

Full Length Research Paper

Antibacterial resistance pattern among *Escherichia coli* strains isolated from Mansoura hospitals in Egypt with a special reference to quinolones

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Extensive use of fluoroquinolone antibacterial in clinical practice has been associated with increasing frequency of quinolone-resistant *Escherichia coli* strains. In the current study, a total of 80 *E. coli* clinical isolates from Mansoura hospitals patients in Egypt were studied for antibacterial susceptibility pattern against 15 different antibacterials. These strains were tested for quinolones resistance by minimum inhibitory concentration (MIC) determination using broth micro-dilution method. The resistance rate of ciprofloxacin and levofloxacin for *E. coli* isolates was found to be 60%. PCR was performed for detection of plasmid-mediated quinolone resistance genes including *qnrA*, *qnrB* and *qnrS*. 30 and 61.3% of *E. coli* isolates were positive for *qnrA* and *qnrB*, respectively, whereas *qnrS* was identified in only 15% of isolates. Quinolone resistance-determining region (QRDR) of *gyrA* and *ParC* genes was characterized for 17 ciprofloxacin and levofloxacin resistant *E. coli* isolates (MIC 12.5-200 $\mu\text{g mL}^{-1}$). Two mutation sites in *gyrA* were detected in 17 tested *E. coli* isolates. However, two mutation sites in *parC* were detected in four *E. coli* isolates. The amino acid change at Ser-83 and aspartic-87 in GyrA were the most common mutation sites identified in the isolates. These results indicated that multiple mechanisms of quinolone-resistance are commonly found in *E. coli* isolated from Mansoura hospitals.

Key words: Quinolone resistance, *gyrA*, *parC*, *qnr* gene.

INTRODUCTION

Quinolones are powerful broad-spectrum antibacterial agents commonly used in both human and veterinary medicine for the treatment of a wide variety of infections. In the last decade, fluoroquinolones have become first and second-line antibacterials of choice for acute respiratory, enteric and urinary tract infections as well as serious systemic infections such as bacteremia (Jamison, 2006). Their extensive use has been associated with raising level of quinolone resistance in different microorganisms

(Robicsek et al., 2006). Fluoroquinolones act by increasing levels of enzyme-mediated DNA cleavage affecting DNA gyrase enzyme which catalyzes the negative supercoiling of DNA and topoisomerase IV enzyme which decatenates or removes the interlinking of daughter chromosomes at the completion of a round of DNA replication allowing their segregation into daughter cell (Ambrozic et al., 2007; Wang et al., 2009). Quinolone resistance has traditionally been attributed mainly to chromosomal mutations

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in the *gyrA* and *gyrB* genes of DNA gyrase and in *parC* and *parE* genes of topoisomerase IV or due to decreased intracellular concentration as a result of decreased permeability of the membrane or over expression of efflux pump systems (Poirel et al., 2006; Oktem et al., 2008; Allou et al., 2009).

Alteration in quinolone resistance determining region (QRDR) is considered as the most important mechanism of quinolone resistance. Mutations in QRDRs of *gyrA* and *parC* are most commonly documented, however resistance is also conferred by mutations in *parE* (Hopkins et al., 2005). These mutations block the action of quinolones resulting in increased level of resistance to fluoroquinolones (Ruiz, 2003). In *Escherichia coli* and related Gram negative bacteria, DNA gyrase is the first target for fluoroquinolones. Alterations in *gyrA* are reported much more often than alterations in *gyrB* (Frank et al., 2011).

Plasmid-mediated quinolone resistance has been also previously described. The study of Martinez-Martinez et al. (1998) was the initial report of this transferable mode of resistance associated with *qnr* gene (now named *qnrA*). This gene was identified for the first time in *Klebsiella pneumoniae* in the United States. Several studies reported a world wide distribution of *qnr* determinants among bacterial isolates (Cheung et al., 2005; Cerquetti et al., 2009). The *qnr* gene encodes a 218-amino-acid protein which protects DNA gyrase and topoisomerase IV activity from the action of quinolones (Tran and Jacoby, 2002; Strahilevitz et al., 2009). The plasmid-mediated quinolone resistance determinants are widely distributed in clinical Enterobacteriaceae isolates around the world. These genes are usually located on mobile elements, including integrons, insertion sequences and transposons (Martinez-Martinez et al., 1998; Robiesek et al., 2006; Yamane et al., 2008). Furthermore, the *qnr* gene carrying plasmids, which are classified as Class I integron-carrying plasmids, usually carry in addition to *qnr* gene multiple resistance determinants providing multidrug resistance to different antimicrobials including: aminoglycosides, B-lactams and sulfonamides (Martinez-Martinez et al., 1998; Tran et al., 2005).

The prevalence and distribution of *qnr* genes were different in various geographical areas. *qnrA* genes have been identified worldwide in a variety of Enterobacterial species. Six variants have been identified (*qnrA1* to *qnrA6*). These genes can increase the MIC of fluoroquinolones up to 32-fold in *E. coli* isolates (Poirel et al., 2006; Allou et al., 2009). In addition, *qnrA* gene enhances the selection of chromosomal encoded quinolone resistance determinants which confer additional resistance to fluoroquinolones. Other plasmid-mediated quinolone resistance determinants *qnrB* (*qnrB1* to *qnrB6*) and *qnrS* (*qnrS1* and *qnrS2*) have been also identified in enterobacterial species, sharing 41 and 60% amino acid identity with *qnrA*, respectively (Nordmann and Poirel, 2005; Shin et al., 2009). Another mechanism of quinolone resistance relies on upregulation of efflux pump which exports

quinolones and other antimicrobials out of the bacterial cell. Although multiple mechanisms of quinolone resistance have been reported from many continents, there are few data from Africa on the molecular basis for quinolone resistance. In the current study, we focused on the prevalence of *E. coli* resistance to quinolones and the frequency of *qnrA*, *qnrB* and *qnrS* among clinical isolates of *E. coli* in Mansoura Hospitals. Also, this study was undertaken to determine the mechanism of quinolone resistance among *E. coli* isolates from Mansoura Hospitals.

MATERIALS AND METHODS

Bacterial strains

Eighty clinical *E. coli* isolates were collected from Mansoura university hospitals in Dakahlia governorate, Egypt during March 2011 to February 2013. The isolates were collected from urine, wound and sputum samples. These isolates were identified using standard biotyping methods (Crichton, 1996).

Antibacterial susceptibility testing

Each strain was screened for susceptibility to fifteen antimicrobials using the Clinical Laboratory Standards Institute (CLSI, formerly NCCLS) disc diffusion method (NCCLS, 2003). Discs used contained ampicillin (10 µg), imipenem (10 µg), ceftazidime (30 µg), ceftriaxone (30 µg), cefoperazone (75 µg), ceftizoxime (30 µg), cefoxitin (30 µg), cefotaxime (30 µg), norfloxacin (5 µg), ciprofloxacin (5 µg), levofloxacin (5 µg), tobramycin (10 µg), gentamicin (10 µg), azithromycin (15 µg) and amikacin (30 µg) (Oxoid). Inhibition zone diameters were interpreted in accordance with CLSI guideline using WHONET software version 5.3 (O'Brien and Stelling, 1995).

Determination of MIC (Minimal inhibitory concentration) for the isolated strains

The minimal inhibitory concentrations of the isolates for ciprofloxacin and levofloxacin were determined using broth microdilution method following the Clinical and Laboratory Standards Institute (CLSI, formerly NCCLS) guidelines (CLSI, 2007). In this method, 96-well microtitre plates were filled with small volumes (200 µl) of serial two-fold dilutions of each tested antibacterial. The final concentrations of each antibacterial in the wells ranged from 1.56 to 200 µg mL⁻¹. The turbidity of overnight culture is adjusted to obtain visually comparable turbidity to that of the 0.5 McFarland turbidity standards, then an aliquot of 20 µl was added to each wells of the microtitre plate. The plates were incubated for 24h at 30°C before determining the results. The MICs were read visually and were defined as the lowest concentration where no viability was observed in the wells of the microplates after incubation. The MICs values indicate resistance > 1 and > 2 µg mL⁻¹ for ciprofloxacin and levofloxacin, respectively.

Screening for the *qnr* genes in clinical strains

Screening was carried out by polymerase chain reaction (PCR) amplification of *qnrA*, *qnrB* and *qnrS* using the primed sets listed in Table 1. DNA templates were prepared by transferring bacteria isolates to distilled water in Eppendorf tubes and then boiling for 10 min then prepared DNA templates were directly used in the PCR

Table 1. Primers used in this study.

Target gene	Name	5'-3' sequence	References
<i>gyrA</i>	gyrA12004	TGC CAG ATG TCC GAG AT	Wang et al., 2001
	gyrA11753	GTA TAA CGC ATT GCC GC	Wang et al., 2001
	EC-PAR-A	CTG AAT GCC AGC GCC AAA TT	Deguchi et al., 1997
<i>parC</i>	EC-PAR-B	GCG AAC GAT TTC GGA TCG TC	Deguchi et al., 1997
	qnrA-1A	TTC AGC AAG ATT TCT CA	Wu et al., 2007
<i>qnrA</i>	qnrA-1B	GGC AGC ACT ATT ACT CCC AA	Wu et al., 2007
	qnrB-CS-1A	CCT GAG CGG CAC TGA ATT TAT	Wu et al., 2007
<i>qnrB</i>	qnrB-CS-1B	GTT TGC TGC TCG CCA GTC GA	Wu et al., 2007
	qnrS-1A	CAA TCA TAC ATA TCG GCA CC	Wu et al., 2007
<i>qnrS</i>	qnrS-1B	TCA GGA TAA ACA ACA ATA CCC	Wu et al., 2007

assay. A reaction mixture containing 0.5 μ M of each primer, 1.5 mM MgCl₂, 0.2 mM dNTPs, 1 U Taq polymerase (Thermoscientific Dream Taq Green DNA polymerase), 5 μ l of template DNA and nuclease free water was added for a total volume of 25 μ l per reaction. PCR reactions were carried out by using Techne progene thermocycler under the following conditions: initial denaturation at 95°C for 3 min, followed by 40 cycles of denaturation at 95°C for 30 s, annealing at 46°C for *qnrA* or 54°C for *qnrB* or 48°C for *qnrS* for 30 s, and extension at 72°C for 1 min; final extension at 72°C for 5 min. PCR products aliquots were analyzed by agarose gel electrophoresis on 1% agarose gel and visualized by ethidium bromide staining

Mutational analysis of the quinolone-resistance determining regions of *gyrA* and *parC*

The QRDR of the *gyrA* and *parC* genes were amplified in seventeen quinolone resistant *E. coli* strains by PCR using the primer pairs listed in Table 1. PCR reactions began with 10 min primary denaturation at 94°C followed by 40 cycles of 94°C for 30s, annealing temperature for 30 s and 72°C for 30 s. *gyrA* and *parC* amplification primers were annealed at 55 and 62°C, respectively.

PCR amplified gene fragments were purified using the PCR Purification Kit (MEGA quick-spin fragment DNA purification INtRON biotechnology, Korea) for subsequent sequencing. Purified PCR products were used as a template in sequencing reactions carried out with the ABI PRISM® BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, USA). The reaction mixtures were analysed on an ABI 3730 DNA analyser (Applied Biosystems, Foster City, USA).

Amplicons were sequenced on both strands and predicted peptide sequences were analysed by the online BLAST of the NCBI website software (<http://www.ncbi.nlm.nih.gov/BLAST/>). Then point mutations were identified by comparing the identified sequences to the corresponding genes by pair-wise FASTA alignments.

Nucleotide sequences of the antibacterial resistance genes from the fragments of *gyrA* and *parC* were deposited in GenBank under the following accession numbers: KF994612, KF994613, KF994614, KF994615, KF994616, KF994617, KF994618, KF994619, KF994620, KF994621, KF994622, KF994623, KF994624, KF994625, KF994626, KF994627, KF994628, KF994629, KF994630, KF994631, KF994632, KF994633, KF994634, KF994635, KF994636, KF994637, KF994638, KF994639, KF994640, KF994641, KF994642, KF994643, KF994644, KF994645, KF994646.

RESULTS

Antibacterial susceptibility testing

All *E. coli* isolates were resistant to ampicillin. On the other hand, they were all sensitive to imipenem. In addition, the highest resistance rate was recorded against ceftazidime (72.5%), ceftriaxone and cefoperazone (71.3%), ceftizoxime and cefoxitin (70%), norfloxacin, ciprofloxacin and azithromycin (67.5%), cefotaxime (65%), tobramycin and levofloxacin (62.5%) and gentamicin (51.3%). In contrast, resistance to amikacin was less common but was seen in only 23.75% of the isolates. Quinolone resistance was always seen in multiple-resistant *E. coli*, as all quinolone resistant *E. coli* were resistant to at least one other antimicrobial (Figure 1).

Determination of minimum inhibitory concentrations (MICs) of ciprofloxacin and levofloxacin against *E. coli* isolates

Ciprofloxacin MICs values for 80 *E. coli* isolates are shown in Figure 2. The susceptibility of isolates to ciprofloxacin showed that 48 (60%) of 80 *E. coli* were resistant while 22 (27.5%) of *E. coli* isolates were sensitive to ciprofloxacin in addition to 10 isolates (12.5%) exhibiting intermediate susceptibility to ciprofloxacin. The distribution of levofloxacin MICs values are shown in Figure 3. 48 isolates exhibited high-level levofloxacin resistance producing MICs of $\geq 12.5 \mu\text{g mL}^{-1}$. However, 25 isolates exhibited susceptibility to levofloxacin. Only 7 isolates showed intermediate susceptibility.

Characterization of quinolone resistance mechanisms in *E. coli*

Multiple horizontally-transmitted quinolone resistance genes were detected among *E. coli* strains isolated from

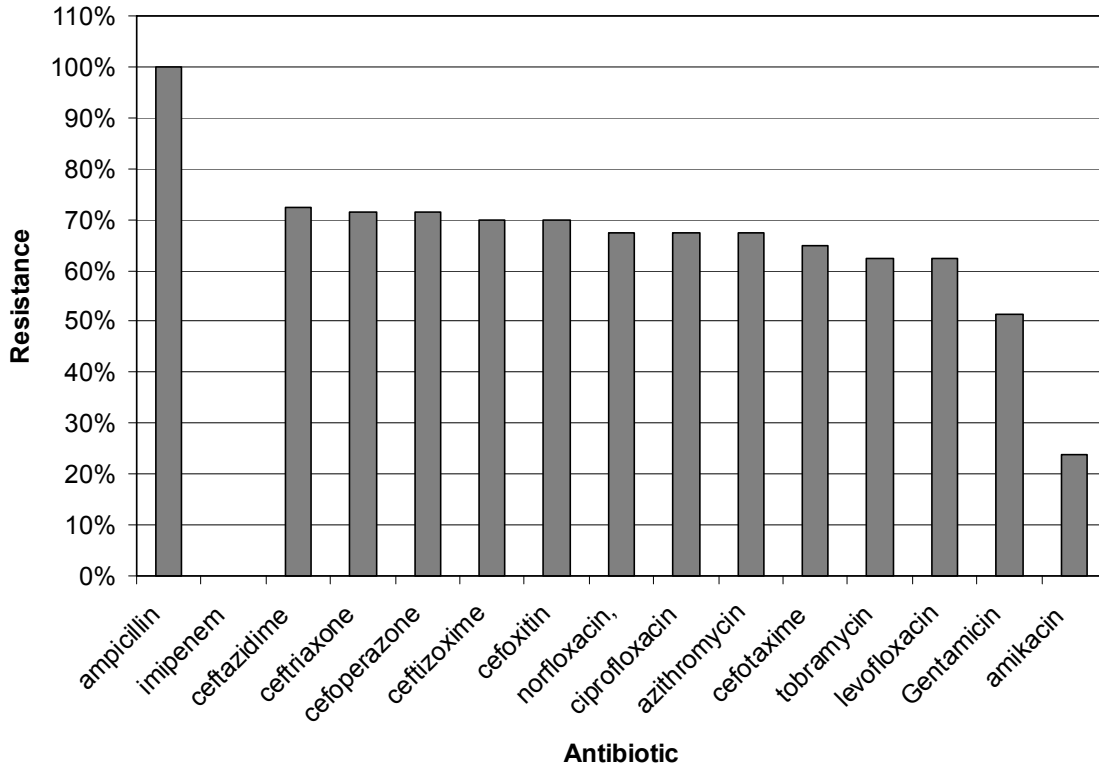


Figure 1. Proportion of *E. coli* isolates resistant to each of the fifteen antimicrobial agents.

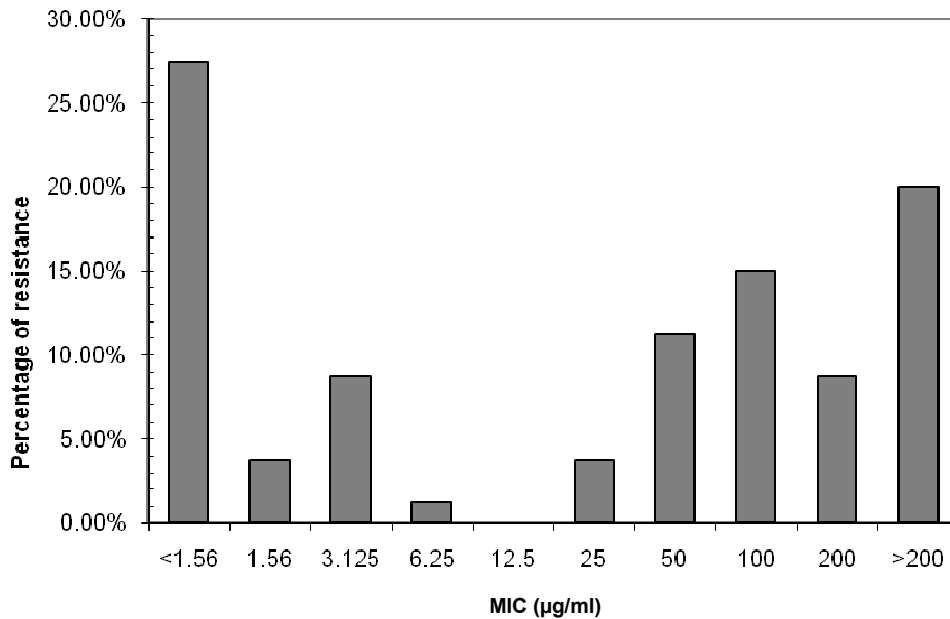


Figure 2. Ciprofloxacin MIC distributions of 80 *E. coli* isolates.

Mansoura Hospitals. By applying PCR technique, *E. coli* isolates were screened for the presence of *qnrA*, *qnrB* and *qnrS* genes as shown in Figure 4. In 80 *E. coli* isolates, 59 carried one or more of horizontally acquired

quinolone resistance gene. These genes were *qnrS* (12 isolates), *qnrA* (24 isolates) and *qnrB* (49 isolates). The tested horizontally acquired quinolone resistance genes were detected in all resistant and intermediate resistant

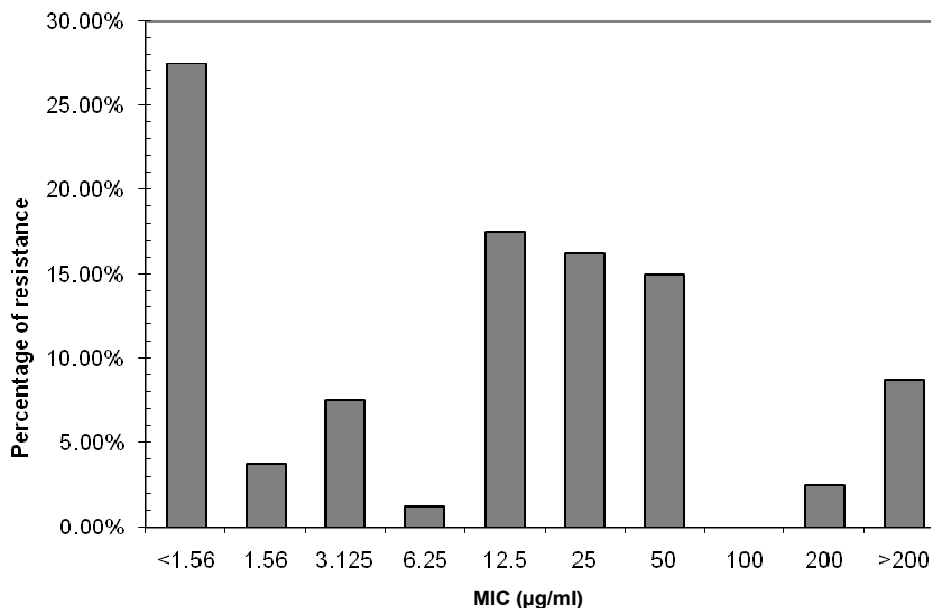


Figure 3. Levofloxacin MIC distribution of 80 *E. coli* isolates.

of the tested isolates. However, 9 of 59 *qnr* positive strains showed low level of resistance to ciprofloxacin and levofloxacin (MIC of 3.125-1.56 µg mL⁻¹). In contrast, only 4 isolates of 16 *qnr* negative isolates had higher level of resistance to ciprofloxacin and levofloxacin (200-12.5 µg mL⁻¹).

Seventeen highly resistant *E. coli* isolates were selected in this study to perform *gyrA* and *parC* sequence. Mutations in *gyrA* and *parC* subunits are summarised in Table 2. Quinolone-resistant isolates had at least two non-synonymous substitutions in the QRDR of *gyrA* and some of these isolates also had one additional mutation in *parC*. Sixteen from the tested isolates having two mutations in *GyrA* had a serine to leucine substitution at position 83 and aspartic acid to asparagine substitution at position 87, one of the most commonly documented resistance conferring mutations. All of these isolates had MIC values of at least 12.5 and 25 µg mL⁻¹ levofloxacin and ciprofloxacin, respectively. Only one isolate had S83L and additional *GyrA* substitution, Aspartic acid with Tyrosine (D87Y) had MIC values of 50 and 200 µg mL⁻¹ levofloxacin and ciprofloxacin, respectively. Four of *E. coli* isolates also harboured the frequently documented non-synonymous mutations in the QRDR of *parC*. These *ParC* substitutions were identified as glutamic acid substituted with glycine E84G, alanine with valine A108V and glutamic acid with valine E84V.

DISCUSSION

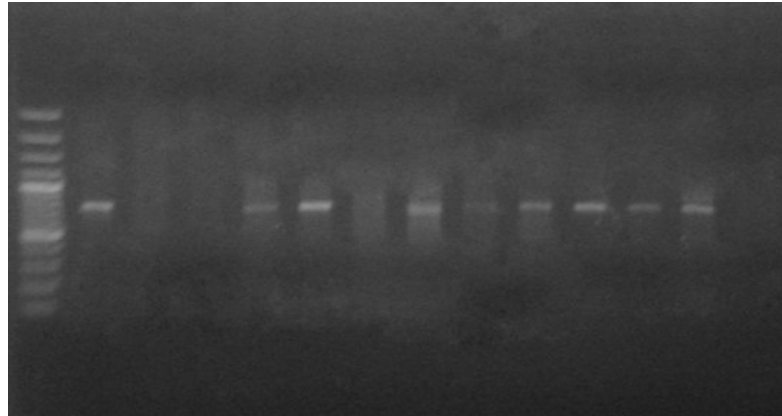
The emergence of fluoroquinolone-resistant *E. coli* is increasing in many parts of the world (Levermore, 2009). In African countries with a high infectious disease burden,

formal and informal health systems depend heavily on broad spectrum orally-administrable antibacterials. In this study, most of *E. coli* isolates from Mansoura hospitals in Egypt were resistant to ceftazidime, ceftriaxone, cefoperazone, ceftizoxime, ceftoxitin, cefotaxime, azithromycin, tobramycin and gentamicin. Fluoroquinolone antibacterials have been recently introduced as an effective alternative to antibacterials that have been compromised by resistance. Our results indicated high level of quinolone resistance with elevated MIC levels identified among the isolated *E. coli* in this study. However, lower resistance rates were markedly identified previously for this class of drugs (Sreela et al., 2011).

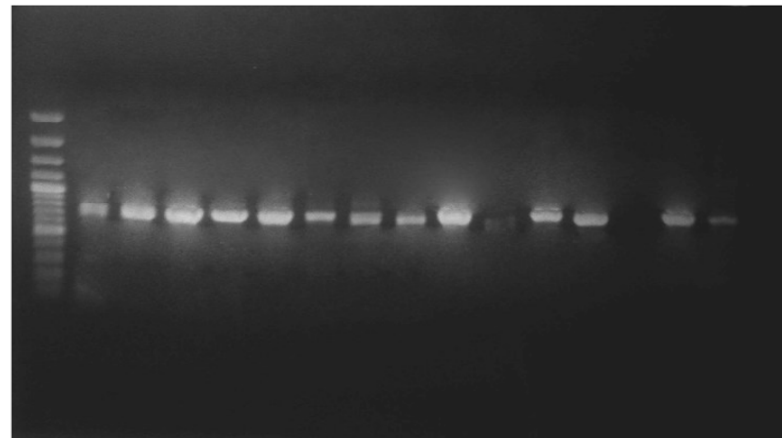
In our study, 60% of *E. coli* isolates exhibited high level of levofloxacin and ciprofloxacin resistance. The rate of *E. coli* resistance to fluoroquinolones is increasing worldwide. In USA, the rate of resistance in *E. coli* isolates was 3.5 and 1.9 to 2.5% for nalidixic acid and fluoroquinolones, respectively (Karlowsky et al., 2003). Quinolones target the bacterial enzymes DNA gyrase and topoisomerase IV, which are essential for cell growth and proliferation. DNA gyrase and topoisomerase IV are both tetrameric enzymes comprising two subunits *gyrA* and *gyrB* in DNA gyrase and two subunits *parC* and *parE* in topoisomerase IV.

The association between mutations of DNA gyrase and topoisomerase IV with fluoroquinolone resistance has been previously established for both Gram-negative and Gram-positive organisms (Frank et al., 2011). Accumulation of alterations in *gyrA* and the simultaneous presence alterations in *parC* play fundamental role in developing high level of resistance to ciprofloxacin in clinical isolates. In Gram negative bacteria, the primary target of quinolones is the *gyrA* subunit of DNA gyrase, and point mutations

A. M 1 2 3 4 5 6 7 8 9 10 11 12 13



B. M 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15



C. M 1 2 3 4 5 6 7 8 9 10 11 12 13

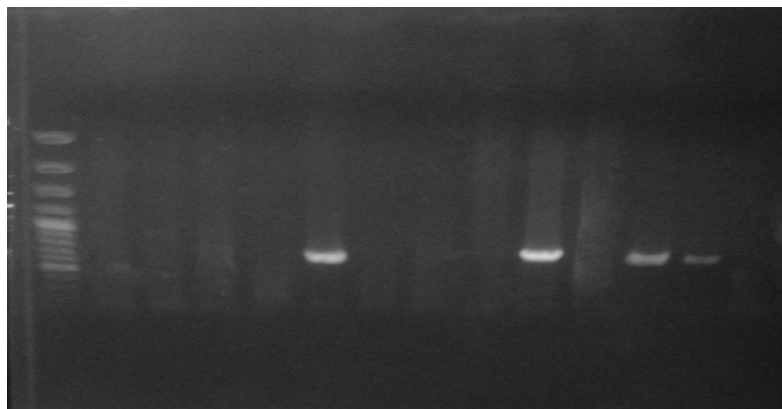


Figure 4. Agarose gel electrophoresis of genes. Lane M is 100 bp ladder molecular weight marker. A. Lanes 1 to 12 were amplicons of *qnrA* gene from *E. coli* isolates No.1, 4, 18, 34, 35, 50, 53, 59, 64, 65, 59, 70; B. Lanes 1 to 15 were amplicones of *qnr B* gene from *E. coli* isolates No. 2, 8, 9, 10, 11, 12, 15, 16, 27, 31, 33, 38, 80, 83, 90; C. Lanes 1 to 13 were amplicons of *qnrS* gene from *E. coli* isolates No. 6, 10, 16, 13, 19, 37, 50, 53, 60, 71, 69, 80, 84.

Table 2. Correlation between susceptibilities of levofloxacin and ciprofloxacin and alterations in GyrA and ParC in 17 *E. coli* isolates.

Number of isolate	Ciprofloxacin MIC ($\mu\text{g mL}^{-1}$)	Levofloxacin MIC ($\mu\text{g mL}^{-1}$)	Horizontally acquired	Mutations in QRDR	
				GyrA	ParC
2	> 200	50	<i>qnrB</i>	S83L,D87N	-
3	> 200	50	<i>qnrA, qnrB</i>	S83L,D87N	E84G
4	> 200	> 200	<i>qnrA</i>	S83L,D87N	-
29	> 200	200	<i>qnr B</i>	S83L,D87N	-
54	> 200	50	<i>qnrA, qnrB</i>	S83L,D87N	-
68	> 200	50	<i>qnrA, qnrB</i>	S83L,D87N	-
77	> 200	25	<i>qnr B</i>	S83L,D87N	-
18	200	50	<i>qnrA, qnrB</i>	S83L,D87N	A108V
38	200	50	<i>qnrA, qnrB</i>	S83L,D87Y	E84G
60	200	25	<i>qnrS, qnrB</i>	S83L,D87N	-
69	200	25	<i>qnrA, qnrS</i>	S83L,D87N	-
33	100	25	<i>qnr B</i>	S83L,D87N	-
36	100	25	<i>qnr B</i>	S83L,D87N	-
41	100	12.5	<i>qnr B</i>	S83L,D87N	E84V
63	100	50	<i>qnrA, qnrB</i>	S83L,D87N	-
67	50	50	<i>qnrA</i>	S83L,D87N	-
53	25	12.5	<i>qnrA</i>	S83L,D87N	-

are mostly observed within a highly conserved domain of the *gyrA* N-terminus, known as the quinolone resistance determining region (QRDR) which is in close proximity with the DNA-binding region near the putative active site tyrosine-122 (Piddock, 1999).

Two positions for mutations in GyrA were identified in 17 highly resistant isolates, representing serine-83 and aspartic-87. These two sites were previously identified as being most often observed and their presence has been validated experimentally in resistant strains (Piddock, 1999). High-level fluoroquinolone resistance was detected in *E. coli* isolates in our study. These isolates harbored two GyrA substitutions and in some isolates another additional substitution in ParC. However, no isolate had a mutation in the *parC* gene without the simultaneous presence of quinolone resistance-associated mutations in the *gyrA* gene.

Our *E. coli* isolates exhibited MIC ranging from 12.5-200 and 50-200 $\mu\text{g/ml}$ of levofloxacin and ciprofloxacin, respectively. Four of these isolates also harbored point mutations in the topoisomerase IV subunit genes *parC* which is previously identified in Gram negative bacteria but at a significantly lower frequency than *gyrA* mutations (Ling et al., 2003). These ParC substitutions were detected in Glutamic acid-84 and Alanine-108 amino acid positions in the protein sequence of ParC. The QRDR polymorphisms most commonly detected in this study are those most frequently reported in the literature in *E. coli* (Namboodiri et al., 2011). It is generally believed that

parC gene mutations arise after *gyrA* gene mutations, as DNA gyrase (rather than topoisomerase IV) is the preferred target of quinolones in Gram negative bacteria (Ling et al., 2003).

This study suggests that in clinical isolates of *E. coli*, DNA gyrase is a primary target of quinolones. In addition, two amino acid changes at Ser-83 and Asp-87 in GyrA were identified in all of high-level quinolone resistant *E. coli* with decreased susceptibility to both ciprofloxacin and levofloxacin. Moreover, the simultaneous presence of the ParC alterations play additional role in developing high-level resistance to quinolones which is supported by lower frequency ParC substitutions in highly quinolone resistant strains.

qnr gene, a naturally occurring gene encoding a pentapeptide repeat protein that confers reduced susceptibility to nalidixic acid or fluoroquinolone can be easily transferred between bacterial isolates due to its presence on mobile genetic elements (Jacoby et al., 2008; Strahilevitz et al., 2009). The first *qnr* gene, now known as *qnrA*, was found to protect *E. coli* DNA gyrase from inactivation by ciprofloxacin (Tran et al., 2005). Other *qnr* genes have been isolated, including *qnrS1* from *Shigella flexneri* (Tran et al., 2005) and *qnrB* (Jacoby et al., 2006).

In this study, the presence of *qnr* genes was investigated in some isolates. *qnr* positive isolates identified at higher level were: *qnrB* (49 isolates) as compared to *qnrS* and *qnrA* (14 isolates and 24 isolates,

respectively). It is important to note that plasmid-encoded *qnr* genes do not confer quinolone resistance by themselves, but facilitate the selection of bacteria bearing higher-level resistance, thereby augmenting the effect of other resistance mutations. The presence of horizontally-acquired genes accounted in part for elevated nalidixic acid MICs in strains that harboured these genes, but not completely. It is therefore possible that other resistance mechanisms, such as *ParE* polymorphisms, other horizontally acquired resistance genes, over-active efflux, or even novel mechanisms are present in some of the isolates (Martínez et al., 1998).

Conclusion

Fluoroquinolones, largely ciprofloxacin and levofloxacin, are considered as antimicrobials of high use in Mansoura hospitals in Egypt. As expected, this study demonstrates that resistance to these drugs is common, present at high numbers in *E. coli* isolates and occurs through multiple mechanisms. The main mechanism is associated with *gyrA* alterations in QRDR. Additionally, horizontally-acquired resistance to the quinolones was also identified at high frequency in this study this is supported by the hypothesis that these genes are present on mobile elements that could be transmitted to different pathogens. Finally, resistance to other antimicrobials except imipenem was also documented in this study which may limit the use of other antimicrobials as alternatives.

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Conflict of interest

The authors declare that they have no conflict of interest.

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