**NOTE** *Bacteriology*

# Prevalence and Molecular Characterization of Fluoroquinolone-Resistant *Escherichia coli* Isolated from Diarrheic Cattle in Korea

Suk-Kyung LIM[1)], Keum-Gi LIM[2)], Hee-Soo LEE[1)], Suk-Chan JUNG[1)], Mun-Il KANG[2)] and Hyang-Mi NAM[1)]*

[1)]*National Veterinary Research and Quarantine Service, Ministry of Agriculture and Forestry, 480 Anyang, Kyonggido and*
[2)]*College of Veterinary Medicine, Chonnam National University, College of Veterinary Medicine, Chonnam National University, Gwangju 500–757, Republic of Korea*

(Received 19 August 2008/Accepted 28 November 2009/Published online in J-STAGE 15 December 2009)

ABSTRACT. A total of 176 *E. coli* isolates were retrieved from 203 diarrheic fecal samples collected from Korean cattle on 117 different farms. The most frequently observed resistance in *E. coli* isolates was to tetracycline (88.6%), followed by streptomycin (80.7%) and ampicillin (64.8%). Resistance to cefazolin, cefoperazone, cefepime and amikacin was very low. Of the 176 *E. coli* strains, forty (22.7%) isolates from 30 farms showed resistance to fluoroquinolones (FQ). All the FQ-resistant strains possessed double mutations at codons 83 and 87 in the *gyrA* gene, and a single mutation mostly at codon 80 in the *parC* gene, except in one isolate. The pulsed-field gel electrophoresis profiles of the FQ-resistant *E. coli* isolates were heterogeneous, but two or three isolates that showed an identical pattern originated from the same or different farms. This study demonstrates that FQ resistance is frequently observed in *E. coli* from diarrheic cattle and that mutations in the quinolone resistance-determining region are the same as those seen in *E. coli* originating from other animal species and humans. The FQ resistance in diarrheic cattle might have been mostly acquired independently, although the possibility of transmission of FQ-resistant *E. coli* within a farm or between farms is plausible.
KEY WORDS: cattle, diarrhea, *E. coli*, fluoroquinolones, resistance.

*J. Vet. Med. Sci.* 72(5): 611–614, 2010

Certain strains of *Escherichia coli* (*E. coli*) can cause infections of intestinal tracts including diarrheal diseases in cattle [22]; fluoroquinolones (FQ) are very effective against most Gram-negative bacteria and are often the choice for treatment of the disease [13]. Increased use of FQ, however, has led to rising rates of resistance to these antimicrobials in *E. coli* worldwide [20]. FQ resistance involves three main mechanisms: target mutations, reduced antibiotic intracellular accumulation by lowering outer membrane permeability or increasing efflux activity and target protection mediated by the Qnr protein [8]. Among them, clinical resistance to quinolones in *E. coli* is mostly associated with mutations in genes that encode subunits of the quinolone target DNA gyrase and topoisomerase IV [15, 21].

Korea is known to be one of the hot spots of antimicrobial resistance [10]. High frequencies of antimicrobial resistance in livestock, especially in poultry and swine, have also been reported in Korea [12]. Since there is no veterinary prescription regulation, indiscriminate and indiscreet use of antimicrobials has been a common practice in this country. In particular, FQ, a broad spectrum antimicrobial agent effective against most bacteria, has been widely used in the treatment of diseased cattle in Korea. While there has been a number of studies on the mechanisms of FQ resistance in *E. coli* that have originated from human clinics in Korea [3, 9], no study has been conducted to elucidate FQ resistance mechanisms in *E. coli* isolates from animals, except chickens, in Korea [11]. The aim of the present study was therefore to determine the prevalence and mechanisms of FQ resistance in *E. coli* isolates from diarrheic beef cattle in Korea. To identify clonal spread of resistance among cattle farms, most of which were located in close geographic proximity, genetic relatedness among the isolates was also determined using pulsed-field gel electrophoresis (PFGE).

From April 2003 to October 2004, a total of 203 diarrheic fecal samples were collected from native Korean beef cattle on 117 farms. The farms were visited, and fresh fecal samples were obtained aseptically from individual cattle. *E. coli* strains were isolated on selective agar (Chromogenic E.coli/coliform agar, Eosin methylene blue agar and MacConkey agar), and one or two colonies per sample were tested for biochemical characteristics using the Vitek system (bioMerieux Vitek, Hazelwood, Mo, U.S.A.) for confirmation of *E. coli*. Susceptibilities of the *E. coli* isolates were tested against 14 different antimicrobials using the standard Kirby-Bauer disk diffusion method [1]. Inhibition zones were interpreted according to the Clinical Laboratory and Standard Institute (CLSI) guidelines [14]. The minimal inhibition concentrations (MICs) of nalidixic acid and ciprofloxacin against *E. coli* isolates were determined by the agar dilution method [14]. *E. coli* (strain) ATCC 25922 was used for quality control.

Detection of mutations in the quinolone resistance-determining region (QRDR) of the *gyrA* gene [15], as well as in the analogous region of the *parC* gene [21], in FQ-resistant *E. coli* strains was performed by polymerase chain reaction (PCR). Amplified products were purified, and both strands were automatically sequenced using the same set of primers as for the PCR. The PFGE was performed according to the CDC PulseNet standardized procedure [5] for typing *E. coli*

* CORRESPONDENCE TO: NAM, H.-M., National Veterinary Research and Quarantine Service, Ministry of Agriculture and Forestry, 480 Anyang, Kyonggido, Republic of Korea.
e-mail : namhm@nvrqs.go.kr

Table 1. Antimicrobial resistance of *E. coli* isolates from diarrheic cattle feces (n=176)

| Class and antimicrobial agents | Concentration disk ($\mu$g) | Diffusion zone breakpoint (mm) | No. of resistant isolates (%) |
|---|---|---|---|
| Penicillins | | | |
| Ampicillin | 10 | ≤13 | 114 (64.8) |
| Cephems | | | |
| Cephalothin | 30 | ≤14 | 20 (11.4) |
| Cefazolin | 30 | ≤14 | 2 (1.1) |
| Cefoperazone | 75 | ≤15 | 0 (0) |
| Cefepime | 30 | ≤14 | 0 (0) |
| Aminoglycosides | | | |
| Streptomycin | 10 | ≤11 | 142 (80.7) |
| Gentamicin | 10 | ≤12 | 29 (16.5) |
| Amikacin | 30 | ≤14 | 3 (1.7) |
| Fluoroquinolones | | | |
| Ciprofloxacin | 5 | ≤15 | 40 (22.7) |
| Enrofloxacin | 5 | ≤16 | 40 (22.7) |
| Norfloxacin | 10 | ≤12 | 40 (22.7) |
| Folate pathway inhibitors | | | |
| Trimethoprim/sulfamethoxazole | 1.25/23.75 | ≤10 | 63 (35.8) |
| Phenicols | | | |
| Chloramphenicol | 30 | ≤12 | 75 (42.6) |
| Tetracyclines | | | |
| Tetracycline | 30 | ≤14 | 156 (88.6) |

Table 2. Amino acid changes in the QRDR of GyrA and ParC proteins in 44 *E. coli* strains (40 $CIP^R$ and 4 $CIP^S$) in response to nalidixic acid and ciprofloxacin

| MICs ($\mu$g/m*l*) | | No. of isolates | Amino acid change in the QRDR[a)] | |
|---|---|---|---|---|
| NAL | CIP | | GyrA | ParC |
| <1 | <1 | 4 | Ser-83 + Asp-87 | Ser-80 + Glu-84 |
| >128 | 2 | 8 | Ser-83→Leu + Asp-87→Asn[a)] | Ser-80→Ile |
| | | 1 | Ser-83→Leu + Asp-87→Asn | Glu-84→Lys |
| >128 | 4 | 1 | Ser-83→Leu + Asp-87→His | Ser-80→Ile |
| | | 9 | Ser-83→Leu + Asp-87→Asn | Ser-80→Ile |
| | | 1 | Ser-83→Leu + Asp-87→Tyr | Ser-80→Ile |
| >128 | 8 | 1 | Ser-83→Leu + Asp-87→Asn | Ser-80→Ile |
| | | 1 | Ser-83→Leu + Asp-87→Tyr | Ser-80→Ile |
| >128 | 32 | 2 | Ser-83→Leu + Asp-87→Asn | Ser-80→Ile |
| | | 1 | Ser-83→Leu + Asp-87→Tyr | Ser-80→Ile |
| >128 | 128 | 12 | Ser-83→Leu + Asp-87→Asn | Ser-80→Ile |
| | | 2 | Ser-83→Leu + Asp-87→Tyr | Ser-80→Ile |
| | | 1 | Ser-83→Leu + Asp-87→Asn | Ser-80→Ile+Glu-84→Gly |

NAL: nalidixic acid CIP: ciprofloxacin Ser: serine Leu: leucine Asp: aspartic acid Asn: asparagine Lys: lysine His: histidine Tyr: tyrosine Ile: isoleucine Glu: glutamic acid Gly: glycine.
a) The designations indicates the change of amino acid and the position number. For example, Ser-83→Leu + Asp-87→Asn indicates the change of a serine for a leucine at position 83 and aspartic acid for a aspargine at position 87.

using a CHEF Mapper apparatus (Bio-Rad Laboratories, Hercules, CA, U.S.A.). Patterns of PFGE were analyzed by computer-assisted analysis (Applied Maths, BioNumerics), and a dendrogram was constructed using the Dice coefficients and unweighted pair group method with the arithmetic mean (UPGMA).

A total of 176 *E. coli* isolates were identified from 203 diarrheic fecal samples of cattle. The results of antimicrobial susceptibility testing of these isolates are shown in Table 1. The most frequently observed resistance was to tetracycline (TET, 88.6%), followed by resistance to streptomycin (STR, 80.7%) and ampicillin (AMP, 64.8%). Few isolates, however, were resistant to cefazolin and amikacin, and all were sensitive to cefoperazone and cefepime. A total of 40 (22%) *E. coli* isolates were resistant to FQ. All ciprofloxacin-resistant strains possessed double mutations at codons 83 and 87 in the *gyrA* gene, and a single mutation mostly at codon 80 in the *parC* gene, except in one isolate, which had a double mutation at codons 80 and 84. In every case, serine was replaced by leucine at codon 83 in the GyrA protein. However, aspartic acid at codon 87 was changed into asparagine (n=34), tyrosine (n=5) or histidine (n=1),

respectively. In the ParC protein, serine at codon 80 was replaced by isoleucine (n=39), and glutamic acid at codon 84 was replaced by lysine (n=1) and glycine (n=1), respectively (Table 2). Highly heterogeneous PFGE profiles were observed among the 40 FQ-resistant *E. coli* strains. A total of 33 subtypes were identified, and the same PFGE patterns were observed among isolates originating from the same farms (CA06 and CA07-K from Farm K; CG14 and CG05 from Farm T) and from different farms (CE15-AD and CE37-Y; CE02-K and CE09-AE; CG14-T or CG05-T and CE32-B and CD08-U; and CB20-A and CC01-AG), respectively (Fig. 1).

*E. coli* isolates from diarrheic cattle showed a high prevalence of resistance to antimicrobials commonly used in livestock, such as tetracycline (TET), streptomycin (STR), and ampicillin (AMP). The tendency of resistance in diarrheic cattle observed in this study was similar to that in healthy cattle reported from Korea [12], although the prevalence of resistance to each antimicrobial in the former was much higher than in the latter. The frequency of FQ resistance in *E. coli* in this study was also marked higher than that in a previous study on healthy cattle during the same period as this study in Korea [12]. Several investigators have also described higher rates of resistance to quinolones in *E. coli* isolates from sick animals than those from healthy animals [7, 16]. This could be the consequence of higher exposure of sick animals to these antimicrobials compared with healthy cattle. Since there is no law or regulation in Korea that requires a prescription from a veterinarian to purchase and use antimicrobials for animals, the possibility of abuse or misuse of the drugs by farmers and animal owners has existed in this country.

Our molecular study on the underlying mechanism of quinolone resistance showed that all 40 quinolone-resistant *E. coli* isolates had three mutations in the *gyrA* and *parC* genes associated with deduced amino acid substitutions, except for one case that had four. All the nucleotide substitutions observed in this study have previously been found in *E. coli* and are also associated with high-level resistance to quinolones [18, 21]. According to the previous studies [19, 21], the typical three mutations observed at codons Ser83 and Asp87 in gyrA and Ser80 in parC should have contributed to the highest level of FQ resistance in the *E. coli* isolates in this study. However, no significant difference in the pattern of amino acid changes was observed among the *E. coli* isolates showing different levels of resistance to ciprofloxacin. Sáenz *et al.* [18] suggested that three amino acid substitutions are associated with moderate or high level of ciprofloxacin resistance and that four substitutions are associated with highest ciprofloxacin MIC; however, no correlation was observed between the number of mutations and the level of resistance to ciprofloxacin in the present study. This result indicates that mutations in the DNA gyrase and topoisomerase genes might not be the only determinants for the resistance phenotype of these strains. For the strains that had three substitutions and showed the highest ciprofloxacin MIC (128 $\mu$g/m*l*) in this study, the highest level of resistance might have been attributed to additional mechanisms such as decreased permeability, efflux and target protection [18].

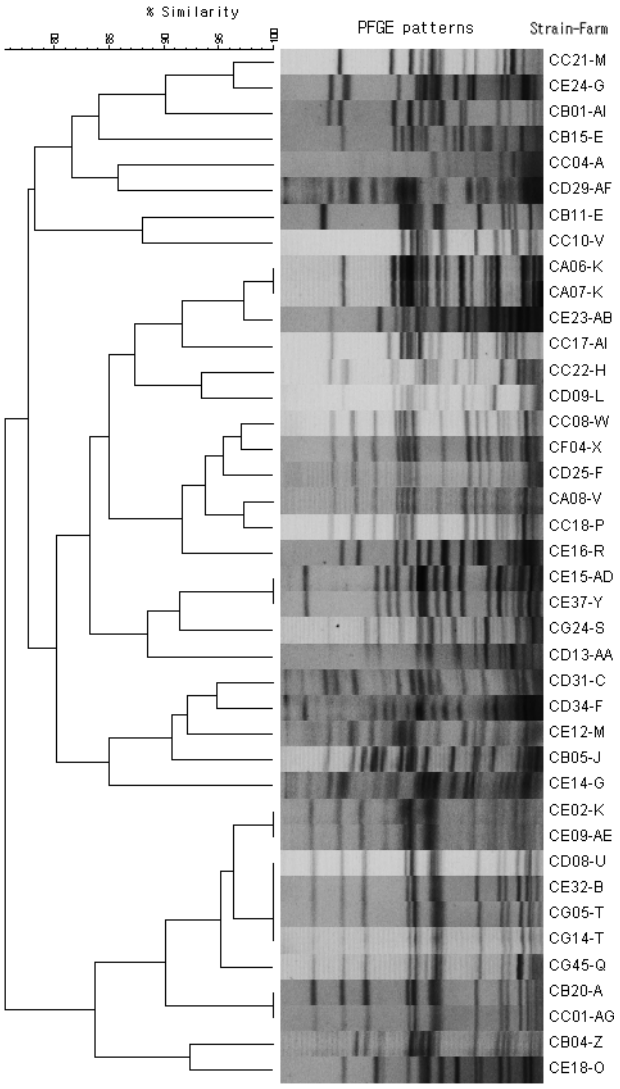


Fig. 1. PFGE patterns and dendrogram showing clustering (by UPGMA and the Dice coefficient) of 40 fluoroquinolone-resistant *E. coli* strains isolated from diarrheic cattle.

Examination of clonal relatedness using PFGE revealed marked genetic diversity, as the 40 FQ-resistant *E. coli* isolates from 30 cattle farms belonged to 33 subtypes. The high degree of genomic diversity among these isolates indicates independent emergence of resistance to FQ among the cattle. Our result is in agreement with a number of studies that have described high genetic variability among quinolone-resistant *E. coli* strains [4, 17]. However, identical PFGE patterns were observed among isolates originating both from the same farm and from different farms, suggesting the possibility of clonal spread of FQ resistant *E. coli* within a farm and between farms. Although there were no

differences in rearing practices among farms study, the sanitary conditions of farms K and T, where each of the two FQ-resistant isolates with identical PFGE patterns were obtained, were relatively poor. It is thus speculated that FQ-resistant *E. coli* contamination of the interior of the cattle house, such as floor and fence, may have infected other cattle at the farm. Meanwhile, farms AD, Y, K, AE, B, U, T, A and AG, for which FQ-resistant isolates with identical PFGE patterns were obtained, were located at distances of approximately 15–30 km, and no movement of animals or personnel (farmers) was found among the farms. There is, however, a possibility of introducing cattle from those related farms through a local livestock market, that has been commonly used by all the farms included in this study. Transmission of resistant bacteria or determinants might occur through close contact between men, animals, animal products, manure and surface water [6], and the level of antimicrobial resistance in an animal population may be related to contact intensity between the animals and a certain environment in which antimicrobials are used [2]. Thus, we cannot completely rule out the possibility that the FQ-resistant bacteria or determinants could have been transmitted by the veterinary practitioner charged with treatment of the sick cattle and with providing management advice for all the cattle farms involved in this study.

ACKNOWLEDGMENT. This study was funded by the National Veterinary Research and Quarantine Service, MAF, Republic of Korea.

REFERENCES

1. Bauer, A. W., Kirby, M. M., Sherris, J. C. and Turck, M. 1966. Antibiotic susceptibility testing by a standardized single disk method. *Am. J. Clin. Pathol.* **45**: 493–496.
2. Catry, B., Lavens, H., Devriese, L. A., Opsomer, G. and De Kruif, A. 2003. Antimicrobial resistance in livestock. *J. Vet. Pharmacol. Ther.* **26**: 81–93.
3. Cheong, H. J., Yoo, C. W., Sohn, J. W., Kim, W. J., Kim, M. J. and Park, S. C. 2001. Bacteremia due to quinolone-resistant *Escherichia coli* in a teaching hospital in South Korea. *Clin. Infect. Dis.* **33**: 48–53.
4. Eom, J.S., Hwang, B.Y., Sohn, J.W., Kim, W.J., Kim, M.J., Park, S.C. and Cheong, H.J. 2002. Clinical and molecular epidemiology of quinolone-resistant *Escherichia coli* isolated from urinary tract infection. *Microb. Drug Resist.* **8**: 227–234.
5. Gautom, R. K. 1997. Rapid pulsed-field gel electrophoresis protocol for typing of *Escherichia coli* O157:H7 and other gram-negative organisms in 1 day. *J. Clin. Microbiol.* **35**: 2977–2980.
6. Greko, C. 1999. Antibiotics as growth promoters. *Acta Vet. Scand. (Suppl.)* **92:** 87–100.
7. Harada, K., Asai, T., Kojima, A., Oda, C., Ishihara, K. and Takahashi, T. 2005. Antimicrobial susceptibility of pathogenic *Escherichia coli* isolated from sick cattle and pigs in Japan. *J. Vet. Med. Sci.* **67**: 999–1003.
8. Jacoby, G. A. 2005. Mechanisms of resistance to quinolones. *Clin. Infect. Dis.* **41** (Suppl. 2): S120–126.
9. Jeong, J. Y., Yoon, H. J., Kim, E. S., Lee, Y., Choi, S. H., Kim, N. J., Woo, J. H. and Kim, Y. S. 2005. Detection of qnr in clinical isolates of *Escherichia coli* from Korea. *Antimicrob. Agents Chemother.* **49**: 2522–2524.
10. Kim, Y., Jung, J., Kim, M., Park, J., Boxall, A. and Choi, K. 2008. Prioritizing veterinary pharmaceuticals for aquatic environment in Korea. *Environ.Toxicol. Pharmacol.* **26**: 167–176.
11. Lee, Y. J., Oh, J. K., Kim, K. S., Tak, R. B., Kim, A. R., Kim, J. W., Im, S. K. and Kim, B. H. 2005. Fluoroquinolone resistance and gyrA and parC mutations of *Escherichia coli* isolated from chicken. *J. Microbiol.* **43**: 391–397.
12. Lim, S. K., Lee, H. S., Nam, H. M., Cho, Y. S., Kim, J. M., Song, S. W., Park, Y. H. and Jung, S. C. 2007. Antimicrobial resistance observed in *Escherichia coli* strains isolated from fecal samples of cattle and pigs in Korea during 2003–2004. *Int. J. Food Microbiol.* **116**: 283–286.
13. Maple, P., Brumfitt, W. and Hamilton-Miller, J. M. 1990. A review of the antimicrobial activity of the fluoroquinolones. *J. Chemother.* **2**: 280–294.
14. National Committee for Clinical Laboratory Standards. 2003. Performance Standards for Antimicrobial Susceptibility Testing; Thirteenth Informational Supplement M100-S13. NCCLS, Wayne, PA.
15. Oram, M. and Fisher, L. M. 1991. 4-Quinolone resistance mutations in the DNA gyrase of *Escherichia coli* clinical isolates identified by using the polymerase chain reaction. *Antimicrob. Agents Chemother.* **35**: 387–389.
16. Orden, J. A., Ruiz-Santa-Quiteria, J. A., Cid, D., Diez, R., Martinez, S. and de la Fuente, R. 2001. Quinolone resistance in potentially pathogenic and non-pathogenic *Escherichia coli* strains isolated from healthy ruminants. *J. Antimicrob. Chemother.* **48**: 421–424.
17. Pereira, A. S., Andrade, S. S., Monteriro, J., Sader, H. S., Pignatari, A. C. and Gales, A. C. 2007. Evaluation of the Susceptibility profiles, genetic similarity and presence of *qnr* gene in *Escherichia coli* resistant to ciprofloxacin isolated in Brazilian hospitals. *Braz. J. Infect. Dis.* **11**: 40–43.
18. Saenz, Y., Zarazaga, M., Brinas, L., Ruiz-Larrea, F. and Torres, C. 2003. Mutations in *gyrA* and *parC* genes in nalidixic acid-resistant *Escherichia col*i strains from food products, humans and animals. *J. Antimicrob. Chemother.* **51**: 1001–1005.
19. Tavio, M. M., Vila, J., Ruiz, J., Martub-Sanchez, A. M. and Jimenez de Anta, M. T. 1999. Mechanisms involved in the development of resistance to fluoroquinolones in *Escherichia coli* isolates. *J. Antimicrob. Chemother.* **44**: 735–742.
20. Threlfall, E. J., Cheasty, T., Graham, A. and Rowe, B. 1997. High-level resistance to ciprofloxacin in *Escherichia coli*. *Lancet*. **349**: 403.
21. Vila, J., Ruiz, J., Goni, P. and De Anta, M. T. 1996. Detection of mutations in parC in quinolone-resistant clinical isolates of *Escherichia coli*. *Antimicrob. Agents Chemother.* **40**: 491–493.
22. Wieler, L.H., Sobjinski, G., Schlapp, T., Failing, K., Weiss, R., Menge, C. and Baljer, G. 2007. Longitudinal prevalence study of diarrheagenic *Escherichia coli* in dairy calves. *Berl. Münch. Tierärztl. Wochenschr.* **120**: 296–306.