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RESEARCH ARTICLE

Molecular Analysis of Quinolone Resistance-Determining Regions in Avian Pathogenic *Escherichia coli*

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Abstract

Fluoroquinolones are potent broad-spectrum antimicrobial agents that are increasingly used to treat *Escherichia coli* infection. Despite initial optimism, resistance to these antibiotics has increased significantly since their introduction into medicine in the late 1980's and early 1990's. Mutational alterations in the fluoroquinolone target enzymes are recognized to be the major mechanisms through which resistance develops. This study devotes to determine the resistance rate of 13 avian pathogenic *Escherichia coli* (APEC) strains recovered from diagnosed cases of avian colibacillosis from different farms in Sharkia Province, Egypt for fluoroquinolone agents and to detect the mutations in the quinolone resistance-determining regions (QRDRs) in some resistant strains under study. Our results showed that minimum inhibitory concentration (MIC) of fluoroquinolones against APEC strains revealed high level of resistance to the veterinary-use fluoroquinolones, nalidixic acid (100%) and enrofloxacin and difloxacin (92.3%) and the human-use ones, including 76.92% to each of ciprofloxacin, norfloxacin and levofloxacin and 69.23% to gatifloxacin. Nucleotide sequence analysis revealed point mutations in QRDR of *gyrA* (Ser-83→Leu, Asp-87→Asn and Asp-87→Gly), and in its homologous region of *parC* (Ser-80→Ile). Mutations outside this region were also found in *gyrA* (Ser-116→Phe), *parC* (Val-46→Leu), both are considered to be first report, and *parE* (Ser-458→Ala). No mutations were observed in *gyrB*. These data indicate that high-level fluoroquinolone resistance in APEC involves the acquisition of mutations at multiple loci in QRDRs and containment strategies to limit the spread of resistance need to be deployed to conserve quinolone effectiveness and promote alternatives to their use.

Introduction

Avian-pathogenic *E. coli* strains cause avian colibacillosis, the most significant infectious bacterial disease of poultry worldwide (Ron, 2006). Since avian colibacillosis is an extraintestinal disease, APEC strains are commonly classified as extraintestinal pathogenic *E. coli* (ExPEC) (Johnson and Russo, 2002). All ExPEC strains share certain virulence attributes enabling their extraintestinal lifestyle, including production of adhesins, toxins, protectins, siderophores, iron transport systems and invasins (Johnson and Russo, 2005). Certain reports have suggested a link between APEC and human diseases, thus, the enhanced control of avian colibacillosis could prove beneficial to both animal and human health (Rodriguez-Siek et al., 2005; Ewers et al., 2007).

Quinolones are a large and widely consumed class of synthetic drugs. First-generation (acidic) quinolones, including nalidixic acid and oxolinic acid, have been only used for treatment of urinary tract infections (Ruiz, 2003). However, modifications of subsequent generations have increased their spectrum and potency, one of these modifications has been the addition of a fluorine atom at position C-6 of drug molecules (Peterson and Schemke, 1998). Expanded-

spectrum quinolones, like ciprofloxacin are highly effective against Gram-negative bacteria, especially *E. coli* (Jaktaji and Mohiti, 2010).

Fluoroquinolones inhibit DNA gyrase and topoisomerase IV activities (Wang et al., 2009). DNA gyrase is a tetrameric enzyme composed of two GyrA and GyrB subunits, encoded by *gyrA* and *gyrB* genes, respectively, while topoisomerase IV is a tetrameric enzyme composed of two ParC and ParE subunits, encoded by *parC* and *parE* genes, respectively (Hawkey, 2003). In *E. coli*, the major target for quinolones is DNA gyrase (Ruiz, 2003), an essential enzyme that is responsible, in part, for the maintenance of DNA topology within the bacterial cell (Wigley, 1995). This enzyme belongs to type II topoisomerase family, which facilitates DNA unwinding at replication forks, while the main action of topoisomerase IV, minor target for quinolones in Gram-negative bacteria, is to decatenate or remove the interlinking of daughter chromosomes at the completion of a round of DNA replication and allows their segregation into daughter cell (Ruiz, 2003; Ambrozic Avgustin et al., 2007).

Resistance to fluoroquinolone drugs emerged following their widespread use, and as such, infections that were previously responsive to therapy may now pose an increased risk of treatment failure (Hopkins et al., 2005; Gagliotti et al., 2008). However, the World Health Organization (WHO) currently considers fluoroquinolones to be critically important antimicrobials, proposing very restricted use in veterinary practice, and a number of countries such as those of the European Union have forbidden some related uses (i.e. use as growth promoters) (Collignon et al., 2009).

Until now, three mechanisms of fluoroquinolones resistance were reported: chromosomal mutations in QRDRs of DNA gyrase and topoisomerase IV genes; decreased drug concentrations within bacteria mediated by activation of efflux pumps and loss of porins; and acquisition of plasmid-mediated fluoroquinolone resistance genes such as *qnr* (*qnrA*, *qnrB* and *qnrS*), *aac(6`)-Ib-cr* (Robicsek et al., 2006) and *qepA* (Yamane et al., 2007). The most important mechanism of fluoroquinolones resistance is alteration in QRDRs of GyrA and ParC subunits (Frank et al., 2011), these important residues are located near the active site of the enzymes (positions 116-130) which is a segment containing the catalytic Tyr-122 residue at their core (Marchetto et al., 2001). DNA gyrase commonly presents substitutions at amino acid position Ser83 and/or Asp87 of the GyrA subunit, while substitutions at residues Ser80 and Glu84 are commonly identified alterations in the ParC subunit of the topoisomerase IV when exposed to DNA sequence analysis (Yoshida et al., 1991; Heisig, 1996; Ozeki et al., 1997).

The presence of a single mutation in the QRDR of *gyrA* usually results in a high-level resistance to nalidixic acid, but additional mutation(s) in *gyrA* and/or in another target such as *parC* is required to obtain high levels of resistance to fluoroquinolones. Thus, it has been proposed that the MIC of nalidixic acid could be used as a genetic marker of resistance for quinolone family in Gram-negative bacteria (Ruiz, 2003).

In reality, resistance-conferring mutations are typically selected in *gyrA* first, and then *parC*. Mutations in the *gyrB* and *parE* genes confer a low-level resistance and are less frequently observed (Yu et al., 2004; Hopkins et al., 2005; Jacoby, 2005).

Unfortunately, data on the prevalence of antimicrobial-resistant veterinary pathogens are sparse, particularly in developing countries, including Egypt, where antimicrobials are overused in veterinary medicine and food animals. Additionally, due to the high incidence of fluoroquinolone resistance in our tested APEC strains recovered from diseased chickens diagnosed with colibacillosis in Sharkia Province, Egypt and the limited amount of information on fluoroquinolone resistance in our country, we determined MICs of some veterinary-use and human-use fluoroquinolones and identified the topoisomerase mutations underlying decreased fluoroquinolone susceptibility in our nalidixic acid-resistant strains.

Materials and Methods

Bacterial strains

Thirteen *E. coli* isolates were recovered from respiratory organs of clinically affected broilers diagnosed with avian colibacillosis from different farms in Sharkia province, Egypt. Presumptive *E. coli* isolates were confirmed by rapid API20 E bacterial identification system (Biomerieux, France). O-serotyping of the isolates was applied at Serology Department, Animal Health Research Institute, Dokki, Giza, Egypt, using polyvalent and monovalent sera (Denka Seiken CO., LTD., 3-4-2 Nihonbashikaya-cho, Chuo-ku, Tokyo, Japan) and the obtained serotypes were O78 (5), O27 (2), O115, O29, O125 and O146 (one for each) and untypable (2). These strains had been previously proved to harbor five virulence genes (*iuc*D, *fim*C, irp_2, *iss* and *tsh*) via PCR to be APEC (Ammar et al., 2011).

Antimicrobial susceptibility testing

Minimum inhibitory concentrations (MICs) of nalidixic acid (NAL) resistant *E. coli* strains were determined against fluoroquinolone agents via broth macrodilution technique using Muller Hinton broth medium (Difco, USA). The concentration of stock solution should be 1000 mg/L or greater and the inoculum size was approximately 5×10^5

CFU/ml. A double fold serial dilution was made for each antimicrobial agent starting from a concentration of 1024 µg/ml (Rankin, 2005). The veterinary-use fluoroquinolone agents as nalidixic acid (NAL), enrofloxacin (ENFX) and difloxacin (DIFX) and the human-use ones as ciprofloxacin (CPFX), norfloxacin (NFLX), levofloxacin (LVFX) and gatifloxacin (GFLX) (Oxoid, UK) were tested. MIC results were interpreted according to National Commitee for Clinical Laboratory Standards (NCCLS) institute (NCCLS, 2002; CLSI, 2011), additionally, MIC50 and MIC90 were calculated using an orderly array method (Hamilton-Miller, 1991).

PCR amplification of DNA gyrase and topoisomerase IV genes

DNA extraction was applied according to a previously published paper (Al-Agamy et al., 2012) with some modifications. Aliquot of 200 µl volume of overnight *E. coli* culture in Luria Beratni broth (Difco, USA) were pelleted by centrifugation at 10.000 rpm for 5 minutes, washed twice with PBS, then resuspended with 50 µl PBS. The template DNA was prepared by boiling for 10 minutes, quickly placed on ice for 10 minutes then centrifuged at 10.000 rpm for 5 minutes. The supernatant containing DNA was collected and stored at 4°C for further PCR applications.

The oligonucleotide primers used in this study and their amplicon sizes are listed in Table 1 (Everett et al., 1996). Uniplex PCR was performed in 25 µl of reaction volume consisting of 12.5 µl of DreamTaq TM Green Master Mix (2X) (Fermentas, USA), 0.1 µl of 100 pmole of each primer (Sigma, USA), 2 µl of template DNA and water nuclease-free up to 25 µl. PCR cycling program was performed in PTC-100 ™ programmable thermal cycler (Peltier-Effect cycling, MJ, Research, INC., UK) as following: initial denaturing step at 95°C for 10 min; followed by 30 cycles of 95°C for 30 s, 55°C for 45 s, and 72°C for 45 s; and a final extension step at 72°C for 7 min (Yang et al., 2004).

Aliquot of each amplicon, along with a 100-bp molecular weight DNA ladder (Fermentas, USA) were subsequently separated by electrophoresis on 1.5% molecular biology grade agarose gel (Sigma, USA) stained with 0.5 µg/ml ethidium bromide (Sigma, USA) on a mini slab horizontal electrophoresis unit (Bio-Rad, USA) at 100 V for 30 min. DNA bands were visualized under UV transilluminator (Spectroline, USA) and photographed.

DNA sequencing and analysis

PCR amplification products were purified using the QIAquick PCR purification kit (QIAGEN, Valencia, CA), each purified amplicon was sequenced in both forward and reverse directions using the amplification primers. The sequencing reaction was performed in an automated sequencer (Macrogen Inc., Korea ABI 3730XL DNA analyzer). DNA sequence data were analyzed by comparison with published GenBank DNA sequences using the NCBI-BLAST program (Altschul et al., 1997). Alignment of the nucleotide sequences was performed by the use of MEGA5 program (Tamura et al., 2011), product version 5.1 (http://www.megasoftware.net). Translation of the nucleotide sequences to amino acid sequences was performed using the ExPASy (Expert Protein Analysis System) Translate Tool (http://us.expasy.org/, Swiss Institute of Bioinformatics SIB, Geneva, Switzerland). Lastly, amino acid sequences were aligned using the MEGA5 program.

Predicted polypeptide products were analyzed for amino acid changes by comparison with wild type *E. coli* complete genome strain K-12 substrain MG1655 (GenBank accession no. NC000913). Topoisomerase genes of *E. coli* references strains were retrieved from the GenBank database and their accession numbers used in this study were listed in Table 2.

Results

Fluoroquinolones resistance phenotypes of APEC strains

Thirteen APEC strains were tested against a panel of veterinary-use and human-use fluoroquinolones in accordance with NCCLS broth macro-dilution method. Among 13 APEC strains, high level of resistance was observed to the veterinary-use fluoroquinolones; nalidixic acid (100%) and enrofloxacin and difloxacin (92.3%). Furthermore, decreased susceptibility was observed among the human-use fluoroquinolones tested, including 76.92% to each of ciprofloxacin, norfloxacin and levofloxacin and 69.23% to gatifloxacin. Additionally, MIC50 and MIC90 values were higher for the veterinary-use fluoroquinolones as compared to the human-use agents (Table 3).

At the level of APEC serotypes, it was detected that O78, the most common serovar in chicken, showed a high level of resistance to all fluoroquinolones tested, followed by O115, O146, O27 (one strain) and both untypable strains, while O125, O29 and the other O27 strain showed resistant only for most of veterinary-use fluoroquinolones tested.

PCR amplification and DNA sequence analysis.

From all tested *E. coli* strains, a 190-bp PCR product, covering the entire QRDR of *gyrA* (Fig 1A), a 204-bp fragment covering the region of *gyrB* (Fig 1B) and 265-bp PCR products encompassing the regions of both *parC* and *parE* (Fig 1C and D, respectively) were obtained.

PCR amplification products of two strains; *E. coli* serotype O125 resistant to veterinary-use fluoroquinolones only and *E. coli* serotype O78 resistant to all fluoroquinolones tested were purified and sequenced on both strands with specific primers.

Genetic analysis of *gyrA*

Solid-phase sequencing of the amplified DNA revealed an amino acid replacement in the QRDR of *gyrA* at codon 83 (serine TCG→leucine TTG) in both analyzed *E. coli* strains. A nucleotide number 248 of *gyrA* is exchanged from C to T, which subsequently exchanged amino acid from serine into leucine. Furthermore, point mutations were predicted at codon 87 (aspartic acid GAC→asparagine AAC) in *E. coli* O125 and aspartic acid GAC→glysine GGC in *E. coli* O78. On the other hand, mutation outside QRDR section of *gyrA* was detected in *E. coli* O78 at codon 116, as first report, due to substitution of serine TCT→phenylalanine TTC, this important residue is located near the active site of the enzymes (positions 116-130) which is also essential for DNA gyrase activity (Fig 2).

Interestingly, silent mutations were recorded in *gyrA* sequences of both strains by three nucleotide changes, none of which results in amino acid substitutions, which were at nucleotides 255 (GTC→GTT, both are valine), 273 (CGC→CGT, arginine), and 300 (TAT→TAC, tyrosine).

Genetic analysis of *gyrB*

In fact, alterations in the B subunit of DNA gyrase, although less common than those in the A subunit, have previously been shown to confer decreased fluoroquinolone susceptibility. In this study, no missense mutations were founded in QRDR of *gyrB* (Fig 3), meanwhile, silent mutation was detected only at nucleotide 1359 (GTC→GTT, valine) in *E. coli* O125 strain.

Genetic analysis of *parC*

Nucleotide sequencing of *parC* region analogous to the QRDR of *gyrA* revealed mutations at codon 80 (serine AGC→isoleucine ATC) in *E. coli* O125 and serine AGC→isoleucine ATT in *E. coli* O78 strain. Furthermore, mutation outside QRDR was also detected in both analyzed strains at codon 46, as first report, due to substitution of valine GTG into leucine TTG adding another site may resulting in increasing resistance to such antimicrobials (Fig 4). No silent mutations were recorded in the region of *parC* at all.

Genetic analysis of *parE*

Mutations in the topisomerase IV subunit B gene, *parE*, have been implicated in resistance to fluoroquinolone antibiotics in *E. coli* at a position analogous to mutations in *gyrB*. Nucleotide sequence analysis of *parE* revealed no changes in the QRDR, meanwhile, a single mutation outside this region at codon 458 (serine TCG→alanine GCG) in *E. coli* O125 strain only was detected (Fig 5). Moreover, eight silent mutations were found in such fluoroquinolone resistant strain at nucleotides 1350 (GTT→GTC, valine), 1386 (CAT→CAC, histidine), 1401 (GCG→GCT, alanine), 1429 (CTG→TTG, leucine), 1446 (TAT→TAC, tyrosine), 1452 (AAA→AAG, lysine), 1464 (CTC→CTG, leucine) and 1473 (GCG→GCT, alanine). In addition, both analyzed strains showed silent mutations at nucleotides 1317 (CCG→CCA, proline) and 1248 (CTC→CTT, leucine), none of these resulted in amino acid substitutions.

The nucleotide and aminoacid sequences of the topoisomerases under study were deposited into GenBank under the following accession numbers: KF531631 and KF546210 for *gyrA*, KF561243 and KF550125 for *gyrB*, KF546211and KF550122 for *parC* and KF550123 and KF550124 for *parE*. The results of topoisomerase mutations are shown in Table 4.

Table 1 Oligonucleotide primers used to amplify QRDRs of topoisomerase genes

PCR target	Primers	Primer sequence (5′-3′)	Position	Amplicon size (bp)
QRDR of *gyrA*	*gyrA*-1 *gyrA*-2	ACGTACTAGGCAATGACTGG AGAAGTCGCCGTCGATAGAAC	166 to 355	190
QRDR of *gyrB*	*gyrB*-1 *gyrB*-2	CAGACTGCCAGGAACGCGAT AGCCAAGTGCGGTGATAAGA	1223 to 1426	204
QRDR of *parC*	*parC*-1 *parC*-2	TGTATGCGATGTCTGAACTG CTCAATAGCAGCTC GGAATA	137 to 401	265
QRDR of *parE*	*parE*-1 *parE*-2	TACCGAGCTGTTCCTTGTGG GGCAATGTGCAGACCATCAG	1498 to 1232	265

QRDR**:** quinolone resistance-determining regions**;** bp: base pair

Table 2 GenBank accession numbers of topoisomerase genes of *E. coli* reference strains

GenBank accession number			
gyrA	***gyrB***	***parC***	***parE***
GQ869708	EU447175	GQ869753	JN565715
DQ447133	DQ386888	GQ869752	JN565717
DQ447125	EF064807	GQ869750	JN565720
DQ447124	EF064814	GQ869740	JN565721
GQ869729	AB083951	GQ869747	AY065820
GQ869728	JQ907518	DQ447147	JN565714
GQ869719	DQ386875	GQ869766	DQ447150
GQ869723	DQ447149	DQ447139	JN565718
GQ869706	EF064803	DQ447138	JN565716

Table 3 MIC range, MIC 50 and MIC 90 of fluoroquinolones of veterinary and human importance against APEC strains under study

Antibiotic	MIC (µg/ml)				% Resistant strains (n=13)
	Resistance breakpoint [a]	Range	MIC 50 [b]*	MIC 90 [c]*	
Nalidixic acid	≥ 32	128-1024	> 512	1024	100
Enrofloxacin	≥2	0.5-128	64	128	92.3
Difloxacin	≥4	0.5-256	128	256	92.3
Ciprofloxacin	≥ 4	1-1024	256	512	76.92
Norfloxacin	≥ 16	1-1024	512	512	76.92
Levofloxacin	≥ 8	2-512	16	512	76.92
Gatifloxacin	≥ 8	1-256	16	256	69.23

MIC: minimum inhibitory concentration

[a] The MIC below which treatment is likely to be successful, values are based on CLSI standards.

[b] The MIC at which 50% of the isolates are inhibited

[c] The MIC at which 90% of the isolates are inhibited.

*Both b and c were calculated by an orderly array method according to Hamilton-Miller, 1991.

Table 4 Mutations in topoisomerase genes of two analyzed APEC strains and their antimicrobial susceptibilities to fluoroquinolones

E. coli **serotype**	**Mutation type***			**MIC (µg/ml)**						
	gyrA	***parC***	***parE***	**NAL**	**ENFX**	**DIFX**	**CPFX**	**NFLX**	**LVFX**	**GFLX**
O125	Ser83-Leu Asp87-Asn	Ser80-Ile Val46-Leu	Ser 458-Ala	≥256	128	128	1	4	2	1
O78	Ser83-Leu Asp87-Gly Ser116-Phe	Ser80-Ile Val46-Leu	-	>512	64	256	512	512	16	16

MIC: minimum inhibitory concentration; NAL: nalidixic acid; ENFX: enrofloxacin; DIFX: difloxacin; CPFX: ciprofloxacin; NFLX: norfloxacin; LVFX: levofloxacin; GFLX: gatifloxacin.

* DNA sequences were analyzed using MEGA5 program and translation to amino acid sequences was performed using the ExPASy Translate Tool program.

Fig 1 Agarose gel electrophoresis pattern for amplification products of *gyrA* (A), *gyrB* (B), *parC* (C) and *parE* (D) genes of 13 APEC strains. M: molecular size markers (100 bp); lanes 1-13: positive for the four topoisomerase genes of all APEC strains. The size in base pairs (bp) of each PCR product is indicated on the right of the bands.

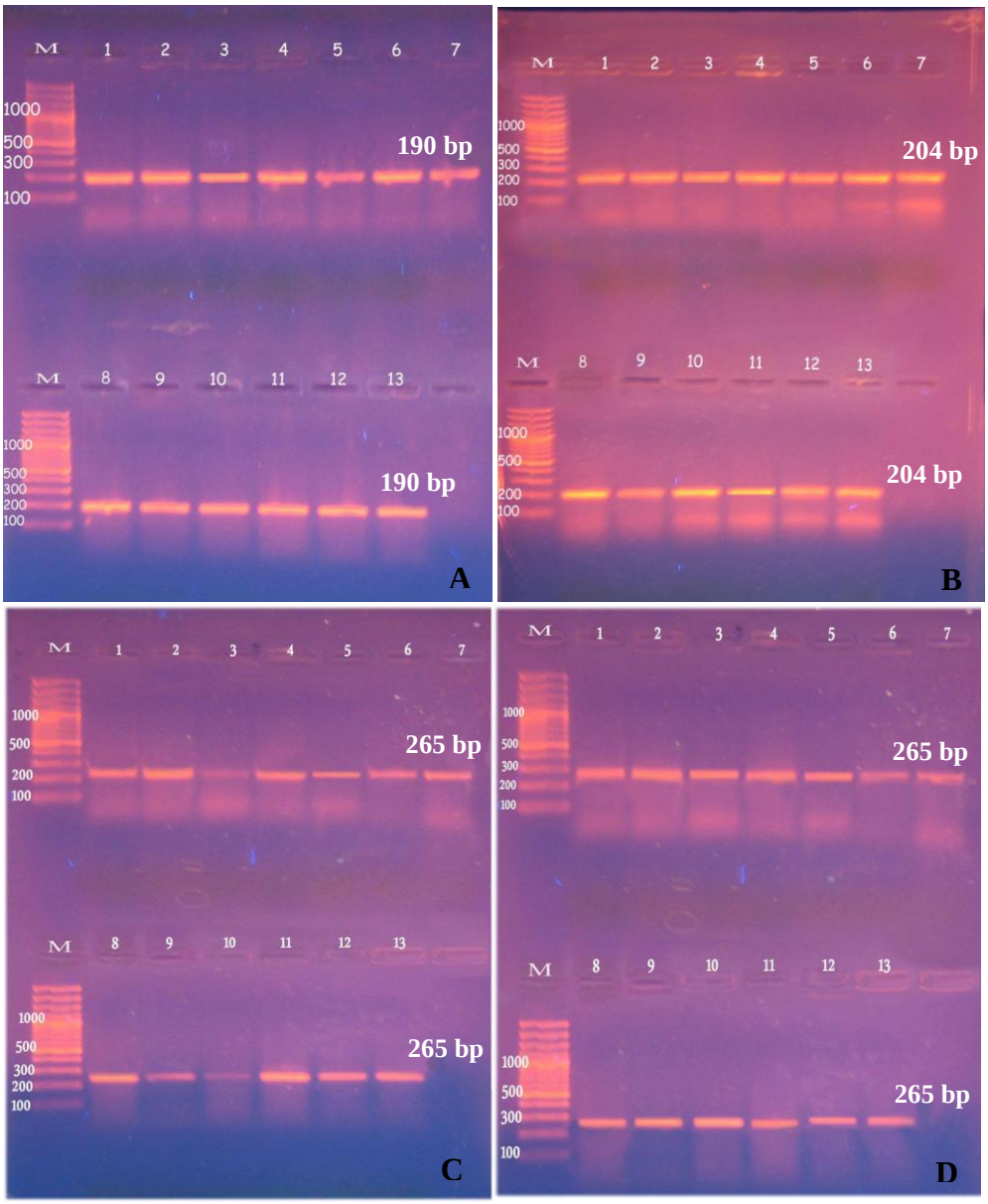

Fig 2 Amino acid sequence similarities for *E. coli gyrA* of the strains under study (APEC O125 and APEC O78) and the reference strains (their accession numbers are listed in the same order in table 2). Dots indicate amino acid positions identical to the corresponding *E. coli gyrA* sequence. Amino acid positions conserved in all sequences are designated by asterisks. Numbers refer to the aminoacid positions in the *E. coli gyrA* sequence. The Ser-83, Asp-87 and Ser-116 codons, in which mutations associated with fluoroquinolone resistance are found, are indicated by the solid bars.

K-12 substr. MG1655	**54:**	V	L	G	N	D	W	N	K	A	Y	K	K	S	A	R	V	V	G	D	V	I	**74**
APEC O125		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
APEC O78		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
YBZN-2		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
C20265-21		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
D-6T		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
47		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
JDCA104		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
YLCNZ		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
YLY7		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
YDCA33		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
YLCN13		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
		*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	
K-12 substr. MG1655	**75:**	G	K	Y	H	P	H	G	D	**S**	A	V	Y	**D**	T	I	V	R	M	A	Q	P	**95**
APEC O125		.	.	.	.	.	.	.	.	**L**	.	.	.	**N**	.	.	.	.	.	.	.	.	
APEC O78		.	.	.	.	.	.	.	.	**L**	.	.	.	**G**	.	.	.	.	.	.	.	.	
YBZN-2		.	.	.	.	.	.	.	.	L	.	.	.	N	.	.	.	.	.	.	.	.	
C20265-21		.	.	.	.	.	.	.	.	L	.	.	.	.	.	.	.	.	.	.	.	.	
D-6T		.	.	.	.	.	.	.	.	L	.	.	.	N	.	.	.	.	.	.	.	.	
47		.	.	.	.	.	.	.	.	L	.	.	.	N	.	.	.	.	.	.	.	.	
JDCA104		.	.	.	.	.	.	.	.	L	.	.	.	.	.	.	.	.	.	.	.	.	
YLCNZ		.	.	.	.	.	.	.	.	L	.	.	.	.	.	.	.	.	.	.	.	.	
YLY7		.	.	.	.	.	.	.	.	L	.	.	.	.	.	.	.	.	.	.	.	.	
YDCA33		.	.	.	.	.	.	.	.	L	.	.	.	.	.	.	.	.	.	.	.	.	
YLCN13		.	.	.	.	.	.	.	.	L	.	.	.	N	.	.	.	.	.	.	.	.	
		*	*	*	*	*	*	*	*		*	*	*		*	*	*	*	*	*	*	*	
K-12 substr. MG1655	**96:**	F	S	L	R	Y	M	L	V	D	G	Q	G	N	F	G	S	I	D	G	D	**S**	**116**
APEC O125		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
APEC O78		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	**F**	
YBZN-2		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
C20265-21		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
D-6T		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
47		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
JDCA104		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
YLCNZ		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
YLY7		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
YDCA33		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
YLCN13		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
		*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*		

Fig 3 Amino acid sequence similarities for *E. coli gyrB* of the strains under study (APEC O125 and APEC O78) and the reference strains (their accession numbers are listed in the same order in table 2). Dots indicate amino acid positions identical to the corresponding *E. coli gyrB* sequence. Amino acid positions conserved in all sequences are designated by asterisks. Numbers refer to the aminoacid positions in the *E. coli gyrB* sequence. No mutations were recorded.

K-12 substr. MG1655	**419:**	E	L	Y	L	V	E	G	D	S	A	G	G	S	A	K	Q	G	R	N	**437**
APEC O125		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
APEC O78		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
LR09		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
SSM4137		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
58		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
183		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
O86a: ECO86aP4		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
6		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
SSM1771		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
178		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
		*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	
K-12 substr. MG1655	**438:**	R	K	N	Q	A	I	L	P	L	K	G	K	I	L	N	V	E	K	A	**456**
APEC O125		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
APECO78		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
LR09		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
SSM4137		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
58		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
183		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
O86a: ECO86aP4		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
6		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
SSM1771		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
178		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
		*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	
K-12 substr. MG1655	**457:**	R	F	D	K	M	L	S	S	Q	E	V	A	T	L	I	T	A	L		**474**
APEC O125		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.		
APEC O78		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.		
LR09		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.		
SSM4137		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.		
58		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.		
183		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.		
O86a: ECO86aP4		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.		
6		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.		
SSM1771		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.		
178		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.		
		*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*		

Fig 4 Amino acid sequence similarities for *E. coli ParC* of the strains under study (APEC O125 and APEC O78) and the reference strains (their accession numbers are listed in the same order in table 2). Dots indicate amino acid positions identical to the corresponding *E. coli parC* sequence. Amino acid positions conserved in all sequences are designated by asterisks. Numbers refer to the aminoacid positions in the *E. coli parC* sequence. The Val-46 and Ser-80 codons, in which mutations associated with fluoroquinolone resistance are found, are indicated by the solid bar.

K-12 substr. MG1655	**46:**	**V**	Y	A	M	S	E	L	G	L	N	A	S	A	K	F	K	K	S	A	R	T	**66**
APEC O125		**L**	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
APEC O78		**L**	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
YLCN13		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
JDCA54		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
YLCNV		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
YDCA66		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
JDCA125		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
C20265-21		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
YLY7		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
D-6T		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
47		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
			*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	
K-12 substr. MG1655	**67:**	V	G	D	V	L	G	K	Y	H	P	H	G	D	**S**	A	C	Y	E	A	M	V	**87**
APEC O125		.	.	.	.	.	.	.	.	.	.	.	.	.	**I**	.	.	.	.	.	.	.	
APEC O78		.	.	.	.	.	.	.	.	.	.	.	.	.	**I**	.	.	.	.	.	.	.	
YLCN13		.	.	.	.	.	.	.	.	.	.	.	.	.	I	.	.	.	.	.	.	.	
JDCA54		.	.	.	.	.	.	.	.	.	.	.	.	.	I	.	.	.	.	.	.	.	
YLCNV		.	.	.	.	.	.	.	.	.	.	.	.	.	I	.	.	.	.	.	.	.	
YDCA66		.	.	.	.	.	.	.	.	.	.	.	.	.	I	.	.	.	.	.	.	.	
JDCA125		.	.	.	.	.	.	.	.	.	.	.	.	.	I	.	.	.	.	.	.	.	
C20265-21		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
YLY7		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
D-6T		.	.	.	.	.	.	.	.	.	.	.	.	.	R	.	.	.	.	.	.	.	
47		.	.	.	.	.	.	.	.	.	.	.	.	.	R	.	.	.	.	.	.	.	
		*	*	*	*	*	*	*	*	*	*	*	*	*		*	*	*	*	*	*	*	
K-12 substr. MG1655	**88:**	L	M	A	Q	P	F	S	Y	R	Y	P	L	V	D	G	Q	G	N	W	G	A	**108**
APEC O125		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
APEC O78		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
YLCN13		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
JDCA54		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
YLCNV		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
YDCA66		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
JDCA125		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
C20265-21		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
YLY7		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
D-6T		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
47		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	

K-12 substr. MG1655	**109**	P	D	D	P	K	S	F	A	A	M	R	Y	T	E	S	R	L	S	K	Y	**128**
APEC O125		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
APEC O78		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
YLCN13		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
JDCA54		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
YLCNV		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
YDCA66		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
JDCA125		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
C20265-21		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
YLY7		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
D-6T		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
47		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
		*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	

Fig 5 Amino acid sequence similarities for *E. coli ParE* of the strains under study (APEC O125 and APEC O78) and the reference strains (their accession numbers are listed in the same order in table 2). Dots indicate amino acid positions identical to the corresponding *E. coli parE* sequence. Amino acid positions conserved in all sequences are designated by asterisks. Numbers refer to the aminoacid positions in the *E. coli parE* sequence. The Ser-458 codon, in which mutation associated with fluoroquinolone resistance is found, is indicated by the solid bar.

K-12 substr. MG1655	**416:**	L	V	E	G	D	S	A	G	G	S	A	K	Q	A	R	D	R	E	Y	Q	**435**
APEC O125		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
APEC O78		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
8		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
24		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
56		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
90		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
4Q		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
7		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
408/65-1		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
26		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
19		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
		*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	
K-12 substr. MG1655	**436:**	A	I	M	P	L	K	G	K	I	L	N	T	W	E	V	S	S	D	E	V	**455**
APEC O125		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
APEC O78		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
8		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
24		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
56		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
90		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
4Q		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
7		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
408/65-1		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
26		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
19		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
		*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	
K-12 substr. MG1655	**456:**	L	A	S	Q	E	V	H	D	I	S	V	A	I	G	I	D	P	D	S	D	**475**
APEC O125		.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
APEC O78		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
8		.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
24		.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
56		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
90		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
4Q		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
7		.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
408/65-1		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
26		.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
19		.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
		*	*		*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	
K-12 substr. MG1655	**476:**	D	L	S	Q	L	R	Y	G	K	I	C	I	L	A	D	A	D	S	D		**494**
APEC O125		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.		
APEC O78		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.		
8		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.		
24		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.		
56		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.		
90		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.		
4Q		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.		
7		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.		
408/65-1		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.		
26		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.		
19		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.		
		*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*		

Discussion

Since the use of fluoroquinolone antibiotics in clinical practice was introduced about two decades ago, quinolone-resistant *E. coli* strains are being isolated with increasing frequency (Al-Agamy et al., 2012). Resistance to fluoroquinolones typically arises by stepwise acquisition of target mutations in the QRDRs of DNA gyrase and topoisomerase IV (Jacoby, 2005).

Perhaps, the most striking finding from this study was the widespread resistance to quinolones and fluoroquinolones. Consequently, fluoroquinolones will become useless in poultry farms within the next few years in Egypt. All tested APEC strains were resistant to nalidixic acid (100%); 92.3% and of the strains were resistant to enrofloxacin and difloxacin representing the veterinary-use agents. Moreover, 76.92% of the strains exhibited a resistance to each of ciprofloxacin, norfloxacin and levofloxacin, and 69.23% were resistant to the newer human fluoroquinolones, gatifloxacin. Similar findings have been reported in a previous study of *E. coli* isolates of both avian and swine origin in China, where all *E. coli* isolates displayed elevated levels of resistance to all fluoroquinolones tested, which were nalidixic acid (100%), difloxacin (95%), enrofloxacin (83%), ciprofloxacin (79%), levofloxacin (64%) and gatifloxacin (72%) (Yang et al., 2004). Somewhat similar results were recorded in another study in Spain, where there was a high frequency of nalidixic acid and ciprofloxacin resistance in *E. coli* isolates recovered from broilers (88% and 38%, respectively) (Sa´enz et al., 2001). The difference in the resistance pattern could be due to differences in isolation dates, localities, rate of consumption of fluoroquinolones …etc.

The association of DNA gyrase and topoisomerase IV mutations with fluoroquinolones resistance has been established for both Gram-negative and Gram-positive organisms (Frank et al., 2011). Accumulation of alterations in *gyrA* and the simultaneous presence of alterations in *parC* play a fundamental role in developing a high level of resistance to ciprofloxacin in clinical isolates (Al-Agamy et al., 2012). Genetic analysis of *gyrA* revealed double mutations in the QRDR at codons 83 (serine→leucine) and 87 (aspartic acid→asparagine or glysine). Moreover, mutation outside this region was also recorded in one analyzed strain at codon 116 (serine→phenylalanine) as first report. In particular, Ser83→Leu and Asp87→Asn or Gly substitutions as favored mutational hot spots in GyrA subunit were commonly reported by other investigators (Hopkins et al., 2005; Uchida et al., 2010; Karczmarczyk et al., 2011; Al-Agamy et al., 2012; Shaheen et al., 2013), and hence, their ubiquity in the strains studied here is not unexpected. Meanwhile, mutation at ser116→phe has not been reported, thus far in naturally occurring quinolone-resistant *E.coli* field isolates, this important residue is located near the active site of the enzymes (positions 116-130) which is also essential for DNA gyrase activity (Marchetto et al., 2001; Jaktaji and Mohiti, 2010). This additional mutation in the ser116 codon of *gyrA* is associated with a greater increase in fluoroquinolone resistance. However, it cannot be concluded that this mutation is responsible for the differences in MICs for fluoroquinolone agents.

Mutations in *parC* were always found together with mutations in *gyrA*. This is due to topoisomerase IV being a secondary target for quinolones in *E. coli* (Hopkins et al., 2005). However, mutations in *parC* play an important role in the formation of highly resistant strains (Hopkins et al., 2005), a single amino acid change in the QRDR of the *ParC* protein was found in both analyzed strains at codon 80 (serine→isoleucine) which was previously reported by several authors (Sa´enz et al., 2003; Yang et al., 2004; Hopkins et al., 2005; Karczmarczyk et al., 2011; Al-Agamy et al., 2012; Shaheen et al., 2013). Furthermore, mutation outside this region was also detected in both strains at codon 46 (valine→leucine). Occurrence of mutation outside the QRDR was reported, but it was at positions 51, 50 (Friedman et al., 2001; Jaktaji and Mohiti, 2010), so the presence of a point mutation at amino acid 46 as a first report in this study suggests the importance of more amino acids in the production of active sites for DNA gyrase.

Mutations in *gyrB* and *parE* have been associated with quinolone resistance (Heddle and Maxwell, 2002); however, the mutation frequency is much lower compared to those for *gyrA* and *parC* (Yu et al., 2004; Hopkins et al., 2005; Jacoby, 2005). In this study, a single mutation was recorded outside QRDR of *parE* at codon 458 (serine→alanine) in one analyzed strain only and have been reported previously (Uchida et al., 2010; Karczmarczyk et al., 2011), meanwhile, no mutations were recorded in *gyrB*.

In summary, the rate of *E. coli* resistance to fluoroquinolones is increasing in Egypt and this finding indicates alarmingly that the fluoroquinolones will become useless within the next few years. This resistance is mainly due to presence of mutations in QRDRs of the fluoroquinolone targets (DNA gyrase and topoisomerase IV), additionally, novel mutations were recorded outside this region in *gyrA* (Ser-116→Phe) and *parC* (Val-46→Leu), as first report in our study, adding essential sites for target enzymes activity.

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