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Fluoroquinolone Resistance and *gyrA* and *parC* Mutations of *Escherichia coli* Isolated from Chicken

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Escherichia coli **is a common inhabitant of the intestinal tracts of animals and humans. The intestines of animals also represent an ideal environment for the selection and transfer of antimicrobial resistance genes. The aim of this study was to investigate the resistance of *E. coli* isolated from chicken fecal samples to fluoroquinolones and to analyze the characterization of mutations in its *gyrA* and *parC* gene related resistance. One hundred and twenty-eight *E. coil* isolates showed a high resistance to ciprofloxacin (CIP; 60.2%), enrofloxacin (ENO; 73.4%) and norfloxacin (NOR; 60.2%). Missense mutation in *gyrA* was only found in the amino acid codons of Ser-83 or Asp-87. A high percentage of isolates (60.2%) showed mutations at both amino acid codons. Missense mutation in *parC* was found in the amino acid codon of Ser-80 or Glu-84, and seven isolates showed mutations at both amino acid codons. Isolates with a single mutation in *gyrA* showed minimal inhibitory concentrations (MIC) for CIP (≤0.5 to 0.75 μg/ml), ENO (1 to 4 μg/ml) and NOR (0.75 to 4 μg/ml). These MIC were level compared to isolates with two mutations, one in *gyrA* and one in *parC*, and three mutations, one in *gyrA* and two in *parC* (CIP, ≤0.5 to 3 μg/ml; ENO, 2 to 32< μg/ml; NOR, 1.5 to 6 μg/ml). However, the isolates with two mutation in *gyrA* regardless of whether there was a mutation in *parC* showed high MIC for the three fluoroquinolones (CIP, 0.75 to 32≤μg/ml; ENO, 3 to 32≤μg/ml; NOR, 3 to 32≤μg/ml). Interestingly, although the *E. coil* used in this study was isolated from normal flora of chicken, not clinical specimens, a high percentage of isolates showed resistance to fluoroquinolones and possessed mutations at *gyrA* and *parC* associated with fluoroquinolone resistance.**

Key words: *E. coli,* fluoroquinolone resistance, *gyrA gene, parC* gene

Fluoroquinolones are a synthetic class of antimicrobial agents, which have been used widely in human and veterinary medicine since their introduction in the late 1980s and early 1990s (Hooper, 1995). This is because fluoroquinolones offer the advantage of oral administration, high potency against many gram-negative organisms, and low host toxicity with some exceptions (Prescott and Baggot, 1993). However, since the mid-1990s, there have been several studies reporting some organisms that were resistant to quinolone (Hoshino *et al.*, 1994; Quabdesselam *et al.*, 1995). Stable resistance at high levels can occur as a result of progressive exposure to increasing subinhibitory drug concentrations. Mutations producing resistance occur by altering target proteins (DNA gyrase encoded by gyrA and gyrB and topoisomerase IV encoded by parC and parE), required for DNA replication and transcription (Cambau and Gutmann, 1993; Blanche *et al.*, 1996), and accumulation of fluoroquinolones in the cytoplasm mediated by reduced cellular permeability and/or enhanced efflux (Han and Wood, 1969).

Escherichia coli is a common inhabitant of the intestinal tracts of animals and humans (Bonten *et al.*, 1990; Murray, 1990). The normal flora is advantageous to the host because it may prevent potential infections with pathogenic bacteria. Nevertheless, the intestine of animals also represents an ideal environment for the selection and transfer of antimicrobial resistance genes (Amara *et al.*, 1995; Bass *et al.*, 1999; Aarestrup *et al.*, 2000; Chin *et al.*, 2005).

The use of antimicrobials, including therapeutically in human and veterinary medicine, or as prophylaxis of growth promotion in animal husbandry, ultimately exerts selective pressure favorable for the propagation of antimicrobial resistant bacteria (Han *et al.*, 2003). Resistant bacteria from the intestines of animals may be transferred

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to meat products resulting from fecal contamination during various stages of the slaughter process and subsequent handling of animal tissue.

The aim of this study was to investigate the resistance for fluoroquinolone of *E. coli* isolated from chicken fecal samples and to analyze the characterization of mutations in its *gyrA* and *parC* gene related resistance.

Materials and Methods

Escherichia coli strains

One hundred and twenty-eight *E. coli* isolates were obtained from normal chicken feces from six broiler and four breeder farms in Korea, from March to November 2003. For the isolation of *E. coli*, 100 fresh fecal samples from healthy chickens at 10 different farms (10 samples/farm) were collected. Fecal samples were placed in sterile plastic specimen tubes on ice and transported to our laboratory for bacterial isolation within 2 days. Feces were plated on EMB agar (Difco, USA) and incubated at 37°C for 24 h. Blue-black colonies with dark centers and greenish metallic sheen were selected randomly. All of the isolates were identified by biochemical test (gram stain, oxidase, TSI, indole, citrate, methyl red, and urea agar) and stored at 4°C in MacConkey agar until use. Only two colonies per colonial morphology were selected for analysis. If the two colonies had the same resistant phenotype, only one of the colonies was used for further analysis.

Fluoroquinolone susceptibility testing

Fluoroquinolone susceptibility was measured in 128 *E. coli* isolates by the disk diffusion method and minimal inhibitory concentrations (MIC) were determined by the agar dilution method. The disk diffusion method was evaluated with disks containing ENO (5 μg), CIP (5 μg), NOR (10 μg) (Sensi-DiskTM, BBL, USA). Mueller Hinton agar (MHA; Biolife, Italy) was added to the plastic culture plates to yield a uniform depth of 4 mm. The density of all isolates tested was adjusted to 0.5 McFarland turbidity (12×10^8 CFU/ml) using a spectrophotometer (Schimadzu, Japan), and inoculated over the agar surface by flooding. Antimicrobial disks were applied using a dispenser within 15 min. after inoculation. Inhibition zone diameters were measured after overnight incubation at 37°C. MIC for CIP, ENO and NOR were deter-mined using the E-test system (AB Biodisk, Sweden). All isolates were inoculated using a swab that had been submerged in a bacterial suspensions adjusted to a cell density of approximately 10^7 CFU/ml. The surface of the plate was swabbed in three directions to ensure a complete distribution of the inoculum over the entire plate. Within 15 min of inoculation the antimicrobial agents' strips were applied and the plates were inverted for incubation at 37°C in air for 18 h. After incubation, the plates were examined, and an elliptical zone of growth inhibition was seen around the strip (Pinheiro *et al.*, 1994). Results were interpreted according to National Commitee for Clinical Laboratory Standards (NCCLS, 2000).

Chromosomal DNA preparation

One milliliter of TSB overnight culture was centrifuged and washed once with 10 mM Tris-HCl-5 mM EDTA buffer (pH 8.0). Pellets were resuspended in 0.5 ml of washing buffer containing 0.3 mg/ml of lysozyme, and incubated at 37°C for 1 h. SDS (0.5%) and proteinase K (100 μg/ml) were added and incubated at 55°C until the solutions became clear. DNA was purified twice by extraction with phenol-chlorform-isoamylalcohol (25:24:1) and precipitated with ethanol. DNA was resuspended in 10 mM Tris-HCl-1 mM EDTA buffer (pH 8.0) and stored at 4°C until use (Lagatolla *et al.*, 1996).

PCR amplification and sequencing of gyrA and parC

GyrA amplification was performed using the following primers: 5'-ACGTACTAGGCAATGACTGG-3' and 5'-AGAAGTCGCCGTCGATAGAAC-3' (Everett *et al.*, 1996). PCR amplification was performed in a final volume of 50 μl containing each primer at a concentration of 0.2 μM, 1 × PCR buffer, 200 μM (each) deoxynucleotide phosphate, 3 mM $MgCl_2$, 2.5 U of *Taq* polymerase (Promega, USA), and approximately 100 ng of chromosomal DNA. PCR reactions performed using a GeneAmp 9700 PCR system (Perkin-Elmer, Applied Biosystems Division, USA) with the following method: 94°C for 5 min, 30 cycles at 94°C for 1 min, 55°C for 1 min. and 72°C for 1 min, and 72°C for 5 min. Amplification of the analogous region from *parC* was achieved by the method described above, using the following primers: 5'-TGTATGCGAT-GTCTGAACTG-3' and 5'-CTCAATAGCAGC-TCG-GAATA-3' (Everett *et al.*, 1996). All PCR products were sequenced at Bioneer Co. (Korea).

Results

The resistance of 128 *E. coli* isolates to three fluoroquinolones was determined (Table 1). The isolates showed high resistance to CIP (60.2%), ENO (73.4%) and NOR (60.2%), and some isolates also showed intermediate resistance to these drugs (7.8-17.2%).

For evidence of amino acid change in *gyrA* and *parC* associated fluoroquinolone resistance, the genes were amplified from chromosomal DNA of all isolates by PCR, and were verified by DNA sequencing. Missense

Table 1. Fluoroquinolone resistance frequency of 128 *E. coli* isolates

Type	No. (%) of resistant isolates		
	Ciprofloxacin	Enrofloxacin	Norfloxacin
Resistance	77 (60.2)	94 (73.4)	77 (60.2)
Intermediate	22 (17.2)	17 (13.3)	10 (7.8)

Table 2. Frequency of mutations and amino acid change within *gyrA* of 128 *E. coli* isolates

Substitution in amino acid position		No. (%) of isolates
83 (Ser)	87 (Asp)	
wt[a]	wt	30 (23.4)
Leu	wt	21 (16.4)
Leu	Asn	64 (50.0)
Leu	Ala	1 (0.8)
Leu	Gly	4 (3.1)
Leu	His	2 (1.6)
Leu	Tyr	6 (4.7)

[a]wild type

Table 3. Frequency of mutations and amino acid change within *parC* of 128 *E. coli* isolates

Substitution in amino acid position		No. of isolates (%)
80 (Ser)	84 (Glu)	
wt[a]	wt	37 (28.9)
Arg	wt	13 (10.2)
Ile	wt	67 (52.3)
Phe	wt	2 (1.6)
wt	Lys	2 (1.6)
Arg	Ile	1 (0.8)
Arg	Lys	3 (2.3)
Arg	Tyr	1 (0.8)
Ile	Lys	1 (0.8)
Gly	Tyr	1 (0.8)

[a]wild type

mutation in *gyrA* was only found in the amino acid codon of Ser-83 and Asp-87 (Table 2). All isolates possessed mutations at codon 83, displaying a substitution of Leu for Ser. Many of the isolates (77 of 128) also possessed mutations at codon 87, displaying substitutions of Asn (n = 64), Ala (n = 1), Gly (n = 4), His (n = 2) and Tyr (n = 6) for Asp. Interestingly, a high percentage of isolates (60.2%) showed mutations at both Ser-83 and Asp-87.

Missense mutation in *parC* was found in the amino acid codon of Ser-80 and Glu-84 (Table 3). Many of the isolates (89 of 128) possessed mutations at codon 80, which is analogous to codon 83 in *gyrA*, displaying substitution of Ile (n = 68), Arg (n = 18), Phe (n = 2) and Gly (n = 1) for Ser. Nine isolates showed a change at codon 84 (analogous to Asp-87 in *gyrA*), resulting in substitutions of Lys (n = 6), Ile (n = 1) or Tyr (n = 2) for Glu. Seven isolates showed mutations at both Ser-80 and Glu-84.

The distribution of MIC for each fluoroquinolone by amino acid mutation in *gyr*A and *par*C are presented in Table 4 to 6. The MIC breakpoint value of resistance to CIP, ENO and NOR for *E. coli* was defined in this article as > 3 μg/ml, 2 μg/ml and 16 μg/ml, in accordance with NCCLS, respectively. Seventy-two isolates showed a MIC greater than the breakpoint value for CIP, and 71 of 72 isolates possessed mutations at both Ser-83 and Asp-87 in *gyrA*. Ninety-four isolates and 55 isolates showed a MIC greater than the breakpoint value for ENO and NOR, respectively. The MIC of ENO was not associated with any amino acid changes in the sequenced area, while 77 isolates with amino acid muatations at both Ser-83 and Asp-87 in *gyrA* showed a MIC greater than 3 μg/ml. All isolates showing a MIC greater than the breakpoint value for NOR also possessed two mutations in *gyrA*. Isolates with a single mutation in *gyrA* showed a MIC breakpoint value for CIP (0.5 to 0.75 μg/ml), ENO (1 to 4 μg/ml) and NOR (0.75 to 4 μg/ml). These MIC were lower compared to isolates with two mutations, one in *gyrA* and a second in *parC*, and three mutations, one in *gyrA* and two in *parC* (CIP, 0.5 to 3 μg/ml; ENO, 2 to 32< μg/ml; NOR, 1.5 to 6 μg/ml). However, isolates with two mutations in *gyrA* regardless of whether there was a mutation in *parC* showed a high MIC for the three fluoroquinolones (CIP, 0.75 to 32 μg/ml; ENO, 3 to 32 μg/ml; NOR, 3 to 32 μg/ml).

Discussion

According to the currently accepted alternating-target model, high-level fluoroquinolone resistance in *E. coli* develops by stepwise acquisition of target mutations (Khodursky *et al.*, 1998; Heisig, 1996; Kumagai *et al.*, 1996). This is essentially mediated by the inhibition of two closely related type II topoisomerases, DNA gyrase composed of the *gyrA* and *gyrB* subunits, and DNA topoisomerase IV composed of *parC* and *parE* subunits (Blanche *et al.*, 1996; Heisig, 1996).

In the present study, 128 *E. coli* isolates from normal chicken feces were analyzed in their mutations of *gyrA* and *parC*. We found that normal fecal *E. coli* showed high resistance to fluoroquinolones and 98 isolates (76.6%) carried a mutation in *gyrA*, resulting in a substitution of Leu for Ser at codon 83. This evidence seems to suggest the first step in the acquisition of fluoroquinolone resistance. Furthermore, we also found that isolates having mutations, in both Ser-83 and Asp-87 of *gyrA*, was associated with higher levels of resistance to fluoroquinolone.

DNA gyrase has been established as the primary target of the quinolones in most gram-negative bacteria. Conversely, DNA topoisomerase IV appears to be the primary target of the fluoroquinolone in gram-positive bacteria, such as *Staphylococcus aureus*, *Streptococcus pneumoniae* and *enterococci* (Ferrero *et al.*, 1994; Blanche *et al.*, 1996; Aarestrup *et al.*, 2000). In the present study, no single *parC* mutation was found without the concomitant presence of a mutation in the *gyrA* gene, suggesting that DNA topoisomerase IV could be a secondary target for quinolones. In addition, the present work has shown that, with some exceptions, a single mutation in Ser-83 of *gyrA*

Table 4. Amino acid change in *gyrA* and *parC* genes of *E. coli* isolates and corresponding MIC of ciprofloxacin

Substitution in		No.	No. of *E. coli* isolates corresponding MIC (μg/ml)[b]												
*gyr*A	*par*C		≤0.5	0.75	1	1.5	2	3	4	6	8	12	16	24	32≤
wt[a]	wt	30	29	1											
S83L	wt	5	4	1											
	S80R	9	2	2	3		1	1							
	S80I	5	3	1	1										
	S80R E84I	1	1												
	E80G E84Y	1	1												
S83L D87N	wt	2													2
	S80I	49					1	1	8	9	14	6	7		3
	S80R	4			1		1		1	1					
	S80F	2							1						1
	E84K	2							1		1				
S83L D87A	S80I	1								1					
S83L D87G	S80I	4		1				1	1	1					
S83L D87H	S80I	2							1	1					
S83L D87Y	S80I	6					2		2		1	1			
S83L D87N	S80R E84K	3							1	1	1				
	S80R E84Y	1										1			
	S80I E84K	1									1				

[a]wild type
[b]The MIC (μg/ml) according to resistance-criteria of NCCLS were shown in shaded area.

is associated with a very lower level of resistance to fluoroquinolones (0.5 - 4 μg/ml), and two mutations at Ser-83 of *gyrA* and Ser-80 of *parC* (0.5 - 3 μg/ml), were associated with a moderate level of resistance. However, mutations at both Ser-83 and Asp-87 of *gyrA* are associated with the highest level of resistance regardless of whether there was a mutation in *parC*.

Mutations in Ser-83 and Asp-87 of *gyrA* are known from previous studies on fluoroquinolone resistance in *E. coli* (Yoshida *et al.*, 1990; Oram and Fisher, 1991; Heisig *et al.*, 1993; Quabdesselam *et al.*, 1995). Kumagai *et al.* (1996) indicated that topoisomerase IV is the primary target of quinolones in *E. coli* with the *gyrA* background. Also, Soussy *et al.* (1993) showed that quinolone-resistant *E. coli* carrying a mutation in the region of *parC* and *parE* required an additional mutation in *gyrA* to express quinolone resistance. On the other hand, some researchers showed that topoisomerase IV is not sensitive to quinolones as much as DNA gyrase (Kato *et al.*, 1992; Peng and Marians, 1993; Hoshino *et al.*, 1994). The results of this study are consistent with those of Kumagai *et al.* (1996), who reported that although the *parC* mutations are shown to confer resistance to quinolones, mutation in topoisomerase IV may be no more than one of the causes for a high level of resistance and there may be other mechanisms of resistance, for example, alteration of the permeability of quinolones across the cell membrane. However, since at least a part of the high level of resistance of some clinical isolates is caused by additional mutations in topoisomerase IV, quinolones, which have inhibitory potency against mutant topoisomerase IV, could be very useful to destroy quinolone-resistant pathogenic

Table 5. Amino acid change in *gyrA* and *parC* genes of *E. coli* isolates and corresponding MIC of enrofloxacin

Substitution in		No.	No. of *E. coli* isolates corresponding MIC (μg/ml)[b]												
gyrA	parC		≤0.5	0.75	1	1.5	2	3	4	6	8	12	16	24	32≤
wt[a]	wt	30	18	5	4	1	2								
S83L	wt	5			4				1						
	S80R	9					3	1	3			1			1
	S80I	5					1	3	1						
	S80R E84I	1					1								
	E80G E84Y	1						1							
S83L D87N	wt	2													2
	S80I	49										1	1	3	44
	S80R	4						1				1			2
	S80F	2													2
	E84K	2													2
S83L D87A	S80I	1													1
S83L D87G	S80I	4						1					1		2
S83L D87H	S80I	2								1	1				
S83L D87Y	S80I	6										1	1		4
S83L D87N	S80R E84K	3													3
	S80R E84Y	1													1
	S80I E84K	1													1

[a]wild type
[b]The MIC (μg/ml) according to resistance-criteria of NCCLS were shown in shaded area.

Table 6. Amino acid change in *gyrA* and *parC genes* of *E. coli* isolates and corresponding MIC of norfloxacin

Substitution in		No.	No. of *E. coli* isolates for which the MIC (μg/ml)[b] was:												
gyrA	parC		≤0.5	0.75	1	1.5	2	3	4	6	8	12	16	24	32≤
wt[a]	wt	30	17	8	1	3		1							
S83L	wt	5		1	1	1	1		1						
	S80R	9				1	2	3	3						
	S80I	5					1	3		1					
	S80R E84I	1						1							
	E80G E84Y	1						1							
S83L D87N	wt	2										1		1	
	S80I	49							1		2	6	18	9	13
	S80R	4						1	1	1		1			
	S80F	2										1			1
	E84K	2											2		

[a]wild type

Table 6. Continued.

Substitution in		No.	No. of *E. coli* isolates corresponding MIC (μg/ml)[b]												
gyrA	parC		≤0.5	0.75	1	1.5	2	3	4	6	8	12	16	24	32≤
wt[a]	wt	30	17	8	1	3		1							
S83L D87A	S80I	1											1		
S83L D87G	S80I	4						1				1	2		
S83L D87H	S80I	2									1		1		
S83L D87Y	S80I	6*								1		3	2		
S83L D87N	S80R E84K	3											3		
	S80R E84Y	1													1
	S80I E84K	1												1	

[a]wild type
[b]The MIC (μg/ml) according to resistance-criteria of NCCLS were shown in shaded area.

bacteria.

Lee *et al.* (2003) reported that the ENO sensitivity of *Salmonella gallinarum* isolates from chicken in Korea reduced significantly from 100% in 1995 to 6.5% in 2001. In addition, Lee *et al.* (2004) showed the incidence of Ser-83 or Asp-87 mutation of *S. gallinarum* rapidly increased from 5.6% in 1995 to 89.1% in 2001. The prevalence of resistant organisms in Korea probably reflects a lack of proper antibiotic policy resulting in prolonged and indiscriminate use of antimicrobial agents. Therefore, routinely screening for high or low-level fluoroquinolone-resistant isolates should allow for the early adjustment of antibacterial chemotherapy.

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