are consistent with previous research on clinical *E. coli* isolates [21, 22]. According to the findings of

this study, most of *E. coli* isolates exhibited a high degree of resistance to the tested antibiotics: Norfloxacin 33 (52.3%), Nalidixic Acid 47 (74.6%), Ciprofloxacin, Levofloxacin and Ofloxacin 35 (55.5%) (Figure 1).

Table III
The results of Antibiotic sensitivity test

No.	Antibiotics	Resistant No. (%)	Sensitive No. (%)	Intermediate No. (%)
1	**Ciprofloxacin**	35 (55.5%)	25 (36.9%)	3 (4.7%)
2	**Levofloxacin**	35 (55.5%)	25 (36.9%)	3 (4.7%)
3	**Ofloxacin**	35 (55.5%)	25 (36.9%)	3 (4.7%)
4	**Norfloxacin**	35 (52.3%)	29 (46.3%)	1 (2%)
5	**Nalidixic Acid**	47 (47.6%)	14 (22.2%)	2 (3.1%)

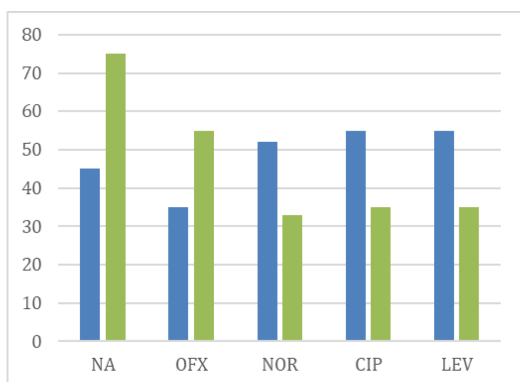

Figure 1.
Number and Percentage of Antibiotic –Resistant *E. coli* isolates

Minimum inhibitory Concentration (MIC)
20 antibiotic-resistant isolates were chosen from a total of 63 isolates for the MIC testing in line with AST and CLSI 2020 criteria for the purpose of determining antibacterial activity by using Vitek 2 compact system. The Results of MIC presented in Table IV. The finding of this study as revealed the lowest inhibition concentration, the inhibition concentration of antibiotic ciprofloxacin ranged from 2 to ≥ 8, the inhibition concentration of antibiotic levofloxacin ranged from 4 to ≥ 16), the inhibition concentration of antibiotic nalidixic acid ranged from ≥ 32 to ≥ 128), the inhibition concentration of antibiotic ofloxacin ranged from ≥ 4 to ≥ 16) and the inhibition concentration of antibiotic norfloxacin ranged from ≥ 32 to ≥ 64.

Table IV
The Results of MIC Test

No. of Resistance Isolate		MIC range (mg/ml)				
		CIP	NOR	OFX	LEV	NAL
1	BA3	≥ 4	≥ 64	≥ 4	≥ 8	≥ 128
2	BA5	≥ 8	≥ 64	≥ 4	≥ 16	≥ 32
3	BA6	2	≥ 64	≥ 8	4	≥ 64
4	BA8	≥ 8	≥ 64	≥ 8	≥ 16	≥ 128
5	BA10	≥ 4	≥ 32	≥ 8	≥ 8	≥ 64
6	BA12	≥ 4	≥ 64	≥ 8	≥ 8	≥ 128
7	BA15	2	≥ 32	≥ 16	2	≥ 64
8	BA17	≥ 4	≥ 32	≥ 8	≥ 8	≥ 128
9	BA18	≥ 4	≥ 32	≥ 16	≥ 8	≥ 64
10	BA19	≥ 4	≥ 64	≥ 8	≥ 8	≥ 128
11	BA22	≥ 8	≥ 32	≥ 4	≥ 16	≥ 64
12	BA24	≥ 4	≥ 32	≥ 8	≥ 8	≥ 64
13	BA25	2	≥ 32	≥ 8	4	≥ 128
14	BA26	2	≥ 64	≥ 4	4	≥ 128
15	BA28	2	≥ 32	≥ 4	4	≥ 64
16	BA30	≥ 4	≥ 32	≥ 8	≥ 8	≥ 64
17	BA34	≥ 4	≥ 64	≥ 8	≥ 8	≥ 32
18	BA35	≥ 4	≥ 32	≥ 8	≥ 8	≥ 64
19	BA38	≥ 4	≥ 32	≥ 8	≥ 8	≥ 64
20	BA47	≥ 4	≥ 64	≥ 8	≥ 8	≥ 64

NA: Nalidixic acid, CIP: Ciprofloxacin, LEVO: Levofloxacin, NOR: Norfloxacin, OFX: Ofloxacin.

Molecular detection of gyrA
The *gyrA* gene was found in *E. coli* isolates linked to fluoroquinolone resistance using the Conventional PCR technique. On the basis of a 54°C annealing temperature, specific primers were employed to amplify 347 bp of the *gyrA* gene (Figure 2). In this investigation, all clinical isolates of *E. coli* linked to antibiotic resistance had fluoroquinolone genes (*gyrA*) as a positive result on antibiotic susceptibility and MIC tests.

Direct sequencing for the gyrA gene.
BioEdit software was used to align the sequences of the *gyrA* gene (acc. app.) by comparing them to a reference sequence of *E. coli* strain SCU-120 (wild

type), which is available in the NCBI's GenBank database (acc. no. NZ_CP054335.1). Local sequences (only forward) of *E. coli* isolates (mutant type) was also used to discover the mutations in the *gyrA* gene associated to fluoroquinolone resistance in *E. coli* isolates (Figure 3 and Figure 4). Before starting the sequence alignment, Genious software eliminated the trimming from the ends of the *gyrA* sequences.

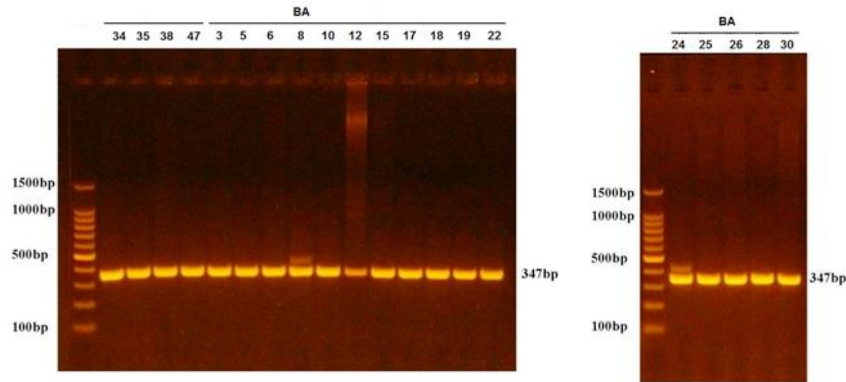

Figure 2.

Agarose gel –electrophoresis (1.5%) Agarose

The amplification of *gyrA* gene in *E. coli* isolates was segregated at 90 volts for 60 minutes and stained with Ethidium Bromide. The 100bp ladder marker BA34-BA30 lane resembles 347 bp of PCR products.

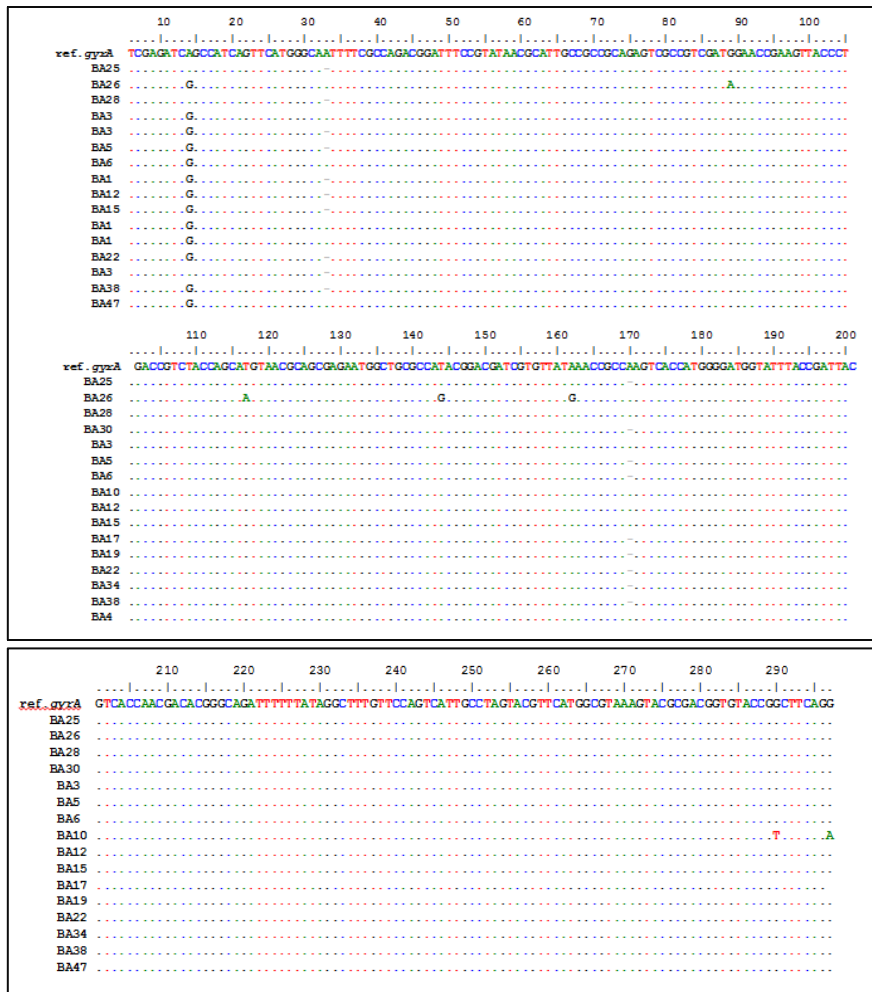

Figure 3.

The results of BioEdit software for the *E. coli* isolates with the appropriate reference sequence of the gyrA gene and changes in each isolate Reference sequence of the *E. coli gyrA* gene from strain SCU-120 (Wild type)

The letters "BA" stand for resistant isolates.

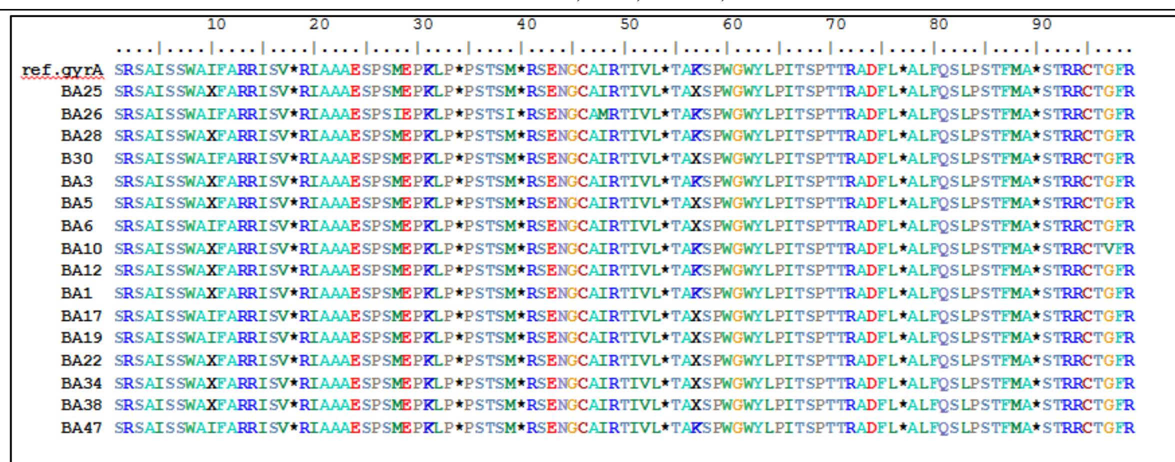

Figure 4.

Alignment of amino acids of the *gyrA* sequences by Bio-Edit software with changes and substitutions in isolate X: any type of amino acid, *: insertion, S = serine amino acid, M = methionine Amino acid, I = isoleucine amino acid, K = lysine amino acid, G = glutamine amino acid, V = valine amino acid, R = Arginine Amino acid

After aligning the *gyrA* sequences, BioEdit software was used to detect changes in amino acids in each isolate using the toggle translation option from the alignment menu, as indicated in the Figure 4.

It is worth mentioning that after DNA sequencing for the 16 isolates that showed resistance to 5 fluoroquinolone drugs, 9 distinct types of mutations at various positions in the *gyrA* gene were noticed as shown in Table V. Mutations in *gyrA* occurred owing to modifications or changes in nucleotide codons at particular locations, with 4 (25% missense mutations), 2 (12.5% deletion mutation), 2 (12.5% silent mutations) and 1(6.25% NON sense mutation) occurring at different positions. In *gyrA*, 9 mutations were found in codons (14, 33, 89, 117, 144, 162, 170, 290 and 297). Because of the existence of numerous mutations, may be lead to resistance of antibiotics In the future, the usage of this class of antibiotics (NA, CIP, NOR, OFX, and LEV) will be restricted.

Table V

Mutations of all amino acid and nucleotide of gyrA gene

No	Mu gyrA	Nucleotide		Amino Acid		Types of Mutation
		Position	Change Codon	Position	Change	
1.	BA3, BA5, BA6, BA10, BA1, BA15, BA17, BA19, BA22, BA26, BA30, BA38, BA47	14	TCA-TCG	6	S-S	Silent
2.	BA3, BA5, BA10, BA12, BA15, BA22, BA25, BA28, BABA38	33	ATT	10	I-X	Deletion
3.	BA26	89	ATG-ATA	29	M-I	Missense
4.	BA26	117	ATG-ATA	39	M-I	Missense
5.	BA26	144	ATA-ATG	48	M-I	Missense
6.	BA26	162	TAA-TAG	54	I-M	Non-Sense Mutation
7.	BA5, BA6, BA17, BA19, BA22, BA25, BA30, BA34, BA38	170	AGT	57	*-*	Deletion
8.	BA10	290	GGC-GTC	97	K-X	Missense
9.	BA10	297	AGG-AGA	99	R-R	Silent

Mu = No. of Mutant isolates, (*) mean insertion, (x) mean any type of Amino Acid, S = serine amino acid, M = methionine Amino acid, I = isoleucine amino acid, K = lysine amino acid, G = glutamine amino acid, V = valine amino acid, R = Arginine Amino acid

Fluoroquinolones (FQs) are a class of synthetic antimicrobials with a wide range of action that are frequently employed in hospitalised patients to prevent and cure *E. coli* related urinary tract infections (UTI) [29-33]. The widespread abuse for these drug by UTI patients has resulted in an increase in FQ resistance in *E. coli* [25]. According to genetic and biochemical investigations. DNA gyrase is primary goal in Gram negative bacteria like *E. coli* [34-37].

In this study the mutations in the *gyrA* gene of the 16 resistant *E. coli* isolates collected from the 63 clinical isolates are shown in Table VI. Nine mutations in the *gyrA* gene at various codons (14, 33, 89, 117, 144, 162, 170, 290 and 297) were found. The amino acid was used to identify nucleic acid changes in these codons. It was noticed that Methionine changed/replaced by to Isoleucine in codon 29 (Met 29 lle), Methionine changed/replaced by to Isoleucine in the

codon 39 (Met 39 lle), Isoleucine changed/replaced by to Phenylalanine in codon 48 (lle 48 Phe), stop codon in 54 (TAA-TAG), Alanine to Valine in codon 97 (Ala 97 Val), Arginine to Arginine in codon 99 (Arg 99 Arg), happened in one resistance isolate, Isoleucine changed/replaced by to Serine in codon 10 (lle 10 Ser) happened in 13 of resistance isolates Isoleucine changed/replaced by to Phenylalanine in codon 10 (lle 10 Phe) happened in 10 resistance isolates, Lysine changed/replaced by to Serine in codon 57 (Lys 57 Ser) happened in 9 resistance isolates. These results of amino acid mutation compatible with previous study [38-41]. The most resistant antibiotics in this investigation were nalidixic acid (74.6%), norfloxacin (52.3%), ciprofloxacin, levofloxacin and ofloxacin (55.5%). Furthermore, the presence of *gyrA* is typically linked with the greatest degree of resistance, which results in the failure of *E. coli* therapy, restricting the use of this class of antibiotics in the future.

Many locations of mutations were discovered in *gyrA* alignment investigations on amino acid sequences of *E. coli* resistant isolates between mutant and wild type, involving Met 29 lle, Met 39 lle, lle 48 Phe, stopcodon 54 , Ala 97 Val, Arg 99 Arg, happened in (12.5%) one resistance isolated, lle 10 Ser, happened in (81%) 13 of resistance isolated, Phe 10 Phe happened in (62.5%) 10 resistance isolates, Lys 57 Ser happened in (56%) 9 resistance isolates. This result of amino acid mutation compatible with different study [42, 43, 44, 45], which demonstrated the mutation that happened in Leu 83 Ser for 30 (100%) resistant isolates of *E. coli*, Asn 87 Asp for 27 (90%) resistant isolate and Asp 87 Tyr for 3 (10%) resistant isolates of *E. coli*, Ala 93 Thr have a third mutation for 5 (17%) resistant isolates of *E. coli,* Ser 93 Ala for one (3%) resistant isolates of *E. coli* and not related with Chenia [31] which demonstrated the mutation that happened in codon Ser 83 Leu and Asp 87Asn [46]. The mutations frequency in the resistant isolates show only one isolates exhibit a significant difference with p-value (≥ 0.05).

Mutations in the Fluoroquinolones (FQs) target enzymes, DNA topoisomerase II (DNA gyrase) and (topoisomerase IV), are known to be the main pathways by which resistance evolves in *E. coli*, the *gyrA* and *gyrB* genes encode the A and B subunits of DNA gyrase, respectively. Topoisomerase IV is similar to DNA gyrase and is made up of two subunits, ParC and ParE, that are encoded by the *parC* and *parE* genes [30].

These enzymes work by stimulating a double strand break in DNA, followed by the passage of another DNA strand across the split and its resealing [44]. Domains in *GyrA* and ParC are involved in DNA strand passing, whereas domains in *GyrB* and *ParE* are involved in ATPase action, which is necessary for enzyme catalysis, in *E. coli*, resistance to fluoroquinolones is caused by chromosomal mutation [39]. The majority of these mutations are located in *gyrA* or *parC* quinolone resistance-determining region (QRDR) [46].

Conclusions

The current study demonstrate that the widespread abuse of Fluoroquinolones antibiotics by Urinary Tract Infections patients has resulted in an increase in Fluoroquinolone resistance in *E. coli* related to mutations in the *gyrA* which encode DNA gyrase.

Conflict of interest

The authors declare no conflict of interest.

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