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Contamination of Chicken Meat with *Salmonella enterica* Serovar Haardt with Nalidixic Acid Resistance and Reduced Fluoroquinolone Susceptibility

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Salmonella **contamination in chicken meat was studied with 100 chicken meat samples purchased from 55 shops located in various regions. A total of 21 isolates of *Salmonella enterica* were isolated from 21 chicken meat samples from four shops located at open markets, whereas there were none from supermarkets with well-equipped cold systems. Among these, 18 isolates were identified as *Salmonella enterica* serotype Haardt (*S.* Haardt) and three isolates were *S. enterica* serotype Muenchen. When the minimal inhibitory concentrations of the *S.* Haardt isolates were assayed with the agar dilution method to determine susceptibility to ampicillin, chloramphenicol, sulfisoxazole, tetracycline, and nalidixic acid, all 18 isolates were resistant to tetracycline and nalidixic acid and nine of these were resistant to ampicillin. These isolates showed reduced susceptibility to eight fluoroquinolones including ciprofloxacin, enrofloxacin, levofloxacin, gatifloxacin, gemifloxacin, moxifloxacin, norfloxacin, and ofloxacin. When quinolone resistance determining regions of *gyrA* and *gyrB* were sequenced, every isolate had the same missense mutation Ser83→Tyr (TCC→TAC) in *gyrA*, whereas no mutation was found in *gyrB*. Pulsed-field gel electrophoresis with XbaI revealed a close relationship among these isolates, suggesting a contamination of raw chicken meat with clonal spread of nalidixic acid-resistant and quinolone-reduced susceptibility *S.* Haardt in chickens. Results in this study show the importance of a well-equipped cold system and the prudent use of fluoroquinolone in chickens to prevent the occurrence of quinolone-resistant isolates.**

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Salmonella spp. are important zoonotic pathogens in humans and animals, widespread in nature, and can colonize or infect a variety of domesticated and wild animals ranging from mammals to birds and reptiles [33]. *Salmonella* spp. are also responsible for extraintestinal pathologies, such as urinary tract infections, abscesses at diverse locations, and bacteremia. Many outbreaks have been traced to ingestion of contaminated animal products [2, 3] rather than person-to-person transmission or direct fecal-oral transmission, in some cases derived from specific farms, flocks, or herds of animals [22]. Most cases of bacterial gastroenteritis are self-limiting, and it has been suggested that in otherwise healthy patients, the administration of antimicrobial agents is not necessary. However, antimicrobial therapy is fundamental for illness control when bacteremia is suspected in infants, elderly people, granulopenic or immunodepressed patients, and also patients with extraintestinal infections in particular. If not treated with an antimicrobial agent, severe life-threatening bacteremia and other deep-seated infections do occur in these people [28]. Increasing rates of antimicrobial resistance in *Salmonella* isolates have been reported from a number of developing and developed countries [4, 8, 15].

Since nalidixic acid was developed as an antimicrobial agent in the 1960s [7], several fluoroquinolones had been developed with stronger antibacterial activity, especially against Gram-negative bacteria including *Salmonella* species [18]. However, there are several reports of treatment failure of fluoroquinolones used on *Salmonella* infections owing to reduced fluoroquinolone susceptibility [9, 17, 19,

21]. Some epidemiological studies have shown that the number of *Salmonella* isolates with reduced fluoroquinolone susceptibility has increased [14] in the clinical environment [9] and livestock industry [13]. In Korea, the nalidixic acid resistance rate in clinical *Salmonella* isolates was 1.8% in 1995–1996 and increased to 21.8% in 2000-2002 [5]. In the case of animal isolates of *Salmonella*, MICs for fluoroquinolone were less than 0.06–0.25 μg/ml in 1995, but have increased to 2–8 μg/ml in 2001 [24].

In this study, antimicrobial-resistant *Salmonella* isolates were isolated from chicken meat and their antimicrobial resistance and similarity among these isolates were characterized.

Materials and Methods

Isolation and Identification of *S.* Haardt

A total of 100 uncut chicken meat samples was purchased from 55 shops, including modernized supermarkets with well-equipped cold systems and open markets in Seoul and Kyung-gi Province where half of the Korean population lives. After a whole uncut chicken was shaken in 400 ml of buffered peptone water in a tightly sealed plastic bag (Whirl Pak), 0.1 ml of the supernatant was mixed with 9 ml of Rappaport Vassiliadis R10 (RV; Merck, Darmstadt, Germany) broth and incubated at 42°C for 24±2 h. Sample that changed the color of RV broth from blue to discolored or green was considered as positive growth for *Salmonella* and further used for the isolation of *Salmonella*. One loopful of material from RV was streaked onto the surface of Rambach (Merck, Darmstadt, Germany) agar plates. Suspected red colonies were serotyped [10] using *Salmonella* antisera (Denka Seiken, Tokyo, Japan). These isolates were identified using the VITEK GNI (bioMerieux, Marcy l'Etoile, France), Easy 24E plus (KOMED, Sung-nam, Korea), or API 20E (bioMerieux) identification systems and 16S rRNA sequencing. Isolates of *Salmonella* spp. were deposited in the Culture Collection of Antimicrobial Resistant Microbes (CCARM) and stored at −70°C on porous beads (Prolab, Austin, TX, U.S.A.) until further use.

Assay of Minimal Inhibitory Concentration

Minimal inhibitory concentrations (MICs) were assayed by the standard agar dilution method according to the Clinical and Laboratory Standard Institute's (CLSI) guidelines [6] to measure the susceptibility to ampicillin, chloramphenicol, streptomycin, sulfisoxazole, tetracycline, nalidixic acid, and eight fluoroquinolones including ciprofloxacin, enrofloxacin, levofloxacin, gatifloxacin, gemifloxacin, moxifloxacin, norfloxacin, and ofloxacin. Mueller-Hinton agar (BBL, Cockeysville, MD, U.S.A.) was used as the culture medium. *Escherichia coli* ATCC 25922 was used as a control in susceptibility testing. All chemicals were purchased from Sigma (St. Louis, MO, U.S.A.) unless otherwise stated.

Polymerase Chain Reaction and Sequencing of Quinolone Resistance Determining Region

Quinolone resistance determining regions (QRDRs) of *gyrA* and *gyrB* were sequenced as reported previously [14, 20]. The QRDR of *gyrA* was amplified with a primer set (5'-GAGGGATAGCGGTTAGATGAG-3' and 5'-TTTTTCCGTGCCGTCATGG-3') with pre-denaturation at 95°C for 10 min, and 30 cycles of 95°C for 1 min, 60°C for 1 min, and 72 °C for 1 min, expecting a DNA fragment with 476 bp. The QRDR for *gyrB* was amplified with a primer set (5'-ACTGGCGGACTGTCAGGAAC-3' and 5'-TCTGACGATAGAAGAAGGTCAAC-3') with pre-denaturation at 95°C for 5 min, and 30 cycles of 1 min at 95°C, 20 sec at 53°C, and 1 min at 72°C, expecting a DNA fragment with 300 bp. After resulting DNA fragments were confirmed on a 1% agarose gel, they were extracted from the gel and purified with the Gel Extraction kit (Qiaquick; Qiagen, Valencia, CA, U.S.A.) and sequenced with Sanger's method [34] in an ABI Prism 310 Genetic Analyzer (PE Applied Biosystems, Foster City, CA, U.S.A.). As a control strain, *S. enterica* serotype Typhimurium NCTC 74 was used.

Pulsed-Field Gel Electrophoresis

Pulsed-field gel electrophoresis (PFGE) was performed following the PulseNet protocol (Center for Disease Control and Prevention, Atlanta, GA, U.S.A.). Genomic DNAs in the plug were digested with 20 Unit XbaI (MBI Fermentas, Hanover, MD, U.S.A.) and the digested genomic DNA fragments were separated in the CHEF-DR III system (Bio-Rad, Richmond, CA, U.S.A.) at 12°C for 20 h with an initial pulse for 5 sec and a final pulse for 40 sec with 6 V/cm and at an angle of 120° using lambda DNA marker (Sigma) as a DNA molecular weight marker. After DNA bands were stained with ethidium bromide, DNA banding patterns were analyzed by using Gel Compar II software (Applied Maths, Kortrijk, Belgium) to calculate Jaccard coefficients of correlation and to generate a dendrogram by the unweighted pair group method using arithmetic averages (UPGMA) clustering.

Results

Isolation and Identification of *Salmonella*

A total of 21 isolates of *S. enterica* were obtained from 100 uncut chicken meat samples purchased from various regions in Seoul and Kyung-gi Province. All of these isolates were obtained from unfrozen chicken meat bought from four open markets where HACCP was not performed. *Salmonella* were not detected in 79 chicken meat samples purchased from modernized supermarkets performing HACCP. Among these *Salmonella* isolates, 18 isolates were serotyped as Haardt (*S.* Haardt) and 3 isolates were serotyped as Muenchen (*S.* Muenchen).

Assay of Minimal Inhibitory Concentration

All 18 isolates of *S.* Haardt were resistant to tetracycline and nalidixic acid (MIC 512 μg/ml) but susceptible to chloramphenicol. Nine of these isolates were resistant to ampicillin and were multidrug resistant. As shown in Table 1, MICs to other fluoroquinolones increased but within a susceptible range according to the criteria set by CLSI: 0.25–0.50 μg/ml for ciprofloxacin, 0.50 μg/ml for enrofloxacin, 0.50 μg/ml for gatifloxacin, 0.25–0.5 μg/ml for gemifloxacin, 0.50 μg/ml for levofloxacin, 1.0 μg/ml

Table 1. Minimal inhibitory concentration of nalidixic acid-resistant and quinolone-reduced susceptibility chicken isolates of *Salmonella enterica* serotype Haardt.

	MIC (μg/ml)													
	AMP	CHL	STR	SSS	TET	NAL	CIP	ENX	GAT	GEM	LEV	MOX	NOR	OFL
CCARM8112	2	4	>128	128	>128	1,024	0.5	0.5	0.5	0.5	0.5	1.0	2.0	1.0
CCARM8113	2	4	128	64	>128	512	0.25	0.5	0.5	0.25	0.5	1.0	1.0	1.0
CCARM8114	>256	4	64	128	>128	1,024	0.5	0.5	0.5	0.5	0.5	1.0	2.0	1.0
CCARM8115	>256	4	64	64	>128	1,024	0.25	0.5	0.5	0.25	0.5	1.0	1.0	1.0
CCARM8116	>256	4	32	128	128	1,024	0.5	0.5	0.5	0.5	0.5	1.0	2.0	1.0
CCARM8117	>256	4	32	128	>128	1,024	0.5	0.5	0.5	0.5	0.5	1.0	2.0	1.0
CCARM8118	>256	4	64	64	>128	512	0.25	0.5	0.5	0.25	0.5	1.0	2.0	1.0
CCARM8120	>256	4	64	128	>128	1,024	0.5	0.5	0.5	0.5	0.5	1.0	2.0	1.0
CCARM8122	2	4	64	128	>128	1,024	0.5	0.5	0.5	0.5	0.5	1.0	2.0	1.0
CCARM8123	2	8	64	128	128	1,024	0.5	0.5	0.5	0.5	0.5	1.0	2.0	2.0
CCARM8124	2	8	64	64	128	512	0.25	0.5	0.5	0.25	0.5	1.0	1.0	1.0
CCARM8125	2	8	64	128	128	1,024	0.5	0.5	0.5	0.5	0.5	1.0	2.0	1.0
CCARM8126	2	8	64	128	128	512	0.25	0.5	0.5	0.25	0.5	1.0	1.0	1.0
CCARM8127	2	8	64	64	128	512	0.5	0.5	0.5	0.25	0.5	1.0	1.0	1.0
CCARM8128	>256	4	64	64	128	512	0.25	0.5	0.5	0.25	0.5	1.0	1.0	1.0
CCARM8129	>256	4	64	128	128	512	0.5	0.5	0.5	0.25	0.5	1.0	1.0	1.0
CCARM8130	>256	4	64	128	128	1,024	0.5	0.5	0.5	0.5	0.5	1.0	2.0	1.0
CCARM8131	2	8	64	64	128	512	0.25	0.5	0.5	0.25	0.5	1.0	1.0	1.0
Control strain	2	2	4	16	0.5	2	0.008	0.016	0.016	0.008	0.016	0.016	0.06	0.032

AMP, Ampicillin; CHL, Chloramphenicol; STR, Streptomycin; SSS, Sulfisoxazole; TET, Tetracycline; NAL, Nalidixic acid; CIP, Ciprofloxacin; ENX, Enrofloxacin; GAT, Gatifloxacin; GEM, Gemifloxacin; LEV, Levofloxacin; MOX, Moxifloxacin; NOR, Norfloxacin; OFL, Ofloxacin; Control strain, *E. coli* ATCC25922.

for moxifloxacin, 1.0–2.0 μg/ml for norfloxacin, and 1.0–2.0 μg/ml for ofloxacin.

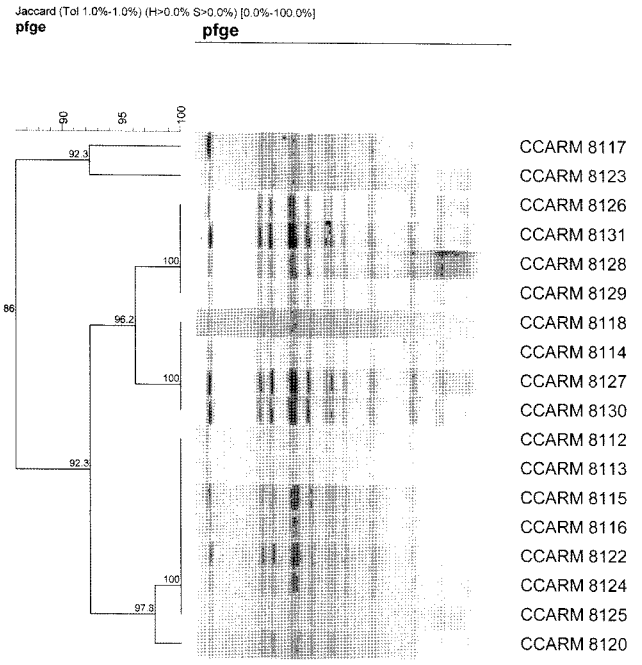

Fig. 1. Pulsed-field gel electrophoresis of nalidixic acid-resistant, quinolone-reduced susceptibility chicken isolates of *S.* Haardt.

Sequencing of Quinolone Resistance Determining Region in GyrA and GyrB

When QRDRs of *gyrA* and *gyrB* were sequenced to determine the mechanism for the reduced susceptibility to quinolone, the same missense mutation, Ser83→Tyr (TCC→TAC), was found in QRDR of *gyrA* in every isolate of *S.* Haardt, whereas no mutation was found in QRDRs in *gyrB*. The control strain *S. enterica* serotype Typhimurium NCTC 74 did not have mutation in QRDRs of *gyrA* or *gyrB*.

Pulsed-Field Gel Electrophoresis

When 12 bands of genomic DNA digested with XbaI were compared, all 18 isolates were closely related with similarity higher than 86% (Fig. 1). Isolates obtained from shops at different locations showed 100% similarity. Ampicillin resistance was not related with similarity among isolates.

DISCUSSION

In a clinical environment, ampicillin, trimethoprim-sulfamethoxazole, and chloramphenicol have been recommended as antimicrobial agents for severe *Salmonella* infections. However, rising rates of resistance to these agents have significantly reduced the efficacy of these agents. As a consequence, fluoroquinolones and expanded-

spectrum cephalosporins have become the recommended antimicrobial agents for invasive *Salmonella* infections in humans. Reports of *Salmonella* resistant to nalixic acid have been increasing, and treatment failures with quinolone have also been reported. Based on these observations, many people suggest to setting a new quinolone resistance criteria for *Salmonella* [9, 12]. Since antimicrobial-resistant bacteria may be transferred to humans through the food chain [1], the antimicrobial resistance mechanisms in *Salmonella* in animals, especially resistance to antimicrobial agents such as quinolone used in humans, is troubling.

Even though precautious measure has been implemented to prevent quinolone resistance, quinolone resistance has been reported worldwide. The alarming increase in quinolone resistance over the past few years among food borne pathogens [32] has led to speculation that this might be due to the use of quinolones in livestock husbandry [26, 30] even though the use of quinolone is only permitted for therapeutic use and not as growth promoters in food animals worldwide. In Europe, none of the fluoroquinolones licensed for humans are approved for animal use except for the treatment of livestock, poultry, and fish. In the United States, only enrofloxacin is licensed, for the treatment of poultry alone [27]. In Korea, enrofloxacin was, until recently, permitted for use in chickens, with the exception of egg-laying chickens.

In Korea, the nalidixic acid resistance rate in clinical *Salmonella* isolates was 1.8% in 1995–1996, but has increased to 21.8% in 2000–2002 [5]. In the case of animal isolates of *Salmonella*, MICs for fluoroquinolone were less than 0.06–0.25 μg/ml in 1995, but they increased to 2–8 μg/ml in 2001 [24]. MICs of chicken isolates assayed in this study were 0.25–2 μg/ml, which were much higher than MICs for fluoroquinolone-susceptible wild-type *Salmonella* isolates (MIC to ciproflorxacin=0.016–0.12 μg/ml) [21]. Even though MICs of the reduced quinolone-susceptible *Salmonella* are categorized as susceptible, these isolates are rendering resistance problem in a clinical environment. Because of this problem, many people have recommended to lower the resistance criteria for quinolone resistance for *Salmonella* [16, 25, 31]. Since high-level resistance can be easily induced by a second mutation in these isolates, preventive measure(s) to stop the further increase in MICs is urgently needed.

Antimicrobial resistance including fluoroquinolone resistance can be spread throughout populations by spread of a particular isolate (clonal spread) [23] or through exchange of genetic materials [11, 29]. Since all *S.* Haardt isolates in this study showed close relationship, even though they were isolated from various shops at various open markets located in different regions, strict sanitary controls are necessary to avoid the spread of nalidixic acid-resistant fluoroquinolone-reduced susceptibility isolates in chickens. In addition, a more efficient veterinary policy, such as continued surveillance and education of veterinary and human medicine practitioners on the appropriate use of antimicrobial agents must be adopted to decrease in humans *Salmonella* infections originated from animals.

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