# ANALYSIS OF *GYRA* MUTATIONS RELATED TO QUINOLONE RESISTANCE IN *ESCHERICHIA COLI* ISOLATES ORIGINATING FROM PET, HUMAN, VEGETABLE AND ICE IN BANGKOK AND VICINITY

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Abstract. *Escherichia coli* was used to investigate quinolone resistance and mutations in *gyrA* gene of *E. coli* isolated from pet (dog and cat), human (pet's owner), vegetable and edible ice in Bangkok and vicinity. Susceptibility test for nalidixic acid (NA) showed similar percent resistance among the sample sources but a lower ciprofloxacin (CIP) resistance was found particularly in human source. Mutations within quinolone resistance determining region of *gyrA* gene analyzed using non-radioactive single-strand conformation polymorphism (SSCP) and sequencing showed 10 different SSCP patterns. *E. coli* isolates from pet, vegetable and ice showed more variety of patterns than strains isolated from human. Four out of 10 SSCP patterns were identified as having mutations in amino acids positions 83 (Ser to Leu) and position 87 (Asp to Asn). These mutations were observed only in NA-resistant strains and combined mutations were observed only in *E. coli* isolated from humans and pets. As only 24% of NA- and CIP-resistant *E. coli* isolates contained *gyrA* mutations, other quinolone resistant mechanisms may be involved. Nevertheless, *gyrA* mutations may be used to monitor nalidixid acid resistance in *E. coli.*

## INTRODUCTION

Quinolones and fluoroquinolones have been extensively applied as wide spectrum antibiotics both in clinical and veterinary medicine (Hopkins *et al*, 2005; Salyers and Shoemaker, 2006). Their mechanism of action is by inhibiting DNA gyrase and topoisomerase IV involved in controlling the topology of the chromosomal DNA during bacterial replication and transcription (Emmerson and Jones, 2003; Hawkey, 2003). Both enzymes are composed of 2 pairs of subunits. The subunits of DNA gyrase are encoded by *gyrA* and *gyrB*, and the corresponding subunits of topoisomerase IV are encoded by *parC* and *parE* (Ruiz, 2003; Hopkins *et al*, 2005; Jacoby, 2005). In gram-negative bacteria, DNA gyrase is a prime target of quinolones rather than topoisomerase IV (Jacoby, 2005). Mutations within the quinolone resistance-determining region (QRDR) of *gyrA* have been observed as a major mechanism of quinolone resistance in *Escherichia coli* (Ruiz, 2003; Hopkins *et al*, 2005).

High incidence of quinolone resistance in gram-negative bacteria has been reported worldwide especially in travelers returning from Thailand ( Hakanen *et al*, 2001, 2006). Use of quinolones in human and animal both for treatment and prevention of infection possibly con-

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tributes to the development of antimicrobial resistance in pathogenic and commensal bacteria (van den Bogaard and Stobberingh, 2000; Swartz, 2002; Belloc *et al*, 2005). Resistant bacteria can reach the general environment, such as water and soil via sewage and manure, and then can be transferrd to human by the food chain and direct contact (Witte, 2000). *E. coli* as commensal gastrointestinal flora is considered to be a good indicator organism not only for fecal contamination in food and water but also for antimicrobial selective pressure (Harwood *et al*, 2000; van den Bogaard and Stobberingh, 2000; Kronvall *et al*, 2005). The prevalence of resistance in indicator bacteria in different populations can be used to monitor transfer of resistant bacteria via food chain and life style (Harwood *et al*, 2000; van den Bogaard and Stobberingh, 2000; Kronvall *et al*, 2005).

The aim of this study was to investigate resistance for quinolones and to characterize mutations in *gyrA* gene of resistant *E. coli* isolated from pets (dog and cat), human (pet's owner), vegetable and edible ice in Bangkok and vicinity.

## MATERIALS AND METHODS

### *E. coli* isolates

A total of 133 *E. coli* isolates were collected from Bangkok and vicinity during March to June 2005. Isolation of *E. coli* was obtained from 4 main sources: fecal samples of pets including dogs and cats (n=34), fecal samples of humans who are pets' owners (n=19), fresh vegetable samples including lettuce, Chinese cabbage, cabbage, coriander and bean sprout (n=59) and edible ice samples (n=21). Fresh vegetable and edible ice were collected from retail markets and isolated using Lauryl tryptose broth and Mac Conkey agar. Membrane filter technique was performed for edible ice samples. *E. coli* isolates were identified by conventional biochemical tests including Gram staining, oxidase test, triple sugar iron agar (TSI), citrate utilization test, urease test, motility test, lysine decarboxylase test (LD) and indole test.

### Antimicrobial susceptibility testing

Quinolone susceptibility was measured in 133 *E. coli* isolates by disk diffusion method, with disks containing nalidixic acid (NA; 20 µg) and ciprofloxacin (CIP; 5 µg). Results were interpreted according to the recommendations of the Clinical and Laboratory Standards Institute (CLSI, formerly NCCLS) guideline (CLSI, 2006). *E. coli* ATCC 25922 was used as control strain.

### PCR amplification of *gyrA* gene

A 248 bp fragment of *E. coli gyrA* gene was amplified by PCR (Pleumsud, 2005). Genomic DNA from each *E. coli* isolate was prepared by phenol-chloroform method. PCR for *gyrA* gene was conducted using the following oligonucleotide primers: 5´-GTACTTTACG CCATGAACG-3´ and 5´- ATTTTCGCCAG ACGGA-3´. Amplification was performed in a final volume of 25 µl containing 2 µl of genomic DNA, 1X *Taq* DNA polymerase reaction buffer, 1.5 mM $MgCl_2$, 200 µM each of deoxynucleoside triphosphate, 20 pmole of each primer and 1U of *Taq* DNA polymerase (Fermentas). Amplification was initiated with denaturation at 95ºC for 5 minutes, followed by 30 cycles of 95ºC for 30 seconds, 55ºC for 30 seconds and 72ºC for 30 seconds with a final extension at 72ºC for 7 minutes.

### Single-strand conformation polymorphism (SSCP) analysis and DNA sequencing

Two µl of PCR product was added to 5 µl of denaturation solution (95% formamide, 0.05% bromophenol blue, 0.05% xylene cyanol and 10 mM NaOH) and 3 µl of 0.5X TBE. Mixture was heated at 95 ºC for 10 minutes and immediately cooled on ice. The solution was analyzed by 10% non-denaturing polyacrylamide gel-electrophoresis (37.5: 1 acrylamide: bisacrylamide) in 0.5X TBE at con-

stant power of 20 mA, 25ºC for 3.5 hours. DNA bands were visualized by silver staining. At least one representative PCR product from each different SSCP pattern was sequenced. Sequences were aligned with reference nucleotide sequence of *E. coli gyrA* gene (GenBank accession no. X57147).

### Statistical analysis

Chi-square method was used to determine significant difference in quinolone resistant *E. coli*. A p-value < 0.05 is considered statistically significant.

## RESULTS

### Prevalence of quinolone resistance

A total of 133 *E. coli* isolates originating from human (pet's owner), pets (dog and cat), vegetables and edible ice were examined for quinolone resistance using disk diffusion method (Fig 1). A relatively high prevalence of resistance in *E. coli* from all sources was observed for nalidixic acid (31.6% human strains, 32.4% pet strains, 35.6% vegetable strains, 57.1% edible ice strains). Lower percent ciprofloxacin resistance was found particularly in *E. coli* from human (5.3%). The percent strains resistant to nalidixic acid and ciprofloxacin were not significantly different when sample sources were compared except for *E. coli* isolates from human and edible ice sources which showed over 25% difference between resistance to nalidixic acid and ciprofloxacin.

### SSCP patterns among sample sources

Mutational analysis of *gyrA* gene within QRDR of 133 *E. coli* isolates using non-radioactive single-strand conformation polymorphism (SSCP) and sequencing showed 10 different SSCP patterns, named as E1 to E10 (Table 1, Fig 2). Each SSCP pattern represented different combinations of nucleotide sequence alterations. Only 4 out of 10 SSCP patterns (E2, E7, E8 and E10) were associated with mutations in amino acids at positions 83 and 87. E7, E8 and E10 contained a single missense mutation, Ser-83-Leu. E2 SSCP pattern contained Asp-87-Asn mutation in combination with Ser-83-Leu. Among nalidixic acid-resistant *E. coli*, strains isolated from pet, vegetable and edible ice were presented by a variety of SSCP patterns (6-7 patterns) whereas strains isolated from human had only 3 patterns (Fig 3). E2 was found only in human and pet strains. A higher frequency of SSCP patterns containing missense mutations was observed in resistant strains from human and pet than from vegetable and edible ice.

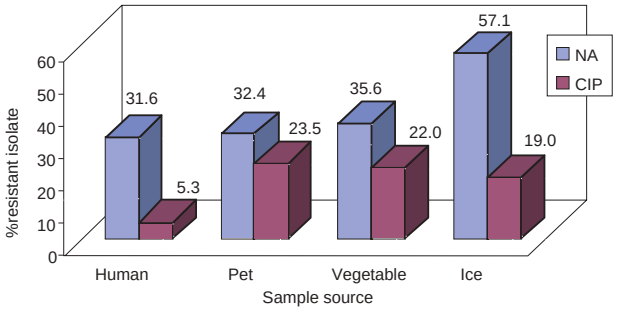


Fig 1–Prevalence of nalidixic acid (NA) and ciprofloxacin (CIP) resistance in *E. coli* isolates originating from human, pet, vegetable and edible ice. Quinolone resistance was determined in 133 *E. coli* isolates using disk diffusion method.

### Association of *gyrA* mutation and quinolone resistance

Ser-83-Leu and Asp-87-Asn mutations were observed only in nalidixic acid resistant strains (Table 1). Moreover, the double mutation of Asp-87-Asn and Ser-83-Leu were detected only in *E. coli* isolated from pets and pet's owners and were not found in ciprofloxacin susceptible strains. All strains representing ciprofloxacin resistance also showed resistance to nalidixic acid. Approximately 24% of nalidixic acid (12/49) and ciprofloxacin (6/25) resistant strains carried either Ser-83-Leu or Asp-87-Asn mutation.

Table 1
Frequency of amino acid change and antimicrobial phenotype of *E. coli* classified by 10 different SSCP patterns.

| SSCP Patterns | NA no. of isolates (%) | | | CIP no. of isolates (%) | | | Amino acid changes |
|---|---|---|---|---|---|---|---|
| | S (n=78) | I (n=6) | R (n=49) | S (n=101) | I (n=7) | R (n=25) | |
| E1 | 67 (85.9) | 4 (66.7) | 16 (32.7) | 78 (77.2) | 3 (42.9) | 6 (24.0) | No change |
| E2 | - | - | 4 (8.2) | - | 1 (14.3) | 3 (12.0) | 83 (Ser→Leu) + 87 (Asp→Asn) |
| E3 | - | - | 1 (2.0) | - | - | 1 (4.0) | No change |
| E4 | 4 (5.1) | 1 (16.7) | 6 (12.2) | 6 (5.9) | 2 (28.6) | 3 (12.0) | No change |
| E5 | 2 (2.6) | - | 9 (18.4) | 3 (3.0) | - | 8 (32.0) | No change |
| E6 | - | 1 (16.7) | - | - | 1 (14.3) | - | No change |
| E7 | - | - | 4 (8.2) | 2 (2.0) | - | 2 (8.0) | 83 (Ser→Leu) |
| E8 | - | - | 3 (6.1) | 2 (2.0) | - | 1 (4.0) | 83 (Ser→Leu) |
| E9 | 5 (6.4) | - | 5 (10.2) | 9 (8.9) | - | 1 (4.0) | No change |
| E10 | - | - | 1 (2.0) | 1 (1.0) | - | - | 83 (Ser→Leu) |

n = Total number of isolates; NA = Nalidixic acid; CIP = Ciprofloxacin; S = Susceptible; I = Intermediate; R = Resistant; Ser = Serine; Leu = Leucine; Asp = Aspartic acid; Asn = Asparagine

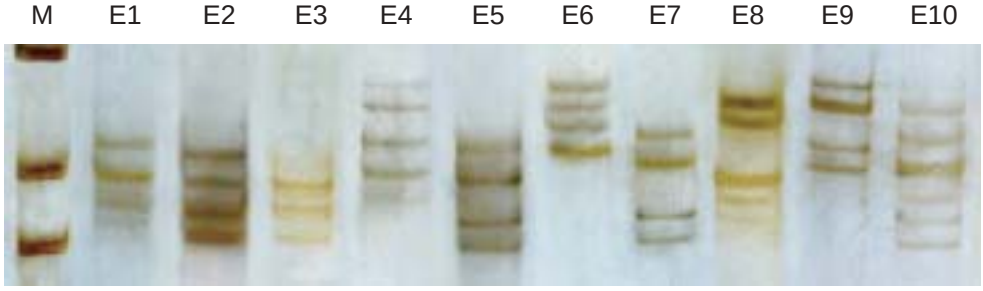


Fig 2–Single-strand conformation polymorphism (SSCP) analysis of *E. coli* showing patterns E1-E10. Pattern types correspond to the following missense mutations in *gyrA*: E7, E8, and E9, Ser-83→Leu; E2, Ser-83→Leu and Asp-87→Asn. DNA marker is indicated in lane M.

## DISCUSSION

Emergence and spread of antimicrobial resistance via food chain, animal and environment are considered to be the possible routes in transferring resistant bacteria and resistance genes to man (van den Bogaard and Stobberingh, 2000). To monitor quinolone resistance dissemination through the cycle of food chain, *E. coli*, conmmensal flora in the intestinal tract of human and animal, are considered to be good indicator bacteria of selective pressure from using antimicrobials in the population (Murray, 1992) and for the expected resistance problems occurring in pathogens (Lester *et al*, 1990).

In this study, vegetable and edible ice were chosen as representatives of the food chain, and domestic pets including cat and dog were chosen as sources of animal samples with close contact with human (pet's

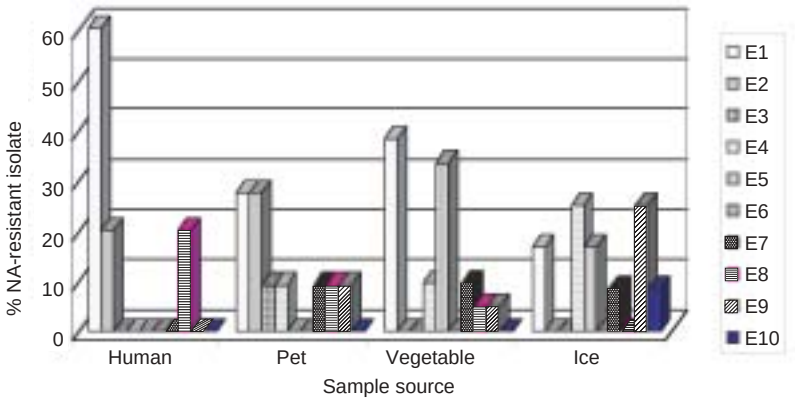


Fig 3–Frequency of SSCP patterns in nalidixic acid (NA)-resistant *E. coli* isolated from human, pet, vegetable and edible ice. Ten different SSCP patterns are indicated by E1 to E10.

owners). Comparison of quinolone resistance among these sample sources showed no significant difference among these sources. Nalidixic acid resistance was more frequently found than ciprofloxacin resistance. In addition, all strains which were resistant to ciprofloxacin were also resistant to nalidixic acid.

Ten different SSCP patterns representing different nucleotide sequence alterations were obtained. Only 4 out of 10 SSCP patterns (E2, E7, E8 and E10) were identified containing missense mutations. The occurrence of different SSCP patterns either containing the same missense mutation or not associated with mutations in codon 83 and 87 resulted from several conbinations of silent mutations ranging from 1 to 19. No typical silent mutation conbination associated with sample sources was observed. Among nalidixic acid resistant *E. coli*, strains isolated from pet, vegetable and edible ice showed more variety of SSCP patterns compared with strains isolated from humans. Different environment and inducers may lead to the variety of mutations in *gyrA* gene and SSCP patterns of *E. coli* isolates originating from different sources. E2 pattern with the double mutations was found only in human and pet strains. A higher frequency of SSCP patterns containing missense mutations was observed in resistant strains from human and pet more often than from vegetable and edible ice. SSCP patterns derived from human would be related to those from pets. Transfer of resistant strains between man and animal may be possible as evidenced by the report of the transfer of ciprofloxacin-resistant *E. coli* strains from turkeys to turkey farmers (van den Bogaard and Stobberingh, 2000). In addition, strepthothricin resistance had been reported from the spread of *E. coli* of nourseothricin-fed pigs to the gut flora of the farm workers, their families and healthy community members (Molbak, 2004). In several studies, food animals or pets were found as potential reservoirs for transmission of *E. coli* infections represented by sharing of phylogenic, pathogenic and genotypic similarities in extraintestinal pathogenic *E. coli* from animals and humans (van den Bogaard and Stobberingh, 2000; Maynard *et al*, 2004). Due to the broad distribution of resistant bacteria and antimicrobial resistance determinants by transfer in the same host species or between different host species, the spread of antibiotic resistance in community (Duerink *et al*, 2007) and environment is of concern.

In the present study, two mutations (Ser-83-Leu and Asp-87-Asn) have been observed in nalidixic acid-resistant *E. coli*. Moreover, Asp-87-Asn appeared in combination with Ser-83-Leu, which was not found in ciprofloxacin susceptible strains. This supports hot spot mutations in *E. coli* and step-wise additional mutations leading to high-level of quinolone resistance (Hopkins *et al*, 2005).

Mutation in *gyrA* was found in as much as 76.6% of fluoroquinolone-resistant *E. coli* isolated from chicken cecal contents in Korea (Lee *et al*, 2005). However, the overall missense mutations in *gyrA* found in this study accounted for approximately 24% of quinolone-resistant *E. coli* strains originating from human, pet, vegetable and edible ice. It suggested that other gene mutations (*gyrB*, *parC* or *parE*) or other resistant mechanisms including the alteration of the permeability of quinolone across the cell membrane and plasmid mediated quinolone resistance (*qnr*) may also play roles in these groups of samples (Hopkins *et al*, 2005).

Spread of quinolone resistance is not only of concern in food chain or local environment but it can also be distributed to many other parts of the world through travel (Hakanen *et al*, 2003). Therefore, the distribution spectrum of antimicrobial resistance should be routinely monitored and *gyrA* mutations may be effective indicators of nalidixic acid resistance.

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