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*Original Article*

# A novel parC mutation potentiating fluoroquinolone resistance in *Klebsiella pneumoniae* and *Escherichia coli* clinical isolates

Marwa Atef Yakout[1], Ghada Hani Ali[1]

[1] *Department of Microbiology and immunology, Faculty of pharmacy, Pharos University in Alexandria, Alexandria, Egypt*

**Abstract**

Introduction: Resistance to fluoroquinolones is mainly due to point mutations that gave rise to amino acid substitutions in the quinolone resistance-determining regions of either *gyrA* or *parC* genes, which may be augmented by plasmid mediated resistance. Accordingly, the main aim of the study was to investigate the mutations in *gyrA* and *parC* genes as well as the *qnrA* and *qnrB* genes acquisition.

Methodology: 193 *Klebsiella pneumoniae* and *Escherichia coli* isolates were collected, identified and MICs for ciprofloxacin, levofloxacin and moxifloxacin were determined. Polymerase Chain Reaction to investigate *qnrA, qnrB, gyrA* and *parC* genes followed by DNA sequencing analysis to identify mutations in *gyrA* and *parC* genes.

Results: The most prominent mutation in *gyrA* gene was ser83leu, followed by asp87asn, and lys154arg. Regarding *parC* mutations, ser80ile was the most detected. Other mutations val141ala and glu84ala were also noted. In addition to a substitution mutation at codon 157 of leucine to tyrosin. To the best of our knowledge this mutation was not previously reported. *qnrB* was the most detected gene, as 64.7% *Klebsiella pneumoniae* and 57.1% *Escherichia coli* were positive. *qnrA* gene was detected in 11% *Klebsiella pneumoniae* and 4% of *Escherichia coli* isolates tested.

Conclusions: This study suggests that the indiscriminate use of fluoroquinolones resulted in the increase of development of resistance either through mutations in the quinolone resistance-determining regions of either *gyrA* or *parC* genes augmented by plasmid mediated resistance. The irrational use of new fluoroquinolones such as moxifloxacin has created selective pressure for the appearance of new mutation.

**Key words:** *Klebsiella pneumonia; Escherichia coli; gyrA*; *parC*; Fluoroquinolones; Mutations.

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## Introduction

Among *Enterobacteriaceae*, *Klebsiella pneumoniae* and *Escherichia coli* are frequently associated with nosocomial infections [1-4]. Fluoroquinolones are considered one of the most potent broad-spectrum agents heavily utilized to treat a range of infections caused by Gram-negative bacteria [5,6]. Ciprofloxacin remains to be one of the most important antibiotics according to the World Health Organization. Levofloxacin as well as moxifloxacin have been used in the treatment of multidrug-resistant (MDR) infections. Despite the development of newer generations of fluoroquinolones with increased potencies, many of these have been abandoned from clinical practice due to safety issues, and the older fluoroquinolones remain the critically available alternatives [6].

Due to the widespread and irrational use of fluoroquinolone, the resistance to fluoroquinolones is becoming particularly serious [2,3]. Resistance to quinolones occurs through multiple mechanisms. Quinolone-resistance-determining regions (QRDRs) mutations are the most common mechanism of quinolone resistance [5,7]. Resistance to fluoroquinolones is mainly due to point mutations that gave rise to amino acid substitutions in the quinolone resistance-determining regions (QRDRs) of either *gyr*A or *par*C genes, or both genes together [8]. Other mechanisms of quinolones resistance are also worth attention [5]. The plasmid-mediated *qnr* determinant protects DNA gyrase and also topoisomerase IV from the inhibitory effect of quinolones [9-11]. Plasmid mediated resistance to quinolones (PMQR) generally results in lower resistance to fluoroquinolones. Nevertheless, its association with mutations in quinolone resistance-determining regions (QRDR) can lead to selection of higher-level -resistant mutants [7, 8,12].

It has been postulated that fluoroquinolones with different molecular structure could have different primary targets and mutation positions within QRDR [13,14].

In an attempt to clarify the effect of different mutations within QRDR as well as the acquisition of *qnr* genes on the resistance to the different fluoroquinolones such as ciprofloxacin, levofloxacin, and moxifloxacin, we investigated quinolone resistance-determining regions (QRDRs) mutations and *qnr* genes availability in *Klebsiella pneumoniae* and *Escherichia coli* in isolates from different hospitals in Alexandria, Egypt.

## Methodology

*Bacterial isolates*

193 clinical strains (100 *K. pneumoniae* and 93 *E. coli*) were collected from four major hospitals in Alexandria, Egypt. They were isolated from urine (n = 68), blood (n = 44), pus (n = 21), sputum (n = 31), broncho-alveolar lavage (n = 28) and chest tube (n = 1). Isolates were identified using standard biochemical methods after Gram stain [15]. The identified stock cultures were preserved at −80°C in 15% glycerol.

*Antibiotic susceptibility testing*

The susceptibility testing of the isolates to the different antibiotics was done by the disk diffusion method according to the CLSI 2016 [15]. *K. pneumoniae* ATCC 35657 and *E. coli* ATCC10408 were used as the respective control strain.

All culture media and antibiotic discs used were purchased from Oxoid (Oxoid Ltd; Basingostok; Hampshire, England).

*MIC determinations*

MICs for ciprofloxacin, levofloxacin and moxifloxacin were determined by the microbroth dilution technique described EUCAST 2017 [16]. Serial two-fold dilutions were prepared in sterile distilled water and were distributed in 96-well polypropylene microtiter plates. The inoculum of each isolate was adjusted spectrophotometrically to $1\times10^8$ CFU/mL ($OD_{600}$ 0.12-0.13) and diluted to create a final concentration of $5\times10^5$ CFU/mL in the microtiter plate. The microtiter plates were incubated at 37 °C for 18 to 20 hours. The MIC was defined as the least concentration of antibiotic giving complete inhibition of visible growth.

*PCR and DNA sequencing*

The bacterial DNA was obtained by suspending colonies in 200 µL sterile distilled water; the suspension was then heated at 100 ºC for 15 minutes followed by centrifugation at 1400 rpm for 5 minutes [17-19]. Colony lysates of selected bacterial isolate were used as the PCR amplification template. The primers for their fluoroquinolone's resistance determining region QRDR and PMQR, in addition to the PCR conditions used in this study are listed in Table 1. The primers were procured from Sigma Oligos, India. The DNA amplification was done in a DNA thermal cycler (Tpersonal Thermocycler biometra, Applied biosystem, USA). 2% agarose gel in Tris-borate-EDTA buffer was used for PCR products separation. Gels were run at a voltage of 100 V for 1 hour, stained in 2 µg/mL ethidium bromide for 10 minutes and visualized under UV transilluminator (BIORAD, Italy).

PCR products for genes encoding QRDR using chain termination method. QRDR mutations were determined by BioEdit sequence alignment editor, using the control sequence for NC_000913.3 for *E. coli* and FO834906.1 for *K. pneumoniae.*

Reversion of mutations was done by subsequent subculture in antibiotic free medium for 7 consecutive days to ensure the stability of mutations.

## Results

*Antibiotic resistance pattern*

According to the Kirby-Bauer disc diffusion method, quinolones resistance among the *K. pneumoniae* isolates tested was as follows: Nalidixic acid (75%), moxifloxacin (72%); levofloxacin (50%); and ciprofloxacin (54%). Whereas, resistance to quinolones among the *E. coli* isolates tested was as follows: Nalidixic acid (80%), moxifloxacin (79%), levofloxacin (67%), and Ciprofloxacin (73%).

**Table 1.** The primers and PCR conditions of the tested fluoroquinolone resistance genes.

| Resistant gene | Primers sequence | Band Size (bp) | Annealing temperature | Ref |
|---|---|---|---|---|
| *gyrA* | F: 5' ATG AGC GAC CTT GCG AGA GAA ATT ACA CCG 3'<br>R: 5' TTC CAT CAG CCC TTC AAT GCT GAT GAT GTC TTC 3' | 630 | 60 °C | [19] |
| *parC* | F:5'ATG AGC GAT ATG GCA GAG CGC CTT GCG CTA 3'<br>R: 5' ACG CGC CGG TAA CAT TTT CGG TTC CTG CAT 3' | 480 | | |
| *qnrA* | F: 5' ATTTCTCACGCCAGGATTTG 3'<br>R: 5' GATCGGCAAAGGTTAGGTCA 3' | 516 | 55 °C | [40] |
| *qnrB* | F: GTTGGEGAAAAAATTGACAGAA<br>R: ACTCCGAATTGGTCAGATCG | 383 | | |

**Table 2.** MIC50 and MIC90 in mg/L of ciprofloxacin, levofloxacin and moxifloxacin against K. pneumoniae and *E. coli* isolates tested.

| | $MIC_{50}$ in mg/L | $MIC_{90}$ in mg/L | EUCAST breakpoints in mg/L |
|---|---|---|---|
| ***K. pneumoniae*** | | | |
| Ciprofloxacin | 32 | 250 | 0.25-0.5 |
| Levofloxacin | 16 | 250 | 0.5-1 |
| Moxifloxacin | 64 | 500 | 0.25 |
| ***E. coli*** | | | |
| Ciprofloxacin | 16 | 500 | 0.25-0.5 |
| Levofloxacin | 16 | 500 | 0.5-1 |
| Moxifloxacin | 16 | 500 | 0.25 |

$MIC_{50}$: MIC at which 50% of the isolates are inhibited; $MIC_{90}$: MIC at which 90% of the isolates are inhibited.

*MIC determinations*

MICs of ciprofloxacin, levofloxacin and moxifloxacin against *K. pneumoniae* and *E. coli* isolates tested ranged from 0.03125 to 500 mg/L, while that of moxifloxacin ranged from 0.0625 to >1000 mg/L. The $MIC_{50}$s of ciprofloxacin and levofloxacin and against both *K. pneumoniae* isolates were 32 mg/L and 16 mg/L, respectively. The $MIC_{90}$s of levofloxacin and ciprofloxacin against *K. pneumoniae* were 250 mg/L, whereas ciprofloxacin and levofloxacin $MIC_{50}$s and $MIC_{90}$s against *E. coli* were 16 mg/L and 500 mg/L, respectively. $MIC_{50}$s and $MIC_{90}$s of moxifloxacin among *K. pneumoniae* isolates were 64mg/L and 500 mg/L, respectively; whereas, *E. coli* isolates showed $MIC_{50}$s and $MIC_{90}$s were 16 and 500 mg/L, respectively (Table 2).

*Prevalence of fluoroquinolone resistance genes*

Prevalence of PMQR genes

*qnrB* was the most frequently detected gene, as 64.7% and 57.1% of *K. pneumoniae* and *E. coli* isolates were positive for *qnrB,* respectively. *qnrA* gene was detected in 11% of the *K. pneumoniae* and 4% of the *E. coli* isolates that were tested. Coexistence of both genes was 5% and 2 % respectively among the tested *K. pneumoniae* and *E. coli* isolates.

QRDR mutations

QRDR mutations in *gyrA* and *parC* was were analysed by PCR, followed by DNA sequencing. The most prominent mutation in *gyrA* gene was at codon 83 (ser83leu), followed by asp87asn. Another substitution mutation at codon 154 was also noted (lys154arg). Regarding *parC* mutations, substitution of serine with isoleucine was the most frequent mutation. Other mutations val141ala and glu84ala were also noted, in addition to a substitution mutation at codon 157 of leucine to tyrosine. To the best of our knowledge this mutation has not been previously reported (Table 3).

## Discussion

Among members of the family *Enterobacteriaceae*, fluoroquinolones resistance is an expanding problem in hospital settings [1-4,20].

**Table 3.** The prevalance of qnrA and qnrB and QRDR mutations among selected *K. pneumoniae* and *E. coli* isolates.

| Sample | Fluoroquinolone resistance pattern | PMQR Genes | QRDR Genes | |
|---|---|---|---|---|
| | | | gyrA mutation | parC mutation |
| *K. pneumoniae 1* | Ciprofloxacin Levofloxacin Moxifloxacin | *qnrA, qnrB* | Ser83leu; lys154arg; asp87asn | ser80ile val141ala |
| *K. pneumoniae 2* | Ciprofloxacin Levofloxacin Moxifloxacin | *qnrA, qnrB* | ser83leu asp87asn | ser80ile |
| *K. pneumoniae 3* | Ciprofloxacin Moxifloxacin | *qnrB* | ser83leu | ser80ile |
| *K. pneumoniae 4* | Ciprofloxacin Moxifloxacin | *qnrB* | ser83leu lys154arg | ser80ile |
| *K. pneumoniae 5* | Moxifloxacin | *qnrB* | ser83leu | leu157tyr |
| *E. coli 1* | Ciprofloxacin, Levofloxacin Moxifloxacin | *qnrA, qnrB* | ser83leu asp87asn | ser80ile val141ala glu84ala |
| *E. coli 2* | Ciprofloxacin, Moxifloxacin | *qnrB* | ser83leu | val141ala leu157tyr |
| *E. coli 3* | Ciprofloxacin Moxifloxacin | *qnrA* | ser83leu | leu157tyr |
| *E. coli 4* | Ciprofloxacin Levofloxacin | *qnrA, qnrB* | ser83leu asp87asn | ser80ile |
| *E. coli 5* | Moxifloxacin | *qnrB* | ser83leu | No mutation |

Based on the molecular structural differences between fluoroquinolones members, different rates of resistances are expected to be observed. In the present study the percentage of *K. pneumoniae* (72%) and *E. coli* (79%) isolates that were resistant to moxifloxacin, was higher than those resistant to ciprofloxacin (73% and 54% respectively), these results were consistent with previous studies [1, 21,22]. On the other hand, levofloxacin seemed to be slightly more active against both *K. pneumoniae* and *E. coli* isolates tested (50% and 67% respectively). Other localities such as China, Taiwan, South Africa, Korea, and the United States have also stated that levofloxacin had higher activity against *K. pneumoniae* and *E. coli* isolates [1,14,23-28].

Resistance to fluoroquinolones is mainly based on the accumulation of several determinants, such as mutations in QRDR as well as the presence of PMQR determinants [21]. Substitution mutations in quinolone resistance-determining region (QRDR) of *gyrA* or *parC* gene are mainly located between residues 67 to 106 and residues 63 to 102 located in *E. coli* [29].

Mutations in QRDR remains the main mechanism of resistance that generally results in high-level of resistance. Based on this fact *gyrA* and *parC* mutations were analyzed. All the tested fluoroquinolone resistant *K. pneumoniae* and *E. coli* isolates had mutations in codons 83 and/or 87 of the *gyrA* gene. Vila *et al*., Kotb *et al.*, Minarini *et al.* and Ruiz *et al.* [8,11,30,31] suggested that the additional mutation in codon 87 is related to increased fluoroquinolone resistance. Low-level fluoroquinolone resistances has been linked to single alteration in the *gyrA* protein, whereas high-level resistance requires the occurrence of double mutations [8,11,32]. Our results are consistent with these findings as isolates that expressed greater level of resistance possessed double mutations in *gyrA* gene as well as *parc* gene [3]. Substitution mutation at codon 80 of the *parC* gene was the most prominent among our isolates and this was consistent with Minarini *et al.*[11] Substitution mutation at codon 141 (Val141Ala) was also common among our isolate, this mutation was previously reported in a study conducted in Brazil by Araújo *et al.*[4] Another substitution mutation at codon 84 of the *parC* gene (Glu84ala) was detected, this mutation was previously described by Minarini *et al.* [11] Mutation at codon (Leu157Tyr) was observed in 2 of our isolates (one *K. pneumoniae* and one *E. coli* isolate). To the best of our knowledge, this mutation has not been previously reported by other studies.

Several studies showed that topoisomerases exhibited different sensitivity and affinity to the different fluoroquinolones, based on differences in their molecular structural [14,33] It has been postulated that different amino acid mutations in QRDR may result in different patterns and rates of resistances to the different fluoroquinolones [14]. The *ParC* subunit of DNA topoisomerase IV is the mainly the primary target of the older quinolones, where *parC* mutation was a major contributor to ciprofloxacin resistance among our isolates [6,34]. The methoxy substituent in moxifloxacin increases the antibacterial activity against resistant *gyrA* mutants among *Enterobacteriaceae* and would require double *parC* and *gyrA* mutations and this was obvious in all moxifloxacin resistant strains [6,13,14]. Notably, moxifloxacin resistance was related to the new mutation Leu157Tyr. This may be explained by the use of newer fluoroquinolones such as moxifloxacin for treating patients and thus increasing selection pressure for resistant mutations with unique mutations being developed.

PMQR is an increasing phenomenon in *Enterobacteriaceae* [4,21]. Generally, the acquisition of *qnr* genes will not render a susceptible wild type strain to become resistant. However, the acquisition of *qnr* genes poses a risk in the dissemination and augmentation of fluoroquinolones resistance among *Enterobacteriaceae* [9,35] Several studies postulated that *qnr* proteins may give rise to higher-level quinolone-resistance, and that the presence of *qnr* genes augments other additional quinolone resistance mechanisms [21,36]. In the light of this theory the PMQR genes (qnr genes) were analyzed. *qnrB* was the most abundant gene among the 193 isolates tested, as 64.7% of *K. pneumoniae* were positive and 57.1% of *E. coli* isolates were positive. On the other hand, 11% of *K. pneumoniae* and 4% of *E. coli* isolates that were tested possessed the *qnrA* gene. Other Egyptian studies that report the prominence of *qnrB* gene are Kotb *et al.* and El-Badawy *et al.* [8,37]. However, these two studies were unable to detect *qnrA* among their isolates. To the best of our knowledge, *qnrA* gene was not detected in other Egyptian studies [8,38-40]. However, *qnrA* gene was detected in some localities worldwide, including Szabó *et al.* in Hungary and Araújo *et al.* in Brazil who detected *qnrA* in their low-level resistance as well as high resistance isolates [4, 8,41]. Nevertheless, combination PMQR genes besides mutations in QRDRs of *gyrA* and *parC* contributes to greater resistance to fluoroquinolones. Our findings are in agreement with other studies such as Szabó and Piekarska *et al.* [3,8,41].

## Conclusions

Fluoroquinolones are one of the most widely prescribed antibiotics in clinical practice. The indiscriminate use of commercially available fluoroquinolones in Egypt has created selective pressure on the development of resistant mutants. This gave rise to the appearance of new unique mutations outside the QRDR that are related to the newer fluoroquinolones. Since only mutations in *gyrA* and *parC* were investigated in this study, other mechanisms of mutations cannot be ruled out. However, the results of this study shed light on the development of new resistance mutations and raise concerns regarding continued surveillance of antimicrobial resistance, and the urgent need for abandoning the irrational use of antimicrobials.

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**Corresponding author**
Dr. Marwa Atef Yakout
Microbiology and Immunology, Faculty of Pharmacy,
Pharos University in Alexandria, Smouha, Mahmoudeya Canal,
Alexandria, Egypt. Pincode.21311
Phone: +201224296850
Email: marwaatef202@gmail.com; marwa.atef@pua.edu.eg

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