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Molecular Characterization of Fluoroquinolone Resistant *Escherichia coli* Isolates from Chickens in Korea

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닭에서 동정된 플르오르퀴놀론 내성 대장균 균주의 분자생물학적 성상에 관한 연구

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Abstract An aim of current study was to investigate the prevalence and the mechanism of quinolone-resistance in *E. coli* isolates obtained from chicken cecum in Korea. In addition, multilocus sequence typing (MLST) was also performed for the molecular characterization of *E. coli* isolates. In an antimicrobial susceptibility test by the disk diffusion method, the 63.5% (54/85) of *E. coli* isolates showed the resistance to quinolone group of antimicrobial agents. All of the 54 *E. coli* isolates showing resistant to quinolone group had sense mutations in *gyrA* gene and point mutations at the 57th, 80th, or 84th residues in *parC* gene were detected in 90.7% of the isolates. Interestingly, *E. coli* ST was closely related to amino acid substitutions in *parE* gene. Our results indicated that the long-term use of antimicrobial agents in food-producing animals was strongly associated with a prevalence of antimicrobial resistance in commensal *Enterobacteriaceae*, suggesting the need for continuous surveillance and monitoring of antimicrobial resistant determinants in bacterial isolates from food animals.

Key Words : Antimicrobial resistance, Chicken, *E. coli*, Food animals, Multilocus sequence typing, Quinolones

요 약 본 연구에서는 한국의 닭에서 분리된 *E. coli* 균주들로부터 퀴놀론계 항생제 내성을 나타내는 균주를 분리·동정하고 그 내성 기전과 유병률에 관하여 조사하였다. 또한 multilocus sequence typing (MLST)을 이용하여 *E. coli* 균주들의 분자생물학적 성상을 분석하였다. 항생제 감수성 테스트에서 63.5% (54/85) 의 *E. coli* 균주들에서 퀴놀론계 항생제 내성률을 보였다. 또한 퀴놀론계 항생제 내성을 보이는 54개 모두에서 *gyrA* 유전자의 sense mutations과 *parC* 유전자의 57th, 80th, or 84th residues에서 점돌연변이를 관찰할 수 있었다. MLST를 통한 분석에서 *E. coli* ST는 *parE* 유전자의 염기치환과 깊은 상관관계를 보이는 것으로 관찰되었다. 이 결과들을 바탕으로 우리가 먹는 가축 및 가금류에 대한 무분별한 항생제 사용은 항생제 내성균의 증가와 유전변이를 초래함을 알 수 있었다. 따라서 식용 동물에 대한 지속적인 감시와 모니터링을 통하여 항생제 내성균의 확산방지를 통제하는 것이 필요할 것으로 사료된다.

주제어 : 가금류, 닭, 대장균, 항생제 내성, 퀴놀론, Multilocus sequence typing

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1. Introduction

Some antimicrobial agents included in quinolones are broad-spectrum antimicrobial agents used extensively in the treatment of various community-acquired and nosocomial infections [6]. Especially, fluoroquinolone resistance bacteria belonged to Enterobacteriaceae have significantly increased worldwide in the past decades [11]. An increment of resistance to fluoroquinolones often causes limited therapeutic options and treatment failures. Among enterobacterial isolates causing community- and hospital-acquired infections, *E. coli* isolates have been the most commonly identified ones from humans and animal intestines and they showed high resistance rate to various antimicrobial agents [7]. In *E. coli*, point mutations in the quinolone resistance-determining regions (QRDRs) are the best-described mechanism of resistance to fluoroquinolones. QRDRs are located on the bacterial chromosome containing DNA topoisomerase II (DNA gyrase) genes (*gyrA* and *gyrB*) and DNA topoisomerase IV (*parC* and *parE*). In addition, in the plasmid-mediated quinolone resistance (PMQR) determinants, five major families of *qnr* genes (*qnrA*, *qnrB*, *qnrC*, *qnrD*, and *qnrS*) and their allelic variants have been identified in *E. coli* isolates from humans [10, 14].

The widespread and long-term use of fluoroquinolones is strongly associated with the resistance development in commensal *E. coli* isolates from humans and animals. The antimicrobial resistant bacterial strains act as potential reservoirs of antimicrobial resistance determinants and play an important role in transferring their determinants among bacterial populations. Especially, the indiscriminating use of antimicrobial agents in food animals for treatments or additives has increased for intestinal bacteria resistant to antimicrobial agent in human. Consequently, the animal intestinal bacteria including *E. coli* may be transferred to humans by food chains, disseminating antimicrobial resistant genes to intestinal bacterial flora in human [8, 16].

However, there are limited studies describing the resistance mechanisms to fluoroquinolones, which include PMQR determinants and mutations in *gyrA*, *parC* and *parE* genes in *E. coli* isolates from food animals. Therefore, we aimed to investigate the prevalence and the mechanisms of resistance to fluoroquinolones in *E. coli* isolates from chickens in the present study. In addition, we analyzed multilocus sequence typing (MLST) for genotyping of *E. coli* isolates and the relationship between sequence types (STs) and fluoroquinolone-resistance determinants of the isolates was also evaluated.

2. Materials and Methods

2.1 Bacterial isolates and antimicrobial susceptibility test

A total of 85 *Escherichia coli* isolates was recovered from ceca of chickens that were grown in the Chungbuk and Gyeongbuk provinces from July to December in 2013. The chicken ceca were obtained from their respective slaughter houses. *E. coli* isolates were identified by using the Vitek 2® automated instrument ID system (bioMérieux, Marcyl'Etoile, France) and conventional methods.

All *E. coli* isolates were subjected to a susceptibility test against 11 antimicrobial agents on Müller-Hinton agar (Difco Laboratories, Detroit, MI, USA) with the Kirby-Bauer disk diffusion method. The following antimicrobial disk (BBL, Cockeysville, MI., USA) were used: amikacin (30 μg), ampicillin (10 μg), cefotaxime (30 μg), ceftazidime (30 μg), cephalothin (30 μg), chloramphenicol (30 μg), ciprofloxacin (5 μg), gentamicin (10 μg), kanamycin (30 μg), nalidixic acid (30 μg), and levofloxacin (5 μg). According to the CLSI M100-S21 guidelines, inhibition zones of all antimicrobial disks were measured and evaluated as susceptible, intermediate or resistant [3]. *E. coli* strain ATCC 25922 was used as a reference strain.

2.2 Characterization of fluoroquinolone resistance determinants

All of *E. coli* isolates showing resistant to quinolone group of antimicrobial agents were subjected to PCR and sequencing assays using specific primers for the detection of mutations associated with QRDR 〈Table 1〉 [1]. Whole-cell (genomic) DNA was obtained from each target strain using a genomic DNA purification kit (SolGent, Daejeon, Korea) according to the manufacturer's instructions. PCR was performed using 50 ng of genomic DNA, 2.5 μL of 10× Taq buffer, 0.5 μL of 10 mM dNTP mix, 20 pmol of each primer, and 0.7 U of Taq DNA polymerase (SolGent) in a total volume of 25 μL. Each target gene was amplified in a GeneAmp PCR System 9600 thermal cycler (Perkin-Elmer Cetus Corp., Norwalk, CT, USA). Thermal cycling conditions consisted of an initial denaturation cycle at 95°C for 5 min, followed by 30 cycles of 95°C for 30 sec, 56°C for 40 sec, and 72°C for 30 sec, with a final extension at 72°C for 5 min. The annealing temperature was 52°C, unless otherwise specified. The amplified products were separated via electrophoresis on 1.5% (w/v) agarose gels containing ethidium bromide, and visualized using a BioDoc-14TM Imaging system (UVP, Cambridge, UK). For sequencing, PCR products were purified with a PCR purification kit (SolGent) according to the manufacturer's protocols.

In addition, multiplex PCR assays using specific primer sets were performed as previously described [2] to identify PMQR genes in the *E. coli* isolates 〈Table 1〉.

〈Table 1〉 Oligonucleotide used in this study

Primer	Sequence (5′-3′)	Gene	Reference
QRDR PCR detection			
gyrA-F	TCTGGATTATGCGATGTCGGTCAT	*gyrA*	[1]
gyrA-R	TCAGCCCTTCAATGCTGATGTCT		
gyrB-F	GCTGAGCGAATACCTGCTGG	*gyrB*	[1]
gyrB-R	TCGGTCATGATGATGATGCTGTGAT		
parC-F	ACTACTCCATGTACGTCATCATGGAC	*parC*	[1]
parC-R	CGCCACTTCGCGCAGGTTAT		
parE-F	GCGGAAGATATCTGGGATCGCT	*parE*	[1]
parE-R	CTGGCTCAGATCGTCGCTGT		
Qnr multiplex PCR detection			
QnrA-F	AGAGGATTTCTCACGCCAGG	*qnrA*	[2]
QnrA-R	TGCCAGGCACAGATCTTGAC		
QnrB-F	GGMATHGAAATTCGCCACTG	*qnrB*	[2]
QnrB-R	TTTGCYGYYCGCCAGTCGAA		
QnrS-F	GCAAGTTCATTGAACAGGGT	*qnrS*	[2]
QnrS-R	TCTAAACCGTCGAGTTCGGCG		
E. coli MLST			
adk-F	ATTCTGCTTGGCGCTCCGGG	*adk*	[17]
adk-R	CCGTCAACTTTCGCGTATTT		
fumC-F	TCACAGGTCGCCAGCGCTTC	*fumC*	[17]
fumC-R	GTACGCAGCGAAAAAGATTC		
gyrB-F	TCGGCGACACGGATGACGGC	*gyrB*	[17]
gyrB-R	ATCAGGCCTTCACGCGCATC		
icd-F	ATGGAAAGTAAAGTAGTTGTTCCGGCACA	*icd*	[17]
icd-R	GGACGCAGCAGGATCTGTT		
mdh-F	CCAGGCGCTTGCACTACTGTTAA	*mdh*	[17]
mdh-R	GCGATATCTTTCTTCAGCGTATC		
purA-F	CGCGCTGATGAAAGAGATGA	*purA*	[17]
purA-R	CATACGGTAAGCCACGCAGA		
recA-F	CGGCAAACTCAACGTTCC	*recA*	[17]
recA-R	CTGACGCTGCAGGTGAT		

F, sense primer; R, antisense primer

2.3 Characterization using MLST

MLST analysis on the basis of sequence variation was performed for the characterization of *E. coli* isolates. MLST scheme [17], which uses 7 housekeeping genes (*adk*, *fumC*, *gyrB*, *icd*, *mdh*, *purA*, and *recA*) was used to determine STs. The reaction conditions of PCR amplification and sequencing of the 7 house keeping genes using specific primers were the same as that of fluoroquinolone - resistance determinant characterization, except annealing temperature, 58℃ 〈 Table 1〉 . A ST number was assigned by comparing the allele sequences to those on the MLSTsite (http://mlst.warwick.ac.uk/mlst/dbs/Ecoli/).

3. Results

3.1 Prevalence of fluoroquinolone resistant *E. coli* isolates from chicken cecum

In total, 85 *E. coli* strains were isolated from ceca of breeding chickens in the Chungbuk and Gyeongbuk provinces. In antimicrobial susceptibility tests, 83 *E. coli i*solates displayed the resistance to nalidixic acid in quinolone group (97.6%) 〈Table 2〉 . In addition, the 63.5% (54/85) of *E. coli* isolates showed the resistance to ciprofloxacin and other quinolone group of antimicrobial agents, levofloxacin, and nalidixic acid as well. Especially, *E. coli* isolates resistant to all three quinolone group were detected in the 39 isolates from the Chungbuk and 15 isolates from the Gyeongbuk province, respectively.

3.2 Point mutations in topoisomerase genes

Based on PCR and sequencing analyses, all of the 54 *E. coli* isolates showing resistant to ciprofloxacin, levofloxacin, and nalidixic acid had sense mutations in gyrA gene. Especially, sense mutations at the 83rd residue (serine to leucine) and at the 87th residue (aspartic acid to asparagine) were most prevalent 〈 Table 3〉 . Point mutations at the 57th, 80th, or 84th residues in parC gene were detected in 49 (90.7%) of the isolates. In 8 isolates, amino acid substitutions at 458^{th} residue (serine to alanine or proline) or 416^{th} residue (leucine to phenylalanine) in *parE* gene were identified. Most isolates (49 isolates, 90.7%) contained either three or four mutations in *gyrA* and *parC* and/or *parE* genes and there were few isolates had a single mutation or double mutations in the *gyrA* gene. In addition, 2 isolates carried *qnrS* gene as well as mutations in *gyrA* and *parC* gene. None of the isolates in our study, however, revealed any mutations in *gyrB* gene or harbored PMQR genes, *qnrA* and *qnrB*.

〈Table 2〉 Antimicrobial susceptibilities of 85 *E.coli* isolats.

Group of antimicrobial agent	Antimicrobial agent	Numbers (%) of susceptible isolates		Numbers (%) of intermediate isolates		Numbers (%) of resistant isolates	
Aminoglycoside	Amikacin	24	(28.2)	35	(41.2)	26	(30.6)
	Gentamicin	42	(49.4)	31	(36.5)	12	(14.1)
	Kanamycin	4	(4.7)	12	(14.1)	69	(81.2)
β-lactam	Ampicillin	13	(15.3)	10	(11.8)	62	(72.9)
	Cefotaxime	80	(94.1)	0	(0)	5	(5.9)
	Ceftazidime	77	(90.6)	7	(8.2)	1	(1.2)
	Cephalothin	9	(10.6)	14	(16.5)	62	(72.9)
Chloramphenicol	Chloramphenicol	32	(37.6)	2	(2.4)	51	(60.0)
Quinolone	Ciprofloxacin	12	(14.1)	19	(22.4)	54	(63.5)
	Levofloxacin	21	(24.7)	7	(8.2)	57	(67.1)
	Nalidixic acid	2	(2.4)	0	(0)	83	(97.6)

〈Table 3〉 Relationship between STs and qunolone resistance-determining region sequence of *E.coli* isolates showing resistant to ciprofloxacin.

Isolate number[a]	Sequence Type	Allele number	QRDR[b] mutationsin				PMQR gene[c]
			gyrA	*gyrB*	*parC*	*parE*	
A22	10	10-11-4-8-8-8-2	S83L, D87N	-	S80I	-	
A58	10	10-11-4-8-8-8-2	S83L	-	-	-	
A37	34	10-11-4-1-8-8-2	S83L, D87N	-	S80I	-	*qnrS*
A64	34	10-11-4-1-8-8-2	S83L, D87N	-	S80I	-	
B35	38	4-26-2-25-5-5-19	S83L, D87N	-	S80I	-	
A16	57	6-31-5-28-1-1-2	S83L, D87G	-		-	
A32	57	6-31-5-28-1-1-2	S83L, D87N	-	S80I	-	
B17	57	6-31-5-28-1-1-2	S83L, D87N	-	S80I	-	
B33	57	6-31-5-28-1-1-2	S83L, D87N	-	S80I	-	
B26	58	6-4-4-16-24-8-14	D87G	-	-	-	
A8	69	21-35-27-6-5-5-4	S83L, D87N	-	S80I	S458A	
A13	69	21-35-27-6-5-5-4	S83L, D87N	-	S80I	S458A	
A53	69	21-35-27-6-5-5-4	S83L, D87N	-	S80I	S458A	
B1	69	21-35-27-6-5-5-4	S83L, D87N	-	S80I	S458A	
A12	88	6-4-12-1-20-12-7	S83L, D87N	-	S80I	-	
A60	93	6-11-4-10-7-8-6	S83L, D87N	-	-	-	
A66	115	4-26-39-25-5-31-19	S83L, D87N	-	S80I	-	
A15	117	20-45-41-43-5-32-2	S83L, D87N	-	S80I	-	
A38W	117	20-45-41-43-5-32-2	S83L, D87N	-	S80I	-	
A39	117	20-45-41-43-5-32-2	S83L, D87N	-	S80I	-	
A41W	117	20-45-41-43-5-32-2	S83L, D87N	-	S80I	-	
A42W	117	20-45-41-43-5-32-2	S83L, D87N	-	S80I	-	
A57	117	20-45-41-43-5-32-2	S83L, D87N	-	S80I	-	
A33	162	9-65-5-1-9-13-6	S83L, D87N	-	S80I	-	
A26	189	10-27-5-10-12-8-49	S83L, D87N	-	S80I	-	
A31	189	10-27-5-10-12-8-49	S83L, D87N	-	S80I	-	
A34	189	10-27-5-10-12-8-49	S83L, D87N	-	S80I	-	
A67	189	10-27-5-10-12-8-49	S83L, D87N	-	S80I	-	
A17	212	6-29-4-18-11-8-6	S83L, D87N	-	E84K	-	
B13	226	10-27-5-8-8-7-2	S83L, D87N	-	S80R	S458P	
B55	226	10-27-5-8-8-7-2	S83L, D87N	-	S80R	S458P	
A56W	302	79-84-71-78-52-57-2	S83L, D87N	-	S57T, S80I	-	
B54	641	9-6-33-131-24-8-7	S83L, D87N	-	S80R	-	
B56	641	9-6-33-131-24-8-7	S83L, D87N	-	S80R	-	
B60	641	9-6-33-131-24-8-7	S83L, D87N	-	S80R	-	
B72	641	9-6-33-131-24-8-7	S83L, D87N	-	S80R	-	
A7	752	10-11-4-8-8-8-49	S83L, D87N	-	S80I	-	
A11	752	10-11-4-8-8-8-49	S83L, D87N	-	S80I	-	
A23	752	10-11-4-8-8-8-49	S83L, D87N	-	S80I	-	
A42	752	10-11-4-8-8-8-49	S83L, D87N	-	S80I	-	
A54	752	10-11-4-8-8-8-49	S83L, D87N	-	S80I	-	
A59	752	10-11-4-8-8-8-49	S83L, D87N	-	S80I	-	
A62	752	10-11-4-8-8-8-49	S83L, D87N	-	S80I	-	
A24	1011	6-4-159-44-112-1-17	S83L, D87N	-	S80R	-	
B6	1140	83-23-164-181-80-1-42	S83L, D87N	-	S80I	-	
B37	1140	83-23-164-181-80-1-42	S83L, D87N	-	S80I	L416F	
B44	2001	200-3-17-6-45-5-191	S83L	-	-	-	
A9	2309	271-26-39-25-5-31-19	S83L, D87N	-	S80I	-	
A238	2847	6-266-83-24-1-1-2	S83L, D87N	-	S80I	-	
A10	N-1[d]	10-27-5-344-12-8-49	S83L, D87Y	-	S80I	-	
A30	N-2	111-11-4-8-8-8-49	S83L, D87N	-	S80I	-	
A40W	N-3	83-23-164-181-80-5-42	S83L, D87N	-	S80I	L416F	
A63	N-4	10-27-69-10-12-8-49	S83L, D87N	-	S80I	-	*qnrS*
B23	N-5	111-27-5-10-12-8-49	S83L, D87N	-	S80I	-	

[a] *E.coli* isolats recovered from ceca of chickens grown in Chungbuk and Gyeongbuk province were defined as A and B, respectively.
[b] Point mutations in the quinolone resistance-determining regions (QRDRs)
[c] Plasmid-mediated quinolone resistance (PMQR) genes
[d] N indicates novel sequence type determined by MLST in this study

3.3 Diverse lineage of fluoroquinolone resistant isolates.

The 54 fluoroquinolone-resistant *E. coli* strains isolated from ceca of chickens were assigned to 22 STs by MLST 〈Table 3〉. In addition, 5 *E. coli* isolates were grouped into novel STs, including N-1 (10-27-5-344-12-8-49), N-2 (111-11-4-8-8-8-49), N-3 (83-23-164-181-80-5-42), N-4 (10-27-69-10-12-8-49), and N-5 (111-27-5-10-12-8-49). As shown in Table 3, ST752 was the most common ST (n = 7) followed by ST117 (n = 6). Four and two isolates corresponded to ST69 and ST226, respectively were positive for four QRDR mutations found in the *gyrA*, *parC*, and *parE* genes.

Fourteen strains isolated in the Gyeongbuk province were corresponded to seven STs (ST38, ST57, ST58, ST226, ST641, ST1140, and ST2001) and N-5. Only ST57 was detected in the Chungbuk as well as in the Gyeongbuk province.

4. Discussion

E. coli represents a part of the normal fecal flora in humans, animals and commensal microorganisms but can cause diverse intestinal and extra-intestinal infectious diseases. Especially, antimicrobial resistant *E. coli* isolates can become a possible source of dissemination of antimicrobial resistance genes among many Gram-negative bacteria [12]. *E. coli* isolates from chicken ceca in this study showed the higher incidence (63.5%) of resistance to quinolone group of antimicrobial agents. In previous studies, the high incidence of resistance for those antibiotics were observed in chicken feces and large intestine swabs (50.1%) from poultry in Korea [4, 9]. Furthermore, the resistant rate (63.5%) of *E. coli* isolates in our study, was considerably higher against those from healthy human stool (1.2%) or clinical specimens (32.8%) [9].

High level of resistance to ciprofloxacin requires *gyrA* gene substitutions which include replacements of serine to leucine at 83 position and aspartic acid to asparagine at 87 position, and at least one mutation either at 80 or 84 position in *parC* gene as well [5]. In our study, 49 (90.7%) of 54 isolates had the three mutations which can induce high level of resistance towards ciprofloxacin. Similar to previous studies, there were no fluoroquinolone-resistant isolates with a single mutation in *parC* gene without double mutation at 83 and 87 positions in *gyrA* gene 〈Table 3〉 [13]. In contrast to previous reports, however, only few *E. coli* isolates (3.7%) were identified to carry *qnrS* of PMQR genes [18, 15].

Except ST57 grouping 4 isolates, there were distinct differences of STs confirmed between the two areas in this study. ST752 was most prevalent in the Chungbuk province, whereas ST641 was the one in the Gyeongbuk province. It is interesting that all of 4 isolates carried substitution at 458^{th} residue (serine to alanine) in *parE* gene corresponded to ST69. Previously, it has been reported that *E. coli* ST648 strains isolated from clinical specimens also carried the same substitution at 458^{th} residue [11]. In addition, ST226 isolates contained mutation at the 458^{th} residue (serine to proline) in *parE* gene. Therefore, it seems that *E. coli* ST might be closely associated with amino acid substitutions in *parE* gene. This suggestion was agreed with a previous study that the substitution at 529^{th} residue (isoleucine to leucine) in *parE* gene was unequivocally related to *E. coli* ST131 isolated from clinical specimens [11].

A major mechanism of fluoroquinolone-resistance in *E. coli* isolates bacteria is known to carry amino acid substitutions in DNA topoisomerase polypeptides. In our study, we confirmed that sense mutations in *gyrA*, *parC*, and *parE* genes for high level of resistance to ciprofloxacin were widely distributed in *E. coli* isolates from chickens in Korea. Therefore, higher incidence of resistance to quinolone was attributed by QRDR mutations on the bacterial chromosome in poultry in

Korea [19, 20]. Moreover, among PMQR determinants, *qnrS* gene was identified together with QRDR mutations in few *E. coli* isolates and it was detected from the Chungbuk province only.

Isolation of bacterial PMQR genes from food animals is very important because the genes could be transmitted to human intestinal flora. Accordingly, our results suggest that continuous surveillance and monitoring will be essential to prevent dissemination of antimicrobial resistant determinants in bacterial isolates from food animals to human and other animal fecal flora.

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