Rapid detection of Escherichia coli gyrA *and* parC *mutants in one-day-old broiler chicks in Iran*

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Veterinaria Italiana 2013, **49** (3), 291-297. doi: 10.12834/VetIt.1304.08
Accepted: 02.09.2013 | Available on line: 23.09.2013

Keywords
Escherichia coli,
Fluoroquinolone resistance,
gyrA,
parC,
Iran,
one-day-old broiler chicks,
PCR-RFLP,
sequencing.

Summary
Vertical and consequently horizontal transmission of quinolone and fluoroquinolone resistant *Escherichia coli* clones following hatch in chickens enables a massive amplification of these clones into a large population. The aim of this study was to determine the antibiotic resistance and susceptibility of Iranian *E. coli* isolates (n=105) from one-day-old chicks to fluoroquinolones and the relation of this resistance with mutations in *gyrA* and *parC* genes using PCR-RFLP. For the first time, EcoRV restriction enzyme was used for rapid mutation screening in *parC* (Ser80Ile). The results showed that the low level of Minimum Inhibitory Concentration (MIC) for ciprofloxacin (0.25-4µg ml^{-1}) and enrofloxacin (0.25-4µg ml^{-1}) corresponded to a single mutation in *gyrA*, while intermediary to high level of MIC for ciprofloxacin (8→64 µg ml^{-1}) and enrofloxacin (16→64 µg ml^{-1}) were related to 2 mutations in *gyrA* or 3 mutations, 2 in *gyrA* and 1 in *parC*. There was a strong positive correlation (R = 0.93, P < 0.001) between MIC levels of enrofloxacin and ciprofloxacin among these isolates. The article concludes by stressing that the rising incidence of enrofloxacin resistant *E. coli* isolates from chicken sources may increase the potential risk of ciprofloxacin resistant *E. coli* acquisition by humans.

Mutazioni nei geni gyrA *e* parC *di* Escherichia coli *in pulcini di un giorno in allevamenti di polli da carne, Iran*

Parole chiave
Escherichia coli,
Resistenza a fluorochinolone,
Gene *gyrA*,
Gene *parC*,
Iran,
PCR-RFLP,
Pulcino,
Sequenziamento.

Riassunto
La trasmissione verticale e orizzontale di cloni di *Escherichia coli* resistenti a chinoloni e fluorochinoloni nei polli dopo la schiusa delle uova provoca una massiccia amplificazione dei cloni in un notevole numero di soggetti. Lo studio ha avuto l'obiettivo di determinare resistenza e suscettibilità ai fluorochinoloni di isolati (n=105) di *E. coli* prelevati da pulcini di un giorno e di verificare, mediante PCR-RFLP, la relazione tra questa stessa resistenza e le mutazioni nei geni *gyrA* e *parC*. Per la prima volta è stato utilizzato l'enzima di restrizione EcoRV per effettuare rapidamente lo screening delle mutazioni presenti in *parC* (Ser80Ile). I risultati hanno evidenziato una singola mutazione in *gyrA* in presenza di un basso livello di concentrazione minima inibente (MIC) per ciprocloxacina (0,25-4 µg ml^{-1}) ed enrofloxacina (0,25-4 µg ml^{-1}). Sono state invece evidenziate 2 mutazioni in *gyrA* o 3 mutazioni, 2 in *gyrA* e 1 in *parC*, in presenza di una concentrazione minima inibente (MIC) intermedia-alta per ciprocloxacina (8→64 µg ml^{-1}) ed enrofloxacina (16→64 µg ml^{-1}). Negli isolati è stata anche riscontrata una significativa correlazione positiva tra i livelli di MIC per ciprocloxacina e enrofloxacina (R = 0,93, P< 0,001). Lo studio evidenzia la relazione tra un crescente numero di isolamenti di *E. coli* resistente ad enrofloxacina nei polli e l'aumento potenziale del rischio di acquisizione di *E. coli* resistente ad ciprofloxacina nell'uomo.

Introduction

Quinolones and fluoroquinolones (FQs) constitute a family of antibacterial agents that damage bacterial DNA via inhibition of type II topoisomerases. These are heterotetrameric enzymes including DNA gyrase with two subunits A and B (respectively encoded by *gyrA* and *gyrB* genes) and DNA topoisomerase IV with two subunits A and B (respectively encoded by *parC* and *parE* genes) (21). These enzymes work together in the replication, transcription, recombination, and repair of bacterial DNA (12). Alterations in quinolone resistance determining regions (QRDRs) via mutations have been recognized as one of the main resistance mechanisms in *Escherichia coli* (22). At the same time, the amino-acid substitution in these regions has significant effects on quinolone and FQ Minimum Inhibitory Concentration (MIC) values (24). In quinolone resistant gram negative bacteria, such as *E. coli*, mutations occurring in *gyrA* (mainly at Ser-83 and ASP-87) and *parC* (mainly at Ser-80 and Glu-84) subunits have been described as a secondary target (12).

E. coli as a component of the bacterial inhabitants of chicken gastrointestinal tract is an indicator of faecal contamination in poultry processing (8). Quinolone and FQ resistant *E. coli* isolates from broilers, broiler meat and humans are closely related and show clonal links, indicating that poultry and their food products can be a source of resistant *E. coli* in humans (10, 11, 14, 17, 18). Quinolone and FQ resistant *E. coli* has been reported in retail chicken products and in both healthy and clinically affected chickens (1, 3, 13, 15, 16, 20, 25). However data regarding FQ resistance in *E. coli* isolated from one-day-old broiler chicks on arrival at the farms are limited. This study was conducted on these chicks to investigate *gyrA* and *parC* mutations as the most important mechanism for FQ resistance in these *E. coli* isolates via DNA sequencing and PCR-RFLP.

Materials and methods

Sample collection

This study was conducted on 21 broiler farms located in the county of Shiraz, in Fars province, Iran. Sampling was done on one-day-old broiler chicks on their arrival at the farms from the hatcheries located in 5 different geographical areas of Iran. Fifteen cloacal samples were taken from each farm using sterile wood applicators. Each 3 swabs were pooled in a sterile tube containing tryptic soy broth (TSB) (Merck, Darmstadt, Germany) and immediately taken to the laboratory.

Bacterial isolation and identification

Samples were spread onto MacConkey agar (Merck, Darmstadt, Germany) plates and incubated overnight at 37°C. One of the pink colour colonies from each plate was picked, spread onto Eosin Methylene Blue agar (Merck, Darmstadt, Germany) plates, and incubated overnight at 37°C. Blue-black colonies with dark centres and greenish metallic sheen were considered as *E. coli* - identified using a panel of biochemical tests (gram stain, oxidase, TSI, indole, citrate, methyl red, and urea agar) - and stored at -70°C in TSB with 30% glycerol until antibiotic susceptibility test and DNA extraction were performed.

Antimicrobial susceptibility test

Resistance of 105 *E. coli* isolates (5 isolates from each farm) to fivequinolone and FQ antimicrobials - nalidixic acid (30µg), ciprofloxacin (5µg), enrofloxacin (5µg), flumequine (30µg) and norfloxacin (10µg) - was investigated using the disk diffusion method in Mueller-Hinton agar (Merck, Darmstadt, Germany) following National Committee for Clinical Laboratory Standards (NCCLS/CLSI) guidelines. The results were interpreted according to NCCLS/CLSI standards at the National Reference Laboratory for Antimicrobial Resistance (5, 6). Quality control was performed using the *E. coli* ATCC 25922 reference strain.

Fluoroquinolone susceptibility test

Minimum inhibitory concentration (MIC) was determined by broth macrodilution method according to CLSI guidelines using enrofloxacin and ciprofloxacin (Bayer AG, Leverkusen, Germany). Susceptibility (≤) and resistance (≥) breakpoints (mg/L) were those defined by the CLSI (2010): 1 and 4 for ciprofloxacin, 0.25 and 2 for enrofloxacin.

PCR amplification and sequencing of *gyrA* and *parC*

A few *E. coli* colonies were transferred to distilled water in an Eppendorf tube and boiled to prepare DNA templates for PCR (23). *GyrA* amplification was performed using 5'- ACGTACTAGGCAATGACTGG-3' and 5'-AGAAGTCGCCGTCGATAGAAC-3'primers. *ParC* amplification was performed using 5'-TGTATGCGATGTCTGAACTG-3' and 5'-CTCAATAGCAGCTCGGAATA-3' primers (7). *GyrA* and *parC* amplicons were amplified using a DNA thermo cycler (MJ mini, BioRad, Hercules, CA, USA) as follows: initial denaturation at 94°C for 5min, followed by 45 amplification cycles (94°C for 45s, 55°C used for *gyrA* and 58°C used for *parC* for 45s, 72°C for 45s) and a final extension cycle (72°C for 5min).

PCR products from 9 *E. coli* isolates with high MIC levels (MIC≥16µg ml^{-1}) were first selected randomly and sequenced (ABI 3730 Capillary DNA analyser - Applied Biosystems, Foster City, CA, USA) in order to find possible existing patterns of mutations in *gyrA* and *parC* genes. Then a PCR-RFLP method, based on the obtained pattern of mutations in *gyrA* and *parC* after sequencing, was used for all isolates (n= 105).

RFLP-PCR for mutation detection in the *gyrA* and *parC* genes

On the basis of the preliminary sequencing results (mutations at Ser-83 and Asp-87 in *gyrA* and Ser80Ile in *parC*) PCR-RFLP was used as a fast and reliable method for mutation detection. For *gyrA*, a sense primer, EC-GYRA-A (5'-CGCGTACTTTACGCCATGAACGTA-3') and an antisense primer, EC-GYRA-*Hinf*I (5'-ATATA ACGCAGCGAGAATGGCTGCGCCATGCGGACAATCG AG-3'') with a mismatch nucleotide (substitution of Thymine with Adenosine) were used to produce a 164-bp DNA fragment as previously described by Ozeki (19). Primer EC-GYRA-*Hinf*I was adjacent to the Asp-87 codon and differed by 1 base (underlined) from the wild type *gyrA* gene sequence to generate a HinfI recognition site. These primers amplified a 164 bp PCR product encompassing the QRDR of the *gyrA* with 2 natural and artificial *Hinf*I restriction sites in the Ser-83 and Asp-87 regions, respectively (19).

The PCR reaction (25µL) was performed in 10mM Tris–HCl, pH 8.4, 50 mMKCl, 2mM MgCl2, 100µM of each dNTP, 20pmol of each primer (Cinnagen Inc., Tehran, Iran), and 2 U Taq DNA polymerase (Cinnagen Inc.) using 2µL of DNA extracted as template. Amplification reactions were carried out using a DNA thermo cycler as follows: initial denaturation step at 94°C for 5min, followed by 45 cycles of denaturation at 94°C for 45s, annealing at 56°C for 1min and extension at 72°C for 1min. The final extension step was carried out at 72°C for 10min. PCR products were digested with *Hinf*I (Jena Bioscience, Jena, Germany) to screen for mutations at positions Ser-83 and Asp-87. Enzyme digestion was performed in a 20µl mixture containing 12µl of the PCR product, 0.5µl (2 U) of enzyme, 2µl B3 buffer and 5.5µl of distilled water at 37°C for 5hrs. After digestion with HinfI, the presence of PCR products was determined by electrophoresis of 10µl of each reaction product in 3% (w/v) Agarose gel with Trisborate EDTA electrophoresis buffer and visualized under UV light.

A 265bp *parC* region containing the QRDR was amplified following the conditions described in (7) and the mutation of Ser80Ile was detected using EcoRV enzyme (Jena Bioscience). Digestion was performed in a final volume of 20µl reaction mixture containing 12µl of the PCR product, 2µl B2 buffer, 0.34µl (2U) enzyme and 5.66µl of distilled water at 37°C for 4hrs.

Statistical analysis

The correlation between MIC values of enrofloxacin and ciprofloxacin was analysed by Pearson correlation test. Analysis was performed using SPSS version 16.0 (SPSS Inc., Chicago, IL, USA).

Results

Susceptibility or resistance to quinolone and FQ agents of 105 *E. coli* isolates from one-day-old chicks were first analysed using Disk Diffusion method (Table I). There was moderate resistance to ciprofloxacin, enrofloxacin and norfloxacin and high resistance to nalidixic acid and flumequine. Susceptibility or resistance to enrofloxacin and ciprofloxacin was also determined by MIC for all *E. coli* isolates. The percentage of resistance, intermediate resistance and susceptibility, of these isolates to enrofloxacin was 41.9%, 34.3% and 23.6%, respectively; while for ciprofloxacin the percentages were 36.2%, 2.9% and 60.9%, respectively. There was a significant positive correlation between MIC levels of enrofloxacin and ciprofloxacin ($R = 0.93$, $P < 0.001$). The distribution of MIC for enrofloxacin and ciprofloxacin by amino-acid mutation in *gyrA* and *parC* are presented in Table II and Table III.

Sequencing results showed that all the sequenced isolates (n=9) shared a similar pattern of mutations in amplified regions of *gyrA* and *parC* genes. Two amino-acid substitutions in the QRDR of *gyrA* protein (Ser83Leu and Asp87Asn) and a single amino-acid substitution in the QRDR of *parC* protein (Ser80Ile) were detected in these sequenced isolates. Two silent mutations (Val-85 and Arg-91) in *gyrA* and 1 nucleotide mutation without amino-acid substitution in Gly-107 in *parC* were also detected in the sequenced isolates. One isolate (KC567240) also had an additional silent mutation (Gln-91) in *parC* (Figure 1).

Accordingly, a total of 105 *E. coli* isolates were analysed by PCR-RFLP method to detect mutations at Ser-83 and Asp-87 in the *gyrA* gene as well as Ser-80 in *parC* (Figure 2).

Table I. *Percentages of* E. coli *isolates (n=105) from one day-old broilers susceptible (S), intermediate (I) and resistant (R) to quinolone and FQs antimicrobial agents by* NCCLS *disc diffusion methods.*

Antimicrobial agent (µg)	Diffusion zone breakpoint (mm)	Number of resistance or susceptible *E. coli* isolates (%)		
		Susceptible	Intermediate	Resistant
Nalidixic acid (30)	≤13	23 (21.9%)	5 (4.8%)	77 (73.3%)
Enrofloxacin (5)	≤16	25 (23.8%)	34 (32.4%)	46 (43.8%)
Ciprofloxacin (5)	≤ 15	62 (59.1%)	6 (5.7%)	37 (35.2%)
Norfloxacin (10)	≤12	59 (56.2%)	7 (6.7%)	39 (37.1%)
Flumequine (30)	≤16	25 (23.8%)	2 (1.9%)	78 (74.3%)

Table II. *Amino-acid changes in* gyrA *and* parC *genes of* E. coli *isolated from one-day-old chicks and corresponding Minimum Inhibitory Concentration (MIC) of enrofloxacin.*

Substitution site		Number of isolates	No. of *E. coli* isolates corresponding MIC (µg ml^{-1})[b]											
gyrA	*parC*		<0.125	0.125	0.25	0.5	1	2	4	8	16	32	64	64<
wt[a]	wt	28	22	3		3								
Ser83	wt	38				7	25	5	1					
Asp87	wt	2			2									
Ser83 and Asp87	wt	2										2		
wt	Ser80	0												
Ser83	Ser80	0												
Asp87	Ser80	0												
Ser83 and Asp87	Ser80	35									15	10	5	5

[a] wild type; [b] MIC (µg ml^{-1}) according to resistance-criteria of *NCCLS* is shown in shaded area.

Table III. *Amino-acid changes in* gyrA *and* parC *genes of* E. coli *isolated from one-day-old chicks and corresponding Minimum Inhibitory Concentration (MIC) of ciprofloxacin.*

Substitution site		Number of isolates	No. of *E. coli* isolates corresponding MIC (µg ml^{-1})[b]											
gyrA	*parC*		<0.125	0.125	0.25	0.5	1	2	4	8	16	32	64	64<
wt[a]	wt	28	22	2	1	2	1							
Ser83	wt	38				11	23	3	1					
Asp87	wt	2			1	1								
Ser83 and Asp87	wt	2									2			
wt	Ser80	0												
Ser83	Ser80	0												
Asp87	Ser80	0												
Ser83 and Asp87	Ser80	35								2	18	7	6	2

[a] wild type; [b] MIC (µg ml^{-1}) according to resistance-criteria of *NCCLS* is shown in shaded area.

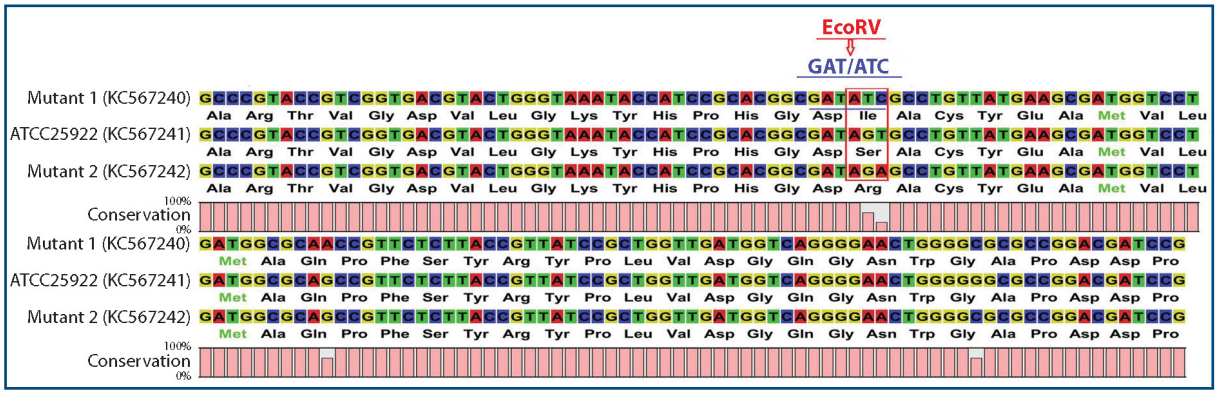

Figure 1. *Simultaneous alignment of partial* parC *nucleotide and amino-acid sequences from 1 susceptible (*E. coli *ATCC25922) and 2 resistant* E. coli *isolates (KC567240 and KC567242) to FQs, showing single location with 2 different patterns of amino-acid substitutions (Ser80Ile or Ser80Arg).* Substitution of Ile instead of Ser produced a cut site (GAT/ATC) for EcoRV enzyme.

Direct sequencing of the PCR-amplified *parC* gene showed a single amino-acid substitution in the Ser-80 codon (Ser80Ile). The point mutation in this area (T→C) led to the creation of a new restriction site (GAT/ATC) for EcoRV enzyme (Figure 1). When 2 DNA fragments of 101 bp and 164 bp were produced from a PCR product (Figure 2), as observed for KC567240, the isolate was assumed to have a T→C mutation at Ser-80. All the *parC* PCR products from *E. coli* isolates (n=105) were examined using EcoRV. Sixty-seven strains for which the MIC of ciprofloxacin was <4µg ml^{-1} showed no

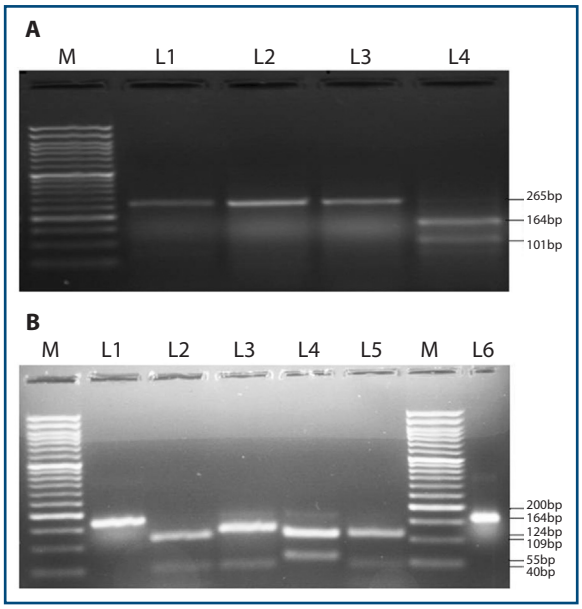

Figure 2. (A) *EcoRV PCR-RFLP patterns of parC.*

L1: PCR product *E. coli* ATCC25922.
L2: *E. coli* ATCC25922 (uncut).
L3: KC567240 PCR product.
L4: RFLP pattern of KC567240 (Ser80Ile): the EcoRV restriction enzyme digestion produced 2 DNA fragments of 164bp and 101bp.
M: DNA size marker (50 bp ladder, Vivantis, Subang Jaya, Malaysia).

(B) *HinfI PCR-RFLP patterns of gyrA.*

L1: sample with mutations at both Ser-83 and Asp-87 (164bp).
L2: *E. coli* ATCC25922 with no mutations at either Ser-83 or Asp-87(109 bp and 40 bp).
L3: sample with a single mutation at Ser-83 (124 bp and 40 bp).
L4: sample with a single mutation at Asp-87 (109 bp and 55 bp).
L5: sample with no mutations (109 bp and 40 bp).
L6: the *gyrA* PCR product (164 bp) of *E. coli* ATCC25922.
M: DNA size marker (50 bp ladder, Vivantis).

restriction site at Ser-80. Among the 38 ciprofloxacin resistant isolates (MIC≥4µg ml^{-1}), 34 isolates had this restriction site at Ser-80 and were assumed to have Ser to Ile substitution in this codon. Four remaining ciprofloxacin resistant isolates were investigated for other mutations in this region by DNA sequencing. One of these isolates with a low level of ciprofloxacin resistance phenotype (MIC=4µg ml^{-1}) had no mutation at Ser-80. However, 3 isolates (mutant2), with MIC≥16µg ml^{-1}, had Arginine (Arg) substitution instead of Serine-80 (Figure 1).

Discussion

In this study, 105 *E. coli* isolates from the normal microflora of one-day-old chicks in Iran were analysed for their enrofloxacin and ciprofloxacin MIC values and mutation detection in QRDRs of *gyrA* and *parC*.

A significant positive correlation was found between MIC levels of enrofloxacin and ciprofloxacin ($R = 0.93$, $P < 0.001$). Additionally, enrofloxacin and ciprofloxacin resistance rates were approximately similar for *E. coli* isolates (41.9% and 36.2%, respectively), even if there were more ciprofloxacin than enrofloxacin susceptible isolates (60.9% and 23.8%, respectively). These results could be attributed to the fact that the susceptibility and resistance break points for enrofloxacin (0.25mg/L and 2mg/L, respectively) are lower than ciprofloxacin (1mg/L and 4mg/L, respectively) (5, 6). In other words, about 76% of these isolates were resistant or intermediate resistant to enrofloxacin, whereas only 39% of all *E. coli* isolates were ciprofloxacin resistant or ciprofloxacin intermediate resistant.

A single mutation in the *gyrA* was associated with a decrease in susceptibility of these *E. coli* isolates to ciprofloxacin and enrofloxacin. Nonetheless, all of these isolates, except 1 for ciprofloxacin and 6 for enrofloxacin, were still susceptible to these 2 FQs. A single mutation in Ser-83 is the most frequent single mutation site in *gyrA* gene. This mutation is the first step in the acquisition of FQ resistance and usually results in high-level resistance to nalidixic acid (22). However, an additional mutation in *gyrA* or *parC* is required to obtain high levels of resistance to second generation FQs such as ciprofloxacin and enrofloxacin (Table II and Table III). This step-by-step acquisition of FQ resistance is in agreement with previous findings (22). Two isolates with single amino-acid substitution in Asp-87 were also detected but this single substitution was not enough to increase MIC values up to enrofloxacin and ciprofloxacin resistance breakpoint. Simultaneous mutations in *gyrA* (Ser-83 and Asp-87) without any amino-acid substitution in *parC* were seen in 2 isolates with high values of MIC for enrofloxacin (32µg ml^{-1}) and ciprofloxacin (16µg ml^{-1}). The emergence of these higher levels of FQ resistance could be associated with plasmid-mediated quinolone resistance (PMQR) mechanisms, such as qnr gene, qepA, oqxAB and aac(60)-Ib-cr, which have been studied and increasingly reported in recent years (21).

Considering these findings, it seems that low level of MIC for ciprofloxacin and enrofloxacin corresponded to a single mutation in *gyrA*, while intermediary to high values of MIC for ciprofloxacin and enrofloxacin were related to 2 mutations in *gyrA* or 3 mutations, 2 in *gyrA* and 1 in *parC*. This was in agreement with previous studies on *E. coli* isolated from chickens (15, 16, 25). Ruiz (22) stated that the most common mutations in ciprofloxacin resistant *E. coli* are present in the QRDR of *gyrA* at positions Ser-83 and Asp-87 and at position Ser-80 and Glu-84 of *parC*. In our study, all 9 preliminary *E. coli* isolates, which were sequenced for GRDR of *gyrA* and *parC*, showed 2 amino-acid substitutions (Ser83Leu and Asp87Asn) in *gyrA* and 1 substitution in *parC* (Ser80Ile). Based on these constant mutation features, PCR-RFLP technique was used for rapid detection of these mutation sites. For the first time mutation RFLP analysis in *parC* (Ser80Ile) was developed using EcoRV in the present study.

Knowledge of the emergence of FQ resistance mechanisms in *E. coli* isolates from one-day-old broiler chicks is limited. This study showed a strong positive correlation between MIC levels of enrofloxacin and ciprofloxacin in *E. coli* isolates of one-day-old broiler chicks. This correlation indicates that most of the enrofloxacin resistant *E. coli* isolates were concomitantly resistant to ciprofloxacin. This correlation is not unexpected, because enrofloxacin is extensively metabolized into ciprofloxacin (2). The history of farms with high FQ-resistant isolates showed that enrofloxacin was used in the rearing or laying period. There is some evidence of vertical transmission of enrofloxacin resistant *E. coli* from healthy broiler breeders (20). Vertical transmission, and consequently horizontal dissemination, has been mentioned as an important approach for distribution of *E. coli* clones in poultry farms. Horizontal transmission following hatch enables a massive amplification of these clones into the large population (9). Vertical transmission of FQ resistant *E. coli* from parents to broilers (4) and horizontal transmission in one-day-old chicks following hatch leads to multiplication of this resistance in large populations (20). The horizontal transmission of these resistant clones in the hatcheries could spread these clones through the integrated broiler operations and lead to clinical outbreaks of FQ-resistant colibacillosis in poultry farms (20). These FQ-resistant *E. coli* may also be transmitted from contaminated chicken meat products to humans (10, 14, 17).

Conclusions

There was a strong positive correlation between MIC levels of enrofloxacin and ciprofloxacin in the *E. coli* isolates considered in this study. The role of chicken as a reservoir to the extension of ciprofloxacin resistance in humans has not been completely quantified. However, the indiscriminate use of FQ antibiotics, such as flumequine and enrofloxacin, should be avoided or minimized, in broiler chickens as well as in other poultry production cycles such as broiler breeders for example.

Acknowledgments

This research was financially supported by a grant from the Shiraz University Research Council.

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