Original Article

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

Marwa Atef Yakout¹, Ghada Hani Ali¹

¹Department 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 gyrA or parC 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; Basingstoke; 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 1×10⁸ CFU/mL (OD₆₀₀ 0.12-0.13) and diluted to create a final concentration of 5×10⁵ 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 °C 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 (72%), levofloxacin (50%), and ciprofloxacin (54%).

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

Table 1. The primers and PCR conditions of the tested fluoroquinolone resistance genes.
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$_{50}$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 2. MIC50 and MIC90 in mg/L of ciprofloxacin, levofloxacin and moxifloxacin against K. pneumoniae and E. coli isolates tested.

| K. pneumoniae | MIC$_{50}$ in mg/L | MIC$_{90}$ in mg/L | EUCAST breakpoints in mg/L |
|---------------|-------------------|-------------------|---------------------------|
| 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.

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

| Sample         | Fluoroquinolone resistance pattern | PMQR Genes | QRDR Genes          |
|----------------|-----------------------------------|------------|---------------------|
| K. pneumoniae 1| Ciprofloxacin, Levofloxacin, Moxifloxacin | qnrA, qnrB | Ser83leu; lys154arg; asp87asn; ser83ile val141ala |
| K. pneumoniae 2| Levofloxacin, Moxifloxacin | qnrA, qnrB | ser83leu; asp87asn; ser83ile |
| K. pneumoniae 3| Ciprofloxacin, Levofloxacin | qnrB | ser83leu; lys154arg |
| K. pneumoniae 4| Ciprofloxacin, Moxifloxacin | qnrB | ser83leu; leu157tyr |
| K. pneumoniae 5| Moxifloxacin, Ciprofloxacin, Levofloxacin | qnrB | ser83leu; val141ala; glu84ala |
| E. coli 1      | Levofloxacin, Moxifloxacin | qnrA, qnrB | asp87asn; ser83ile |
| E. coli 2      | Ciprofloxacin, Moxifloxacin | qnrB | ser83leu; leu157tyr |
| E. coli 3      | Ciprofloxacin, Moxifloxacin | qnrA | ser83leu; leu157tyr |
| E. coli 4      | Levofloxacin, Moxifloxacin | qnrA, qnrB | asp87asn; ser83ile |
| E. coli 5      | Moxifloxacin | qnrB | 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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