Molecular Characterization of Quinolone Resistant Urinary Isolates of *Escherichia coli*

Younes Abd El Gawwad Haggag*1, Amal Eissa Saafan1, Ahmed Osama El-Gendy1, Enas Mamdouh Hefzy2 and Sameh AbdelGhani1,3

1Department of Microbiology and Immunology, Faculty of Pharmacy, Beni-Suef University, Beni-Suef, Egypt.
2Department of Microbiology, Faculty of Medicine, Fayoum University, Fayoum, Egypt.
3Department of Pathology and Medical Laboratory, School of Medicine, Louisville University, KY, United States of America.

Abstract

This study aimed to investigate the genetic basis for quinolones and fluoroquinolones insusceptibility in *E. coli* isolated from outpatients suspected with urinary tract infections (UTIs). Fifty-one nalidixic acid unsusceptible *E. coli* isolates were collected from adult patients with UTIs. Antimicrobial sensitivity testing was performed by disc diffusion test, and minimum inhibitory concentrations of nalidixic acid and ciprofloxacin were determined by E. test strips. PCR amplification and DNA sequencing were carried out for the detection of alterations in the quinolone resistance determining region (QRDR) of *gyrA* and *parC* genes and screening for plasmid-mediated quinolone resistance (PMQR) genes (*qnr, aac(6)-ib-cr, qepA* and *oqxAB*). Genetic analysis of the QRDR revealed amino acid substitutions in the codons 83 and 87 of the *gyrA* gene with or without alterations at codons 80 and 84 of the *parC* gene. Most of the quinolone-resistant isolates (94%) had at least one of the PMQR genes. QRDR mutations in chromosomal genes encoding *gyrA* and *parC* have the principal role in the development of quinolones and fluoroquinolones resistance in *E. coli*. The accumulation of amino acid alterations in *gyrA* and the concurrent mutations in *parC* lead to the emergence of a high-level of insensitivity toward fluoroquinolones. The detected PMQR determinants are widespread in the community, and they are associated with the development of resistance to many other categories of antibiotics. This is the first report to detect *oqxAB* in *E. coli* in Egypt.

**Keywords:** *E. coli*, Urinary tract infection, Egypt, oqxAB

*Correspondence: youneshagag85@yahoo.com; +2-0822162133

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INTRODUCTION

In 1885, Theodor Escherich isolated E. coli from the faeces of neonates and described it as bacterium coli commune. It was later named Escherichia coli. Although some strains of E. coli are significant pathogens of humans and animals, other strains are harmless commensals of the intestinal tract. The pathogenic strains are separated into two groups: those causing diseases inside the intestinal tract and others capable of infection at extra-intestinal sites as the uropathogenic E. coli (UPEC) which is the most common organism implicated in urinary tract infections (UTIs). 

Quinolones are synthetic antibacterial agents with excellent action against Enterobacteriaceae. In 1962 the first quinolone, nalidixic, acid was discovered. This detection led to the rise of a variety of quinolones. Structural modification by adding a fluorine atom at C-6 position led to the invention of fluoroquinolones which are more systemically active. The bactericidal activity of quinolones comes from the inhibition of DNA gyrase and topoisomerase IV; both are involved in DNA replication. The extensive usage of quinolones was responsible for spreading quinolone resistance in different microorganisms.

Quinolone resistance is caused mainly as a result of amino acid alterations in quinolone resistance determining regions (QRDRs) of DNA gyrase and topoisomerase IV. Other mechanisms include decreased membrane permeability, overexpression of efflux pumps and plasmid-mediated quinolone resistance (PMQR) genes.

The first PMQR mechanism the qnr gene (currently qnrA1), was detected in 1998. Qnr genes protect DNA gyrase and/or topoisomerase IV from fluoroquinolone action. Secondly, the aac (6’)-ib-cr which is a variant from the aminoglycoside acetyltransferase (aac(6’)-lb) enzyme. This enzyme acts by N-acetylation of the amino nitrogen on the unsubstituted piperazinyl substitute of ciprofloxacin, and norfloxacin. The third PMQR mechanism includes active efflux pumps: the OqxAB and the QepA, which reduce sensitivity to hydrophilic fluoroquinolones such as ciprofloxacin.

This study was designed to detect the mechanisms of quinolone resistance among E. coli isolates from patients with UTIs in Egypt.

MATERIALS AND METHODS

Clinical Isolates

Two hundred and eighty -left over, remnants- urine samples from adult patients suspected with UTIs from outpatient sections at the clinical microbiological laboratory at Fayoum University Hospital and other private laboratories of medical analysis from September 2014 to August 2015 at Fayoum governorate, Egypt, were screened for E. coli by conventional bacterial identification tests and confirmed by Microbact Identification Test, (Oxoid, UK).

Antibiotic sensitivity testing

The isolates were subjected to antimicrobial sensitivity tests to nalidixic acid, ciprofloxacin, ampicillin, amoxicillin/clavulanate, ceftazidime, cefoxitin, cefotaxime, ceftriaxone, gentamicin, amikacin, and imipenem (Oxoid, UK) according to routine laboratory protocols of clinical and laboratory standards institute (CLSI). The MIC of nalidixic acid has been proposed to could be used as a phenotypic marker of insensitivity for quinolones in Gram-negative bacteria, so that the MIC of nalidixic acid was used to detect quinolone resistance among isolates. The MICs of nalidixic acid and ciprofloxacin were determined by E.test (Liofilchem, Italy). E.coli ATCC 25922 strain was used as a negative control.

Phenotypic detection of Extended-Spectrum Beta-lactamases

The phenotypic revealing of ESBLs was proceeded by the double-disc potentiation test. It was performed by centralizing an Amoxicillin/Clavulanic (20/10µg) disc with cefotaxime(30µg), cefpodoxime(10µg), ceftazidime(30µg) and cefepime(30µg) discs strategically placed 20 to 30 mm away on Muller Hinton agar which already had a lawn of the test organism. After overnight incubation, an extension of an inhibition zone toward the AMC disc was considered positive.

Genetic analysis of resistance mechanisms

DNA extraction

DNA was extracted by using genomic pure link extraction kits (Invitrogen, USA), and according to the manufacturer’s extraction guidelines.

PCR of the QRDR of gyrA and parC genes

QRDR of the gyrA and parC genes of selected isolates with different ciprofloxacin MICs were amplified by PCR using specific primers (Table 1). A volume of 5µl DNA was added to the 20µl
reaction mixture containing 12.5µl PCR reaction mix (Sigma, USA), 1µl of each primer and 5.5µl dH2O. Reaction mixed without DNA template served as negative controls.

**PCR for detection of PMQR genes**

For all PMQR genes, the following reaction mixture was used: 1µl template DNA was added to 24µl PCR master mix (Sigma, USA), which contained 12.5µl PCR reaction mix, 1µl of each primer and completed to 25µl with dH2O. Specific primers used are listed in Table 1.

**Sequencing**

All PCR yields were exposed to electrophoresis in 1% agarose gel having ethidium bromide 0.5 mg/L. After purification, Amplified fragments were sent to Macrogen Company (Macrogen Inc., Seoul, Korea) for sequencing. Nucleotide sequences were determined on both strands of PCR amplification products. Investigation and comparison of nucleotide sequences was performed using programs accessible at the NCBI web site (http://www.ncbi.nlm.nih.gov).

### Table 1. Oligonucleotide primers used for PCR amplification and sequencing of resistance genes

| Gene   | Primer name | Sequence (5’ to 3’ ) | Annealing temperature | Amplicon size (bp) | Reference |
|--------|-------------|----------------------|-----------------------|--------------------|-----------|
| gyrA   | gyrA6-F     | CGACCTTGGCAGAGAACAT | 55                    | 620                | 38        |
|        | gyrA631-R   | GTTCCATCAGCCTTCAA   |                       |                    |           |
| parC   | parC-F      | TGAATTAGGAAAAGCCTA  | 55                    | 559                | 39        |
|        | parC-R      | GCCACCTCAGCAGTTATG  |                       |                    |           |
| Aac(6’)-Ib | aac(6’)-Ib-F | TTGCAGTCATATGAGCTGA | 58                    | 482                | 40        |
|        | aac(6’)-Ib-R | CTCGAATGCCTGCGGTGT |                       |                    |           |
| qnrA   | qnrA-F      | ATTTCTCAGCAGGAATTG  | 55                    | 516                | 6         |
|        | qnrA-R      | GATCGCGAAGGTTAGGTC  |                       |                    |           |
| qnrB   | qnrB-F      | GATCGTTGAAAGCGAAGAG | 55                    | 469                | 6         |
|        | qnrB-R      | ACGATGCCGTGCTTTCCC  |                       |                    |           |
| qnrS   | qnrS-F      | ACGACATTCGTCAACTGCA | 55                    | 417                | 6         |
|        | qnrS-R      | TAAATTGGCACCCGTAGGC |                       |                    |           |
| qepA   | qepA-F      | GCAAGTCCAGCAGGGTAG  | 60                    | 199                | 10        |
|        | qepA-R      | CTTCCTGCCCCGAGTTG   |                       |                    |           |
| oqxA   | oqxA-F      | CTGGGCGGGATAGICT    | 57                    | 392                | 11        |
|        | oqxA-R      | CCACCTCAGGGGAGACGA |                       |                    |           |
| oqxB   | oqxB-F      | TTCTCCCCCGGGAGAATAC | 64                    | 512                | 11        |
|        | oqxB-R      | CTCGGCCATTTTGGCGCTA |                       |                    |           |

**Fig. 1.** Antibiotic Sensitivity pattern of isolated *E.coli* strains from urine samples of patients with UTIs (N=155)
**RESULTS**

Out of all uropathogens recovered (n=280), 155 (55.4%) were identified as *E. coli*. They were serially numbered from Y1 to Y155.

**Antibiotic sensitivity test results**

The sensitivity pattern of isolated *E. coli* strains is shown in Fig. 1.

Fifty-one (32.9%) nalidixic acid-resistant; MIC>16µg/ml *E. coli* isolates were recorded from the isolated *E. coli* strains.

All of the nalidixic acid-resistant isolates were resistant to ampicillin, sensitive to imipenem.

| Antibiotic              | No. of resistant isolates | %    |
|-------------------------|---------------------------|------|
| Nalidixic acid (30µg)   | 51                        | 100.0|
| Ampicillin (10µg)       | 51                        | 100.0|
| Cefoperazone (75µg)     | 40                        | 78.4 |
| Cefotaxim (30µg)        | 39                        | 76.5 |
| Ceftazidime (30µg)      | 39                        | 76.5 |
| Ciprofloxacin (5µg)     | 37                        | 72.5 |
| Norfloxacin (10µg)      | 37                        | 72.5 |
| Ofloxacin (5µg)         | 37                        | 72.5 |
| Levofloxacin (5µg)      | 37                        | 72.5 |
| Amoxicillin/Clavulanic  | 36                        | 70.6 |
| (20/10µg)               |                           |      |
| Ceftriaxone (30µg)      | 35                        | 68.6 |
| Gatifloxacin (5µg)      | 35                        | 68.6 |
| Cefotaxime (30µg)       | 34                        | 66.7 |
| Gentamicin (10µg)       | 30                        | 58.8 |
| Amikacin (30µg)         | 14                        | 27.5 |
| Imipenem (10µg)         | 0                         | 0.0  |

**Phenotypic detection of ESBLs**

The double disc potentiation test showed synergy between amoxicillin/clavulanic and one or more of the following 3rd, 4th cephalosporin: cefotaxime, ceftazidime, aztreonam, and ceftriaxone in all isolates suggesting the production of ESBLs.

**PCR and sequencing of gyrA and parC**

Sequenceing of QRDR of the gyrA and parC genes revealed mutations at the positions 83 (ser→Leu) and 87 (Asp→Asn) in gyrA and the positions 80 (Ser→Ile) and 84 (Glu→Val) in parC. At least one amino acid substitution in the gyrA gene at the codon 83 (Ser83Leu) was identified in each quinolone resistant isolate (Table 4). and all of them were multi drug-resistant (MDR). Thirty-seven isolates (62.5%) were sensitive to amikacin, according to the results of Ciprofloxacin MIC12, 12 (23.5%) of isolates were sensitive to ciprofloxacin; MIC<1µg/ml, 2 (3.9%) were intermediately resistant; MIC=1, and 37 (72.5%) were resistant and highly resistant to ciprofloxacin; MIC >1. The Distribution of MIC values of ciprofloxacin is shown in Table 3.

**Detection of PMQR genes**

At least one PMQR gene was detected in most of the nalidixic acid-resistant *E. coli* isolates (94%). *QnrS* gene was located in 12 (23.5%) isolates. The sequencing of the *qnrS* gene was identical to that of the variant *qnrS1*. Forty-five of the 51 isolates (88.2%) were positive to *aac (6)-Ib-cr*. The sequencing of the *aac (6')-Ib-cr* was identical to the –cr variant. *QepA* gene was detected in twenty-four of the 51 isolates (47.1%). The *oqxAB* gene was identified in three isolates (5.9%). This is the first report for detecting *oqxAB* gene in quinolone-resistant urinary isolates in Egypt. The sequencing of the *oqxAB* gene was identical to that of the *oqxAB1* (Fig. 2).

**DISCUSSION**

The resistance of *E. coli* to fluoroquinolones which is one of the most widely used medicines for the treatment of UTIs is prevalent. Fluoroquinolone treatment is no longer effective in more than half of patients in many countries in the world15.
In the present study, 55.4% of all uropathogens were *E. coli*. This result agrees with that reported in previous studies in which *E. coli* was the leading reason of UTIs\(^3,16,17\).

Also, sensitivity pattern of the isolated *E. coli* strains agrees with previous studies\(^18,19\).

All of the isolated *E. coli* strains were resistant to ampicillin and sensitive to imipenem; this result is consistent with that reported by Mendoca *et al.*\(^20\). Imipenem appears to have a broader range of activity than other tested antimicrobial agents. The explanation for that is probably the fact that these are potent drugs used only in hospital settings and not as first-line therapies, so they are of limited use.

Ciprofloxacin resistance observed was higher than that described in prior studies from Egypt\(^21\). This growing in ciprofloxacin insusceptibility rate may be due to the extensive usage of quinolones, the extended use of small doses of the more effective fluoroquinolones such as ciprofloxacin, also the inappropriate use...

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### Table 4. Results of the sequencing of QRDR of selected isolates

| Strain | MIC.CIP. | Mutations | PMQR Determinant |
|--------|----------|-----------|------------------|
|        |          |           | gyrA              | parC             |
| Y10    | 0.064    | S83L      |                  |
| Y77    | 1        | S83L      |                  |
| Y44    | 0.25     | S83L      |                  |
| Y16    | 0.5      | S83L      |                  |
| Y13    | 32       | S83L,D87N | aac(6)-Ib-cr     |
| Y52    | 32       | S83L,D87N | S80I             |
| Y63    | 16       | S83L,D87N | S80I,aac(6)-Ib-cr|
| Y66    | 2        | S83L,D87N | S80I             |
| Y81    | 2        | S83L,D87N | S80I             |
| Y1     | 32       | S83L,D87N | S80I,E84V,aac(6)-Ib-cr|
| Y6     | 32       | S83L,D87N | S80I,aac(6)-Ib-cr|
| Y7     | 32       | S83L,D87N | S80I,aac(6)-Ib-cr|
| Y15    | 32       | S83L,D87N | S80I,aac(6)-Ib-cr|

CIP, ciprofloxacin; S, serine; L, leucine; D, asparagine; N, aspartic acid; I, isoleucine; E, glutamic acid; V, valine.

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**Fig. 2.** Distribution of PMQR genes among nalidixic acid-resistant *E. coli* strains isolated from patients with UTIs (No. of isolates = 51).

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**Table 4.** Results of the sequencing of QRDR of selected isolates. The table shows the MIC for ciprofloxacin (CIP), gyrA and parC mutations, and the PMQR determinants for selected strains. The results indicate the presence of various PMQR determinants, including aac(6)-Ib-cr, qnrS, qepA, and oqxAB. The sensitivities and resistances are listed for each isolate, providing insight into the mechanisms of resistance.
ESBL production was recorded for all quinolone-resistant isolates. This finding is consistent with that reported by another previous study\(^4\). Also, a study conducted in Europe reported quinolone resistance in 92% of ESBL-producing isolates, this high prevalence of quinolone-resistant ESBL-producing *E. coli* strains had become a significant health problem, making treatment of bacterial infections more challenging and resulting in higher morbidity and mortality rates\(^3\).

In this study, all quinolone-resistant *E. coli* isolates collected were MDR (Resistance to one or more antibiotics in three or more antibacterial classes)\(^6,22\). MDR bacterial infections are relatively common now in the community as well as the hospitals\(^5\). This high predominance of MDR isolates of *E. coli* has been detected in many developing countries\(^22,28\). The high incidence of MDR and ESBL production among these *E. coli* isolates may be attributed to many reasons. The most apparent reason is plasmids which carry the genes encoding ESBLs, and also carry the genes encoding multi-resistance to several antibiotics, including quinolones, further contributing to the spreading of resistance to several antimicrobials through inappropriate use of quinolones, this announces that these plasmid transmitted genes are clinically significant\(^23\).

Insusceptibility to quinolones and fluoroquinolones happens essentially because of amino acid substitutions in *gyrA* and *parC* genes\(^13,27\).

Sequencing of QRDR of the *gyrA* and *parC* genes revealed mutations in *gyrA* gene at two codons: the first is codon 83 where serine is substituted by leucine; the second is codon 87 where Asparagine is substituted by Aspartic acid. The secondary target for quinolone-resistance is the *parC* gene. In the current study, Point mutations at the codons 80 and 84 in *parC* gene were detected in many of the isolates, where Serine was substituted by Isoleucine at codon 80 and Glutamic acid is substituted by Valine at codon 84. One mutation in the *gyrA* gene at codon 83 was detected at least in each quinolone resistant *E. coli* isolate. Additional mutations in other positions in the QRDR of *gyrA* and *parC* were observed in highly quinolone and fluoroquinolone resistant isolates. The reported mutations at the QRDR in this study agree with many previous studies\(^9,13,27,37\) and prove that this is the main quinolone and fluoroquinolone resistance mechanism in *E. coli* and other bacterial species.

MICs of ciprofloxacin were different among *E. coli* isolates with the same types of amino acid alterations in *gyrA* and *parC*. This diversity may be due to other mechanisms involved in fluoroquinolone resistance such as decreased membrane permeability and overexpression of efflux pumps\(^41\).

PMQR genes play an important role in the resistance to quinolones and fluoroquinolones because of their horizontal transfer and they increase the mutant prevention concentration of quinolones, thus enabling the development of mutants with a higher level of resistance to fluoroquinolones by favoring selection of amino acid substitutions in the QRDR genes\(^8,9\). PMQR genes are commonly distributed among *Enterobacteriaceae*\(^28\).

The first PMQR gene is *qnr*. *Qnr* genes bind to DNA gyrase and/or topoisomerase and so they protect them from quinolones. The first detected *Qnr* gene in Egypt was *Providencia* spp.\(^28\). In the current study, *qnr1* was the only detected *qnr* gene, it was detected in 23.5% of the isolates. This result agrees with previous studies from Egypt that described that *qnrS* is the most prevalent *qnr* determinant in Egypt\(^31,32\). But other Egyptian studies reported *qnrB* in a higher rate than the *qnrS*\(^32,33\). This may be due to the different bacterial strains and clinical infections included in that studies.

The second PMQR gene detected was the *aac(6\(^{'\})\)-Ib-cr*, which is a variant of the gene *AAC(6\(^{'\})\)-Ib*. This enzyme reduces only quinolones with piperazinyl nitrogen (ciprofloxacin and norfloxacin) activity by acetylation\(^8\). Screening for *aac(6\(^{'\})\)-Ib-cr* shows a high predominance among the quinolones resistant *E. coli* isolates where 88.2% of the isolates were positive for it. This result agrees with that reported by Yang *et al.*\(^34\).

The most predominant PMQR gene found in these isolates was *aac(6\(^{'\})\)-Ib-cr*. This result was expected because all the isolates were ESBL producers, and many studies have reported its prevalence among other populations of *E. coli*, mostly among those produce ESBL enzymes\(^8\). It
was found that all qnr positive isolates were also harboring aac(6\')-Ib-cr gene. The co-existence of the qnr and aac(6\')-Ib-cr genes on the same plasmid may be the explanation for that\textsuperscript{19}. Recently, qepA and oqxAB, multidrug efflux pumps genes, were discovered and reported as the third PMQR mechanism involved in quinolone resistance\textsuperscript{10,11}. In the present study, the prevalence of qepA among the isolates (47.1\%) was higher than that in a previous study from Egypt\textsuperscript{16}, this high prevalence may be attributed to that most of the isolates were ciprofloxacin resistant with a high MIC.

The OqxAB efflux pump is encoded by the oqxA and oqxB genes, which are located in the same operon. In this study, the plasmid mediated oqxAB was detected in 5.9 \% of the E. coli isolates. This result agrees with that reported by Yuan et al.\textsuperscript{17}.

CONCLUSION

This study concluded that fluoroquinolone resistance is in growing rate in Egypt. This data supports our understanding of the molecular mechanisms of quinolone and fluoroquinolone insensitivity due to the accumulation of amino acid substitutions in the QRDR of gyrA and parC genes and PMQR determinants. Due to the improper and increased usage of wide-spectrum antibacterials and quinolones, it is essential to restrict the use of these antibiotics to be prescribed only when necessary and only when approved by a physician. The detected high prevalence of PMQR determinants through this study in Egypt, because of unrestrained quinolone use, is troublesome. Additionally, other insensitivity genes usually are harbored by these plasmids, causing multiple resistances among isolates which can complicate therapeutic management of infections and also can be transferred to other bacterial species. This study is the first report to detect plasmid mediated oqxAB in E.coli isolates in Egypt.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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None.

AUTHORS’ CONTRIBUTION

All authors listed have made substantial, direct and intellectual contribution to the work, and approved it for publication.

DATA AVAILABILITY

All datasets generated or analyzed during this study are included in the manuscript.

ETHICS STATEMENT

This study was approved by the Research Ethical Committee at Fayoum Faculty of Medicine, Fayoum, Egypt.

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