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RESEARCH PAPER

Molecular basis of ciprofloxacin (fluoroquinolone)-resistant in clinical isolates of *Escherichia coli*

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A B S T R A C T:

It has recently been shown that antibiotic resistance is considered as one of the extremely imperative worrisome in medicine, with tremendously resistant pathogens of numerous species of bacteria demonstrating difficult to treat. The aim of this study was to determine mutations in DNA gyrase subunits GyrA and GyrB, and topoisomerase IV subunits ParC and ParE in clinical isolates of *Escherichia coli*, which are important determining factors for paramount levels of fluoroquinolone (ciprofloxacin) resistance. This article was achieved through five months survey for the occurrence of ciprofloxacin resistant *E. coli* in clinical samples from outpatient clinics in Kalar city. Fifty seven samples were collected included (4) wound swabs, (1) conjunctiva, (2) vaginal and (2) otitis media swabs, as well as (48) urine samples from the period March to August, 2018. The collected samples were cultivated on selective and differential media for *E. coli* isolation. Classical biochemical tests and molecular basis (16SrRNA) were performed for the identification of 14 isolates of *E. coli*. These isolates were tested for antibiotic sensitivity (17 different antimicrobials agents were tested, included ciprofloxacin). The isolates showed ciprofloxacin resistance and were checked for mutations in the quinolone resistance-determining regions (QRDR) of *gyrA*, *gyrB*, *parC*, and *parE* genes by polymerase chain reaction and DNA sequencing. Subsequently, amino acid substitutions were detected by Clustal Omega. Two main mutations in *gyrA*, in addition to a range of extra mutations, were identified in resistant isolates. There were no mutations in the QRDR of each of *gyrB*, and *parC* of CIP-resistant isolates, except a single mutation in *gyrB* out of QRDR, and only in one isolate. However, one main mutation in *parE*, as well as two extra mutations were identified in two resistant isolates. The current study has demonstrated the occurrence of CIP-resistant *E. coli* in clinical specimens, with half of them being unsusceptible to ciprofloxacin, among those, 85.7% were also resistant to at least three antibacterial classes.

KEY WORDS: CIP (fluoroquinolone)-resistant *E .coli*, 16SrRNA, chromosomal mutations, *gyrA*, *gyrB*, *parC*, and *parE* genes
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1.INTRODUCTION :

One of the most urgent universal troubles in medicine is antimicrobial resistance, with the evolving of pathogenic species that are extensively drug- resistance demonstrating obstacle to management (Redgrave *et al.*, 2014). Broad-spectrum antibiotics quinolones and fluoroquinolones (FQs) are powerful inhibitors of DNA gyrase and topoisomerase IV, that are two bacterial type II topoisomerases, and are essential enzymes engaged in numerous important and key cellular activities comprising DNA replication (Drlica, 1990; Hooper and Wolfson, 1993; Hooper, 2001; Hoshino *et al.*, 1994; Pruss *et al.*, 1986); have entered the clinics since the end of 1980s for the eradication of extreme or resistive infections (Redgrave *et al.*, 2014). FQs have been extensively used in human and animal medicine (Aldred *et al.*, 2013), and ciprofloxacin (CIP) is the predominant one in human medicine (Hopkins *et al.*, 2005). In spite of the widely and optimistic treatment of *Escherichia coli* infections with fluoroquinolones, resistance to these antibiotics developed and increased

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significantly from an early stage of their initiation in medicine (Jiménez Gómez *et al.*, 2004).

Both of DNA gyrase and topoisomerase IV are heterotetramers, comprising of two A (GyrA) subunits and two B (GyrB) subunits, encoded by the *gyrA* and *gyrB* genes for the former, and two C (ParC) and E (ParE) subunits encoded by *parC* and *parE* genes for the two laters, respectively (Ruiz *et al.*, 1997). When either of these enzymes stimulates impermanent double-stranded breaks, from the beginning, they adhere covalently to the DNA double helix to construct enzyme-DNA complexes prior of breaking the bound DNA, then passing another piece of DNA via this break, and reconnecting the proper DNA (Drlica *et al.*, 2008). On the other hand, neither DNA gyrase nor topoisomerase IV will be able to re-ligate the DNA substrate when FQs bind to either; in this case the broken pieces of DNA bound to the enzyme are described as cleaved complexes (Drlica *et al.*, 2008). This binding of FQs takes place within the enzyme at the target site of helix-4 of either GyrA or ParC (Redgrave *et al.*, 2014).

Different mechanisms of resistance to FQs were discovered over the time in various species of bacteria, especially in *Salmonella* and *E. coli*. The two main genetic mechanisms of resistance were included chromosomal mutations and plasmid-mediated, which only the plasmid one is interchangeable (Hopkins *et al.*, 2008). Moreover, the most popular and well-known mechanism of resistance that confer highest level of resistance to FQs is the mutations in one or more of the genes (*gyrA*, *gyrB*, *parC*, and *parE*) of topoisomerases II, the primary and secondary target of these antibiotics (Redgrave *et al.*, 2014). A short DNA sequence in these genes known as quinolone resistance-determining region (QRDR) is the region where mutations arise from (Yoshida *et al.*, 1990; Yoshida *et al.*, 1991). Mutations in the QRDR of these genes lead to substitution in the amino acid in this region which in turn alter the structure and configuration of the target protein and ultimately changes the binding affinity of FQs to the target enzyme, resulting in drug resistance (Hooper, 2000; Piddock, 1999).

Earlier research studies have concluded that the occurrence of mutations in *parC* gene in addition to both of *gyrA* and *gyrB* genes have been attributed to the attainment of fluoroquinolone resistance (Nakamura *et al.*, 1989; Hooper and Wolfson, 1993; Ouabdesselam *et al.*, 1995; Vila *et al.*, 1996; Vila *et al.*, 1994). (Jiménez Gómez *et al.*), in 2004 mentioned that the mechanism of resistance to FQs has been mainly ascribed to mutations in both of *gyrA* and *parC* genes, but with lesser frequency in *gyrB* and *parE* genes. More precisely, mutations in the QRDR of *gyrA* and *parC* or in the associated genes for instance *gyrB* and *parE*, or in all of those genes together may also be engaged in increased levels of resistance to FQs (Hopkins *et al.*, 2005).

The aim of this study was to explore the presence of CIP-resistant *E. coli* in clinical specimens and to characterize representative resistant isolates with respect to the susceptibility to antimicrobials, and the presence of chromosomal mutations in the QRDR of *gyrA*, *gyrB*, *parC*, and *parE* genes.

2. MATERIALS AND METHODS

2.1 Sampling and Bacterial Identification

From March to August 2018 fifty-seven (57) clinical samples were gathered from patients who visited the private outpatient clinic in Kalar city. The clinical specimens included; (4) wound swabs (4), (1) from conjunctiva, (2) vaginal swabs, (2) otitis media, and (48) urine. Fresh swabs transferred to the laboratory of microbiology from the private clinic, and they had been cultured directly onto MacConkey agar plates. Urine samples were collected in sterile disposable container and labeled properly, then by the use of platinum microbiological loop 1µl of non-centrifuged urine samples were streaked onto plates of MacConkey agar. Cultured plates incubated at 37ºC for 18 to 48 hours. Lactose fermenter (LF) isolates were sub-cultured to prepare pure cultures. Initially, Gram reaction and colonial characteristics of the bacterial isolates were studied, then traditional biochemical tests (Cheesebrough, 2006; Willey *et al.*, 2008) included catalase, oxidase, indole, methyl red, Voges Proskauer, citrate, urease, nitrate reduction, H_2S, gas, heamolysis on blood agar and coagulase, applied on the presumptive *E. coli* colonies (isolates). PCR primers (Table. 1) designed by Tawfeeq *et al.* (2017) for the amplification of 16S rRNA was used for the confirmation of *E. coli* colonies.

2.2 Antimicrobial susceptibility testing

Kirby-Bauer disk diffusion method (Bauer *et al.*, 1966) was performed to test 17 different

antibiotics against *E. coli* isolates. Antibiotic discs included Piperacillin (100µg) Amikacin (30µg), Meropenem (10µg), Trimethoprim (10µg), Ciprofloxacin (10µg), Nitrofurantoin (300µg), Levofloxacin (5µg), Cephalexin (30µg), Moxifloxacin (5µg), Ofloxacin (5µg), Norfloxacin (10µg), Doxycycline (10µg), Cefixime (5µg), Cefpirome (30µg), Cefpodoxime (10µg), Cefotaxime (30µg), Amoxicillin/Clavulanic acid (30µg) were distributed on plates of Mueller-Hinton agar according to the newest version of the guidelines justified in the Clinical Laboratory Standards Institute (CLSI) by Patel *et al.* (2016) for the purpose of initial detection of ciprofloxacin-resistant *E. coli.*

2.3 Genomic DNA extraction

Colonies from fresh cultures of the presumptive *E. coli* isolates were utilised for the extraction of genomic DNA. Boil preparation (a single colony from each isolate was carefully suspended in 50µl of deionized water, then heated for 10 min at 95°C and spun down at 10,000 × g for 5 min. supernatant was used directly as a DNA template for PCR) or methodology described in *AccuPrep* Genomic DNA Extraction Kit (Bionner) was followed precisely.

2.4 Primers

All the primers that were used in the current study synthesized by Humanizing Genomics (Macrogen) are available in (Table. 1) For the molecular identification of *Escherichia coli* primers designed (Tawfeeq *et al.*, 2017) for the amplification of *Escherichia coli* 16SrRNA based on the complete annotation sequence of *E. coli* obtained from National Centre for Biotechnology Information (Accession No. J01859.1) and *E .coli* ATCC25922 as a reference strain.

In order to identify mutations in QRDR of *gyrA*, *gyrB*, *parC*, and *parE* genes in CIP resistance isolates of *Escherichia coli*, they were amplified by PCR using oligonucleotide primers (Table. 1). These primers were designed based on *gyrA*, *gyrB*, *parC*, and *parE* sequences of *E. coli* K-12, GeneBank accession numbers; X57174.1, D87842, M58408.1, and M58409.1, respectively.

2.5 PCR amplification (16SrRNA and QRDR of *gyrA*, *gyrB*, *parC*, and *parE* genes)

All the PCR assays were carried out in 25µl final reaction volume, composed of 2µl of DNA template (genomic DNA), 0.5µl of each primer (forward and reverse) for 16SrRNA, 12.5µl of One PCRTM master mix (GeneDirex), consisted of Taq DNA polymerase, dNTPs, PCR buffer, enhancer, gel loading dye, and fluorescence dye. Total volume of the reaction mixture was completed through the addition of the required amount of nuclease free water. Prepared PCR mixtures were spun down shortly for 5-10 seconds in a micro-refrigerated centrifuge, then placed in thermal cycler (TCY, Crealcon, NL) and exposed to the following cycling parameters: initial denaturation at 94°C for 4minutes, followed by 35 cycle of denaturation at 94°C for 30 seconds, annealing at 55°C for 1minute and extension at 72°C for 2minutes and a final extension step at 72°C for 5minutes.

Same procedure and PCR parameters were followed for the amplification of QRDR of *gyrA*, *gyrB*, *parC*, and *parE* genes, except that specific primers (Table. 1) for each of these genes were used, and the annealing temperature was at 51.5°C/1minute, and 54°C/minute for the multiplication of *gyrA* and *parC*, and *gyrB* and *parE* genes, respectively.

2.6 DNA analysis by agarose gel electrophoresis

To visualize the amplified DNA fragments, 1.5% agarose gel electrophoresis either containing ethidium bromide or prime safe dye (GeneAid) at 100 volts for 90 minutes, and at room temperature was used. Amplicon size determined by comparison with 100 bp DNA ladder (GeneDirex). This form of DNA analysis uncovered amplification of the expected 648-bp fragment for the *gyrA* gene, 447-bp fragment for the *gyrB*, 395-bp for the *parC*, and 266-bp for the *parE* gene, sequentially. All these fragments were contained the quinolone resistance-determining region of *gyrA*, *gyrB*, *parC*, and *parE* genes, respectively.

2.7 Gel purification

PCR products (fragments) in section 2.6 were gel purified using *PrimPrep* Gel Purification kit to remove primers, free nucleotides, and any unrelated bands (in case if there is any), in order to avoid their interaction with the subsequent steps, according to the manufacturer instructions.

2.8 DNA sequencing

Samples of the gel purified PCR products were processed as early as possible for DNA sequencing in South Korea.

2.9 Blast alignment and Clustal Omega (Clustal W)

Sequenced DNA fragments of the QRDR of *gyrA, gyrB, parC,* and *parE* genes were aligned using blast alignment with their sequences in the original DNA template. ExPASy program was used for the translation of DNA sequences of these genes into protein sequences. Then mutations in the amino acid sequences of each of their correspondence proteins revealed by Clustal Omega when compared with original (non-mutated) proteins of these genes.

3. RESULTS AND DISCUSSION

Out of 57 clinical samples collected for this study, 48 (84.21%) of them were urine samples, 4 (7.01%) wound swabs, 2 (3.5%) for each of otitis media and vaginal swabs, and the rest was 1 (1.75%) for the case of conjunctivitis (Table. 2). Since this work was about *Escherichia coli*, in particular, those resistant to ciprofloxacin with the detection of the expected mutations in the target enzymes, gyrase and topoisomerase, that confers *Escherichia coli* isolates un-susceptibility towards ciprofloxacin, a fluoroquinolone antibiotic, the focus was only on *E. coli* strains isolated from the tested clinical samples.

Out of the 47 samples that were collected, only 14 *E. coli* isolates were obtained, and they were all from the 48 samples of urine, while there was no any *E .coli* in the other samples (Table. 2). As usual, classical biochemical tests recommended in the diagnostic text books were performed for the identification of the isolates. Later on, isolates confirmed in terms of molecular biology via PCR amplification of 627bp of 16SrRNA (Table. 1 and Figure. 1).

Data from an agar diffusion technique which was performed for studying the susceptibility testing of *E .coli* isolates toward 17 antibiotics from different antimicrobial classes displayed that 12(85.7%) *E .coli* isolates were multidrug resistant. Gosling *et al.* (2012) stated that 88.1% of *E .coli* strains were multidrug resistant as well. However, in the current study only 7(50%) isolates were CIP resistant.

PCR amplification was performed on the 14 isolates of *E .coli*, however, gel purification of PCR products and sequencing were applied on all the 7 isolates of *E .coli* which were CIP-resistant in order to identify mutations in *gyrA, gyrB, parC*, and *parE* genes (Figures 2a, and 2b). According to a procedure described by Oram and Fisher (1991), a DNA fragment of 648 bp, covered a sequence of nucleotides from 24 to 671 of the QRDR of *gyrA* was amplified. For the amplification of nucleotide sequence from 995 and up to 1442 of QRDR of *gyrB* to obtain a piece of DNA with 447 bp a procedure described previously by Vila *et al.* (1994) was followed. Moreover, to amplify DNA fragments of 395 bp and 266 bp of QRDR of each of *parC* and *parE,* methodology described by Vila *et al.*, (1996), and Sorlozano *et al.*, (2007), respectively, were followed. However, it should be mentioned that although the primers that were used in this study were constructed by previous researchers, due to the fact of the confirmation that they are undoubtedly covering the QRDRs of the genes under investigation, but they all were checked to confirm that they have been constructed based on the *E .coli* K-12 genes sequences database, as described precisely in section 2.4.

DNA sequences (FASTA) of *gyrA, gyrB, parC*, and *parE* genes for the each of the 7 isolates of CIP-resistant *E .coli* were obtained from South Korea after a period of time. The sequence of each of the genes in each of the 7 isolates was blast aligned and compared with the corresponding sequence of QRDR of the reference strain, *E .coli* K-12. Then, ExPASy program was used to translate DNA sequences into amino acid sequences, which utilized in Clustal Omega program to reveal and identify the places where mutations in the amino acids sequences of GyrA, GyrB, ParC, and ParE proteins occurred that confers resistance to FQs (CIP).

One main mutation, encoding Ser83Leu was detected in the QRDR of *gyrA* in one of *E. coli* isolates that has been studied. This result is similar to those observed by Sorlozano *et al*., (2007) and Gosling *et al.*, (2012) to some extent. They have displayed the second main mutation as Asp87Asn, but in the current study this mutation was Asp87Val, which agreed with observations reported by (Oram and Fisher), in 1991, as they detected similar change. Vila *et al.*, in 1994 mentioned that amino acid changes observed in

eight mutants of *E. coli* isolates at amino acid 87 was Asp to Asn, while it was Asp to Tyr in other three mutants. Amino acid mutations Asp_{87}→Asn, or Gly or Tyr in *gyrA* were found in 7, 2, and 1 clinical isolates of isolates *E. coli*, respectively (Bachoual *et al.*, 1998). Other researchers clarified that the first main mutation, Ser83Leu, is responsible for conferring low level of resistant to FQs (Chapman and Georgopapadakou, 1988); (Oram and Fisher, 1991; Yoshida *et al.*, 1988). Furthermore, the second main mutation, the Asp87 codon of *gyrA* is associated with a significant increase in the resistance to FQs (Vila *et al.*, 1994). More precisely, Vila *et al.* (1994) concluded that Ser83Leu change fosters a high level of resistance toward nalidixic acid but a low level of resistance against ciprofloxacin, and Asp87Asn change possibly act a complementary role in developing high levels of resistance to ciprofloxacin in the strains. Gosling *et al.*, (2012) showed that 90% of CIP-resistant isolates had the two main mutations (Ser_{83}→Leu + Asp_{87}→Asn), while the rest of the strains (10%) had single mutation (Ser_{83}→Leu). They also showed that half of their strains had silent mutations, as extra mutations. In the current study, Ala85Val, Arg98Threo, Ile117Arg, Pro118Lys, and Pro120Val were observed as additional mutations that were located the quinolone resistant determining region of *gyrA* in two of the isolates (Figure. 3). Similar results were observed by Yoshida *et al.*, (1990), as they described additional mutations such as Ala67, Gly81, Ala84, and Gln106 for the QRDR of *gyrA*. Randall *et al.*, (2005) explained these extra mutations as they probably representing the variation in strains of *E. coli*, the condition which has also been seen in *Salmonella*.

Within the *gyrB* gene of *E. coli* two quinolone resistance-determining sites, Asp426 and Lys447 are present where mutations occured (Ruiz *et al.*, 1997; Yamagishi *et al.*, 1986; Yoshida *et al.*, 1991). Mutation in the first point Asp_{426}→Asn confers resistance to either the old quinolones (nalidixic acid) or to the new fluoroquinolones (ciprofloxacin), whereas mutation in the second point Lys_{447}→Glu increased the susceptibility to the new fluoroquinolones while confers resistance to nalidixic acid (Yoshida *et al.*, 1991); Hooper and Wolfson, 1993). This study did not detect any single or double mutations in the QRDR of *gyrB*, which is consistent with the results of (Jiménez Gómez *et al.*, 2004), while it contradicts the results presented by (Vila *et al.*, 1994), as they determined only a single amino acid change, Lys447Glu at the GyrB protein in one of the clinical isolates of *E. coli* out of 27. Concomitantly, Vila *et al.*, (1994) and Quabdesselam *et al.*, (1995) added that this is clearly and strongly indicating the predominance of mutations in *gyrA* over *gyrB*. However, a single amino acid change from Glycine to Arginine was determined out of QRDR (Figure 3), and in one of the strains, which has not been previously described.

Although Gosling *et al.*, (2012) mentioned that FQ- resistant in clinical strains of *E. coli* has often been attributed to the presence of two mutations in *gyrA* and one mutation in *parC*, in this study, none of the 7 strains of CIP-resistant *E. coli* possessed single or double mutations in the DNA sequence of *parC* that acquired detectable amino acid substitutions in the QRDR of ParC. The current observations were in agreement with conclusions reported by (Jiménez Gómez *et al.*, 2004). In contrast to the present study, Chen *et al.*, in 2001 showed that (63%) of the clinical isolates of *E. coli* exhibited a single mutation Ser80Ile of ParC. Moreover, Bachoual *et al.*, (1998) showed that all the clinical strains of *E. coli* were carried one amino acid substitution in ParC; either Ser80Ile, or Ser80Arg, or Glu84Lys in 8, 2, and 1 of the isolates. Mutations in the QRDR of *parC* in the *E. coli* isolates observed by Sorlozano *et al*., (2007) encoded Ser80Ile, Ser80Arg, Glu84Lys, Glu84Gly, and Glu84Val, respectively. Interestingly, Vila *et al.*, (1996), proved that feasible amino acid changes in both of ParC and GyrA prompt an increased level of FQ resistance.

With regard to *parE*, present study showed the presence of a single mutation in the QRDR of *parE*, encoding Ser458Ala in one of the isolates, in addition to two other mutations outside the QRDR, which they are Serine to Phenylalanine and Leucine to Proline in another isolate of *E. coli*. This result is suggesting that *parE* gene mediate or contribute to the development of fluoroquinolone resistance. Sorlozano *et al*. (2007) found a single mutation in the QRDR of *parE* gene of 10 isolates of *E. coli*, encoding Sr458Ala. However, Lindgren *et al.* (2003) reported a different mutation located outside the quinolone resistant determining region, occurred in the identical codon as that for Ser458Thr. In the contrary, each of Ruiz *et al.* (1997) and Jiménez Gómez *et al.* (2004), independently, did not find

any changes in the QRDR of *parE*. However, the last group of researchers did not eliminate the possibility of the occurrence of other substitutions outside QRDR. This is due to the fact that they have investigated the presence of homology between QRDR of *gyrB* gene in the B subunit of DNA gyrase represented by Asp426 and Lys447 with the residues Asp420 and Lys441 of ParE, which would be the most significant candidate for study in *parE* gene in case if it plays a role in the evolution of quinolone resistance. Therefore, based on this assumption, Sorlozano *et al*. (2007) concluded that mutations in *parE* may be linked to quinolone resistance. As the current study detected mutations outside the QRDR of *parE*, which is concomitant with the observations of other researchers, Hopkins *et al.* (2005) recommended the performance of further investigation to confirm their contribution to quinolone resistance.

Table 1. Sequences, symbols, and product size of reference primers

Primers	Primer Sequence (5'--------3')	Product Size (bp)	Reference
16SrRNA-F	GAAGACTGACGCTCAGGTGCGAA	627	(Tawfeeq *et al.*, 2017)
16SrRNA-R	CCGTGGCATTCTGATCCACGATTA		
gyrA-F	TACACCGGTCAACATTGAGG	648	(Oram and Fisher, 1991)
gyrA-R	TTAATGATTGCCGCCGTCGG		
gyrB-F	CTCCTCCCAGACCAAAGACA	447	(Vila *et al.*, 1994)
gyrB-R	TCACGACCGATACCACAGCC		
parC-F	AAACCTGTTCAGCGCCGCATT	395	(Vila *et al.*, 1996)
parC-R	GTGGTGCCGTTAAGCAAA		
parE-F	TACCGAGCTGTTCCTTGTGG	266	(Sorlozano *et al.*, 2007)
parE-R	GGCAATGTGCAGACCATCAG		

Table 2. Percentage of clinical specimens, and the prevalence of (CIP-resistant and CIP-sensitive) *E. coli*

Specimen	No. (% of clinical specimen)	Total no. of *E. coli*	CIP-resistant *E. coli*	CIP-sensitive *E. coli*
Urine	48 (84.21%)	14(100 %)	7(50%)	7(50 %)
Wound swab	4 (7.01)	0	0	0
Otitis media	2 (3.50%)	0	0	0
Vaginal swab	2 (3.50%)	0	0	0
Conjunctiva	1 (1.75%)	0	0	0
Total	57	14	7	7

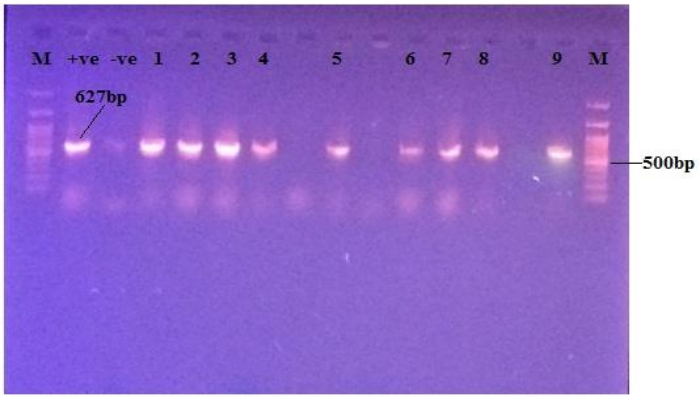

Figure 1. Identification of *E. coli* isolates in terms of molecular biology. M: 100bp DNA marker, +ve; positive control (16SrRNA of *E .coli* ATCC25922), -ve; negative control. Lanes 1 – 9; PCR amplification of 627bp of 16SrRNA of *E. coli* strains.

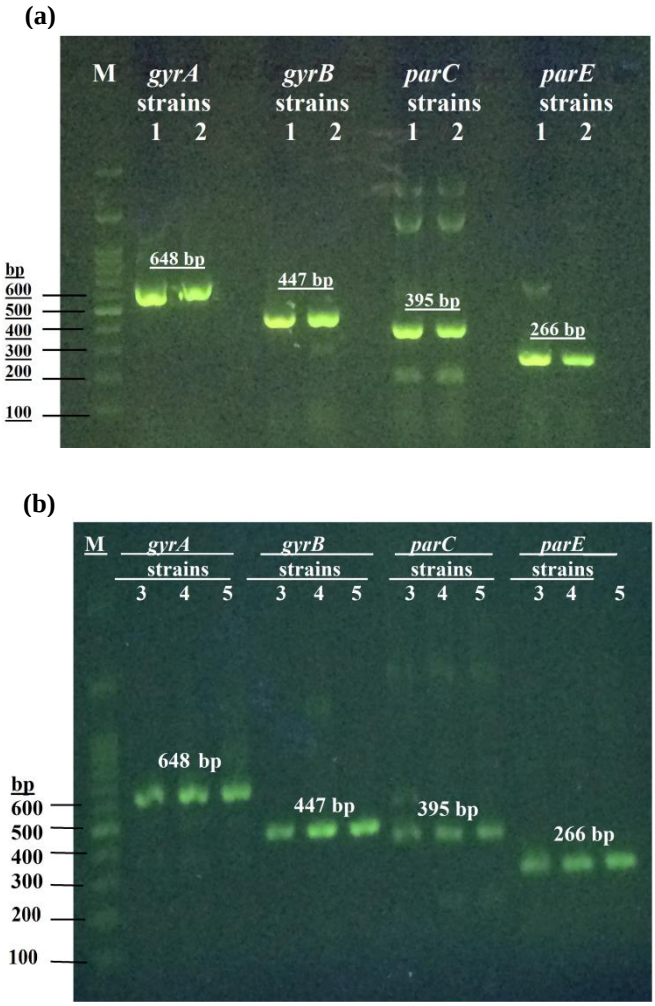

Figure 2. PCR fragments of QRDR of *gyrA*, *gyrB*, *parC*, and *parE* from CIP-resistant *E. coli* isolates 1, and 2 in **(a)**, and isolates 3, 4, and 5 in **(b)**. M: 100bp DNA marker, from left to right; lanes 1 & 2 are PCR products (648 bp) of *gyrA*, lanes 4 & 5 are PCR products (447 bp) of *gyrB*, lanes 7 & 8 are PCR products (395 bp) of *parC*, lanes 10 & 11 are PCR products of *parE*, lanes 3, 6, and 9 left blanks in **(a)**.

```
gyrA        YTGQHStopGRAEELLSGLCDVGHCWPCAARCPRWPEAGTPSRTLRHERTRQStop----     56
A-Ref.      --------PLTPIWIMetRCRSLLAVRCRMetSEMetAStopSRYTVAYFTPStopTYSt     52
A-A         --------PIILSWIAMetSG--HCWPCAARCPEMetAStopSRYTVAYFTPStopTYSt     50
A-M         ---------------------------------------------------TPStopTYSt     10
                                    ****

gyrA        ----------------LEQSLS--------------------------topKICPCRWSt     74
A-Ref.      opAMetTGTKPIKNLPVSLVTStopSVNTIPMetVTWRFMetTRSSAWRSHSRCVTCWSt     112
A-A         opAMetTGTKPIKNLPVSLVTStopSVNTIPMetVTWRSMetTRSSAWRSHSRCVICWSt     110
A-M         opAMetTGTKPIKNLPVSLVTStopSVNTIPMetVTRRSMetTRLSVWRSHSRCVICWSt     70
                 :.   *                              . *   ***

gyrA        opRNRStop------------IPSPWStopLGGLStopHDRPHGAAILAALYAGRRSGSt     122
A-Ref.      opTVRVTSVLSTATLRRQCVIRKSVWRKLPMetNStopWPISKKRRSISLITMe------     166
A-A         opTVRVTSVLSTATLRRQCVIRKSVWRKLPMetNStopWPISKKRRSISLITMe------     164
A-M         opTVRVTSVPSTATLRRQCVIRKSVWRKLPMetNStopWPISKK----------------     114
                  **  * *                * * . *:   ****    :
        ********************************************************************************

B-A         AFSEStopSAVDSRStopTNCWRNTCWKTQPTRKSWSAKLSMetLPVPVKLRVARVKSto      60
gyrB        ------------------------------------SSQTKDKLVSSEVKSAVEQQM--e      22
B-M         ---------------------------------------VLStopVISGStopQQM--e      18
                                  .          :

B-A         pPAVKVRWIStopLACRAN------WQTARNAIRRFPNCTLWKGTPRAALRSRGVTARTR      114
gyrB        tNELLAEYLLENPTDAKIVVGKIIDAARAREAARRAREMe--tTRRKGALDLAGLPGK-L      79
B-M         tNELLAEYLLENPTDAKIVVGKIIDAARAREAARRAREMe--tTRRKGALDLAGLPGK-L      75
              : ..::   *  .:           **:* **  :    .   :.**   *: .:

B-A         RFCRStopRVKSSTLRKRASIRCS------------------LLR----KWRR-StopS-      150
gyrB        ADCQERDPALSELYLVEGDSAGGSAKQGGNRKNQAILPLKGKILNVEKARFDKMetLSSQ      139
B-M         ADCQERDPALSELYLVEGDSAGGSAKQGRNRKNQAILPLKGKILNVEKARFDKMetLSSQ      135
             *:.  * :..  * :  *   *                  :*.    :: : .*  *

                        B-A       ------PRLAVVAGRE   160
                        gyrB      EVATLITALGCGIGR-   154
                        B-M       EVATLITALGCGPGRE   151
        ********************************************************************************

A-C         ------------GSVStopLGStopCQRQIStop-----KIGPYRRStopR---TGS---      37
parC        KPVQRRIVYAMetSE------LGLNASAKFKKSARTVGDVLGKYHPHGDSACYEAMetVL      54
M-C         ---------RMetStopSDWAStopCQRQFKKSARTVGDVLGKYHPHGDSACYEAMetVL      51
                   *           .. ::..        :* *:         : .

A-C         -topIPSARRYRLLStopSD-GPDGA-------------------TVLLPLSAGStopWS      76
parC        MetAQPFSYRYPLVDGQGNWGAPDDPKSFAAMetRYTESRLSKYSELLLSELGQGTADWV      114
M-C         MetAQPFSYRYPLVDGQGNWGAPDDPKSFAAMetRYTESRLSKYSELLLSELGQGTADWV      111
              * : ** *:.   .  .**.                      :**   . .*  *

A-C         GELGRAGRSEIVRGNALHRIPVVEIFRAAIERAGAGDGStopLGAKLRRHFAGAENATCP      136
parC        PNFDGTLQEPKM-etLPARLPNILLN-----------------GT---------------      141
M-C         PNFDGTLQEPKM-etLPARLPNILLN-----------------GTTKRRStopILN----      149
                  ::. : :.  :  .   *:* : :                  *:

                        A-C       SAKHFAStopQGTTK    151
                        parC      ---------------    141
                        M-C       ----VSAPSPPGKGK    160
        ********************************************************************************

 A-E        ------------------------------------------------------------Stop       4
 parE       LNCWRRWRFPAPSAVCVRPKKWCVKSStopPAARRCLANWLIVPRRTLTVPSCSLWKVTP       60
 M-E        ------------------------------------------------------------R       1


A-E          RADLPAGARSRI-----SGDHATERStopDPStop-------HLGSLFRRSA----GF--       46
parE        QADLPSRRAIANIRRSCHStopKVRSLTPGKSLPTKCWLRRKC--TIFRStopRSVSILT       118
M-E         RRGSASRRAIANIRRSCHStopKVRSLTPGKFLPTKCWPRRKCMetIFRStopRSVSILT       61
            : .  :            .   . **  *.              ::** :     .:

A-E         ---AGSARYFGSDRYRSStopQRRSEPASLWQNLYPRGCGL-------------------       84
parE        ATIStopASFVMetAKS---VSSRMetR----TLMetVCTLPRCSALCSStopNISARWS       171
M-E        ATIStopASFVMetAKS---VSSRMetR----TLMetVGTLP------------------       96
                    :     *  :  :*    . * *      .*      *

                         A-E       ---StopWLAHC--     93
                         parE      topNTVTFTSHCHR     185
```

M-E ---------- 96

Figure 3. CLUSTAL OMEGA. Alignment of multiple amino acid sequences derived from the nucleotide sequences of *gyrA*, *gyrB*, *parC*, and *parE* genes of CIP-resistant *E. coli* isolates in comparison to the original amino acid sequences of GyrA, GyrB, ParC, and ParE of *E. coli* K-12.

4. CONCLUSIONS

In summary, we have demonstrated the role of mutations in *gyrA* gene of DNA gyrase, and *parE* gene of the topoisomerase IV in the resistance of *E. coli* isolates against fluoroquinolones, especially ciprofloxacin. This illustrates the conclusion that the mechanism of targeting of either DNA gyrase or topoisomerase by fluoroquinolones depends on both of the bacterial species and specific FQ, in a manner that DNA gyrase is targeted by FQs in Gram-negative bacteria, while topoisomerase IV is preferentially targeted in Gram-positive bacteria (Drlica *et al.*, 2008). Although of this preference, if the primary target of FQs has been substituted to resistant allele, they will bind to the secondary target and exhibit antibacterial action (Redgrave *et al.*, 2014).

In addition to the target-site mutations in DNA gyrase, in case if it is accompanied by mutations in topoisomerase IV or not, is considered an important factor for determining high levels of resistance to fluoroquinolones, there are extra mechanisms implicated in conferring resistance to FQs that were not been included in the present study. Mutations that results in downregulation of the outer membrane porin proteins that are present in the cell wall of Gram-negative bacteria, and works like a barrier for hydrophilic molecule (Chenia *et al.*, 2006; Strahilevitz *et al.*, 2009) are known in the isolates of diverse species with quinolone resistance (Danilchanka *et al.*, 2008; Everett *et al.*, 1996). Chromosomal multidrug efflux pump that are actively expels the antibiotics out of the bacterial cells, and, to a lesser degree, the occurrence of plasmid-mediated quinolone resistance (PMQR) genes (Redgrave *et al.*, 2014), are regarded as complementary mechanisms that cannot be ruled out and possibly enhance the emergence of resistance and associate to the selection of isolates with FQ resistant during the term of management with these group of antibiotics.

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Conflict of Interest

There is no conflict of interest.

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